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Review Article

Research Review on Non-structural Protein 3 (NS3) of Classical Swine Fever Virus and Its Potential Applications in Vaccine Development

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Abstract

Classical Swine Fever Virus (CSFV) is a virus that poses a serious threat to the pig farming industry, and its non-structural protein 3 (NS3) plays a key role in the virus's replication, pathogenicity, and immune evasion. In recent years, with the deepening research on CSFV NS3, its important roles in viral biology and immunology have gradually been revealed. NS3 is not only involved in the replication process of CSFV but also engages in complex interactions with the host immune system, promoting the virus's immune evasion. However, despite numerous studies exploring the functional mechanisms and structural characteristics of NS3, the specific applications of CSFV NS3 in vaccine development still face shortcomings and challenges. This article aims to review the latest research progress on CSFV NS3, analyze its potential as a vaccine target, and provide new ideas and directions for future vaccine development and virus control strategies.

Keywords: Classical Swine Fever Virus, CSFV, Immune evasion, Non-structural protein 3, NS3, Research progress, Vaccine development

Introduction

The Classical Swine Fever Virus (CSFV) is a highly contagious virus that primarily affects domestic pigs, leading to severe economic losses and the collapse of the pig farming industry. This virus belongs to the *Flaviviridae* family, and its transmission routes include direct contact, airborne spread, and indirect transmission through contaminated feed and equipment. Reports indicate that the prevalence of CSFV has not only caused significant economic losses to the pig farming industry but has also had a profound impact on the global pork supply chain [1,2]. In some countries, outbreaks have led to large-scale culling measures, further exacerbating the plight of the pig farming industry. Therefore, control measures against the swine fever virus are particularly important.

Non-structural protein 3 (NS3) is a key component in the CSFV life cycle, involved in the virus's replication and transcription processes. NS3 is not only one of the virus's main enzymes but also plays a crucial role in the interaction between the virus and host cells. Studies have shown that NS3 can suppress the host's immune response, helping the virus evade immune surveillance [3]. Furthermore, the functional and structural characteristics of NS3 make it a potential target for vaccine development and therapeutic interventions, making in-depth research on the biological properties of NS3 essential for understanding the pathogenic mechanisms of CSFV and developing effective control strategies.

The importance of studying NS3 is reflected not only in basic scientific research but also in providing new

insights for controlling swine fever outbreaks. Through in-depth research on NS3, scientists can reveal its specific roles in the virus's life cycle, thereby developing targeted interventions such as vaccines or antiviral drugs [4] *(Fig. 1)*. For example, vaccine development targeting NS3 may enhance the immune response in pigs, increasing their resistance to CSFV and thereby reducing the occurrence and spread of outbreaks. Therefore, NS3 is not only key to understanding the biology of CSFV but also an important target for formulating effective control strategies.

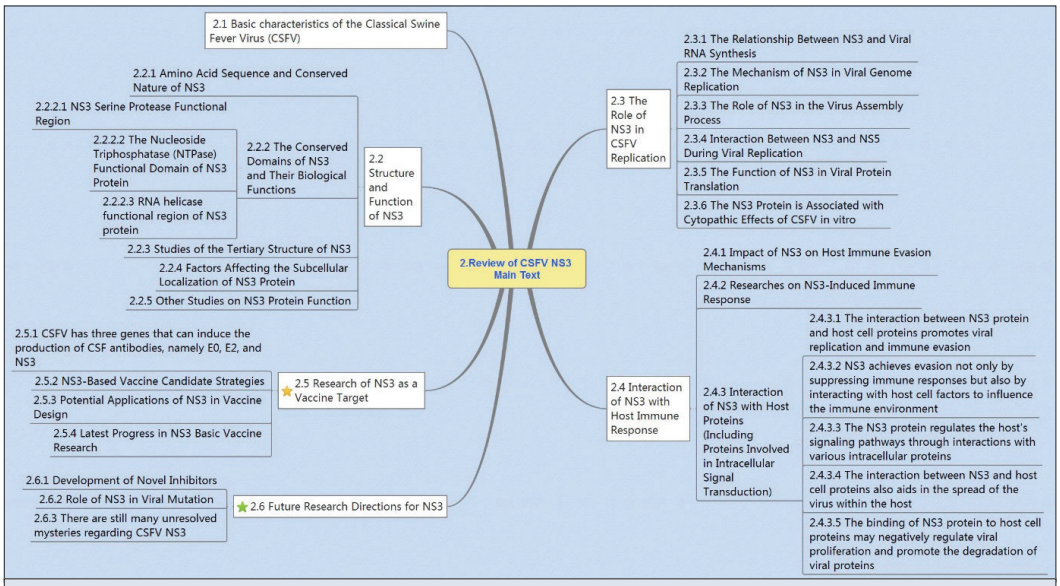

Fig 1. Study scheme on the review of NS3 protein of Classical Swine Fever Virus

Basic Characteristics of the CSFV

CSFV is an important animal virus belonging to the genus *Pestivirus* in the *Flaviviridae* family. Its genome is a positive-sense single-stranded RNA, approximately 12.300 nucleotides in length. The CSFV genome encodes a polyprotein composed of 3.898 amino acid residues, which is cleaved by cellular and viral proteases to produce 12 major protein products, including N^{pro}, C, E^{rns}, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B *(Fig. 2)*. These proteins play crucial roles in the virus's replication, assembly, and evasion of the host immune response.

The E2 and E^{rns} proteins of CSFV play a key role in the virus's immune evasion. The E2 protein is the main immunogen, capable of inducing the host to produce neutralizing antibodies, while the E^{rns} protein helps the virus escape by suppressing the host's antiviral immune response. Additionally, the p7 protein of CSFV is believed to play an important role in the virus's assembly and release process, with functions in regulating the intracellular environment and promoting the formation of the viral envelope.

The transmission routes of CSFV mainly occur through direct contact with infected pigs, contaminated environments, and biological vectors. After infection, CSFV can trigger an immune response in pigs, leading to various clinical symptoms, including high fever, reduced appetite, and bleeding tendencies, which can result in significant mortality in severe cases. This virus has caused serious economic losses globally, especially in the pig farming industry, making research and vaccine development for CSFV of significant practical importance. In recent years, researchers have focused on the variants of CSFV and their prevalence in different regions to better understand the epidemiological characteristics of the virus and to formulate effective prevention and control measures.

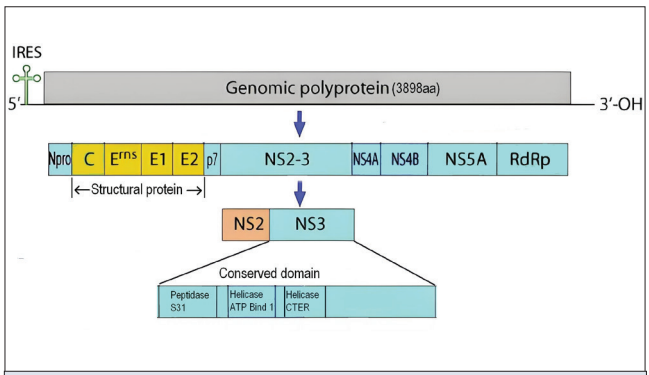

Fig 2. The genome map of CSFV. The genomic structure comprises three parts: the 5'-untranslated region (UTR) containing the internal ribosome entry site (IRES), the polyprotein open reading frame (ORF), and the 3'-UTR. In the large polyprotein ORF, Non-structural proteins are labeled in blue, and structural proteins are color-coded in yellow

Structure and Function of NS3

The NS3 protein exists mainly in two forms after classical swine fever virus (CSFV) infects host cells: as

an NS2-3 protein complex and as a monomeric NS3. The NS2-3 complex is a key molecule in the viral life cycle, particularly in the replication and assembly of viral particles [5]. According to research, the molecular weight of the NS3 protein is approximately 80 kDa, and its formation results from the cleavage of the NS2-3 protein complex by NS2. The NS3 protein plays an important role in viral replication and is also involved in the processing of precursor proteins, providing necessary conditions for viral maturation and assembly.

The NS3 protein of CSFV is a multifunctional enzyme that possesses both RNAse and protease activities. NS3 is primarily responsible for viral replication and the processing of precursor proteins, which are essential for viral maturation and assembly. Structurally, NS3 consists of two main domains: NS3a and NS3b, where NS3b is mainly associated with the replication of viral RNA, while NS3a plays a role in viral cell infection and interaction with the host immune response. The functions of NS3 are closely related to its structure, making the study of its amino acid sequence, domains, and tertiary structure crucial. The high conservation of the NS3 amino acid sequence indicates its importance in the viral life cycle. A deeper understanding of the structure and function of NS3 will aid in the development of antiviral drugs targeting flaviviruses.

The multifunctional characteristics of NS3 make it a central subject in the study of CSFV biology, and a thorough understanding of its structure and function is significant for developing antiviral strategies.

Amino Acid Sequence and Conserved Nature of NS3

The amino acid sequence of the NS3 protein exhibits a high degree of conservation among different viruses in the Flavivirus family, which is an important basis for its function. Studies have shown that the amino acid sequence of NS3 contains multiple functional regions that play key roles in the virus's life cycle. Conserved amino acid residues are not only crucial for the structural stability of NS3 but are also closely related to its enzymatic activity. For example, the serine protease activity of the NS3 protein depends on specific amino acid sequences, and the conservation of these sequences provides important evidence for the development of inhibitors targeting NS3. Furthermore, the conserved amino acid sequences may also influence the subcellular localization of NS3 and its interactions with host factors, thereby affecting viral replication and pathogenicity.

These conserved features make NS3 a potential target for vaccines and antiviral drugs, and studying the variations in its amino acid sequence and their impact on function is significant for understanding the virus's adaptability and evolution *(Fig. 3)*.

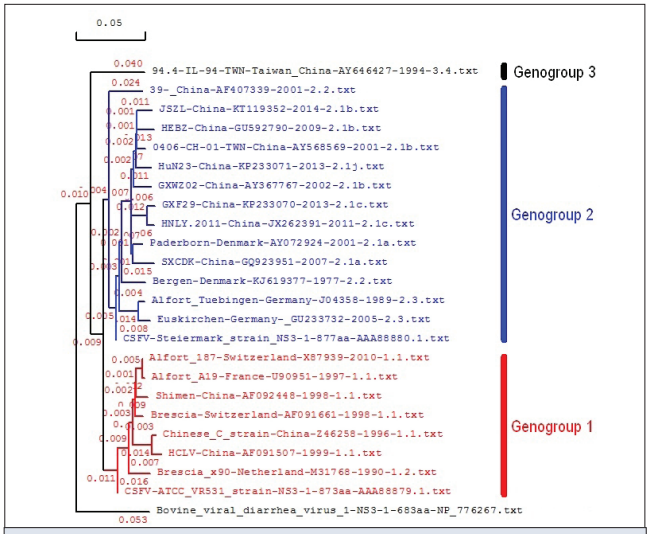

Fig 3. The phylogenetic tree created with the Maximum Likelihood method based on CSFV NS3 protein sequences from different clinical isolates. The phylogenetic tree was constructed by the Maximum Likelihood method with DNAMAN version 8 software after full sequences alignments, and the branch length indicated at the branch nodes were evaluated using 1.000 bootstrap replications. For CSFV, GenBank accessing numbers are listed after the name of each virus isolate's name and location. Besides, the Bovine Viral Diarrhea Virus (BVDV) NS3 protein sequence was used as an outgroup

The Conserved Domains of NS3 and Their Biological Functions

The functions of the NS3 protein can be divided into several domains, including the serine protease domain, the nucleoside triphosphatase domain, and the RNA-activated helicase domain. Each domain carries out specific biological functions, allowing NS3 to play multiple roles in the viral life cycle. The serine protease domain is responsible for the cleavage of viral polyproteins, the nucleoside triphosphatase domain is involved in RNA synthesis and energy metabolism, while the RNA helicase domain plays a crucial role in the replication and transcription of viral RNA [6]. These domains of the NS3 protein not only participate in viral replication and assembly but may also promote the survival and spread of the virus by regulating the immune response of host cells. The interactions and coordinated functions of these domains are essential for the efficient replication of the virus. Therefore, in-depth research on the domains of NS3 and their functions is of great significance for understanding the pathogenic mechanisms of CSFV and developing targeted therapeutic strategies.

NS3 Serine Protease Functional Region

The serine protease functional region of NS3 is one of its most critical domains, responsible for cleaving viral precursor polypeptides to generate mature viral proteins. The catalytic mechanism of this functional region involves several conserved amino acid residues, including serine, histidine, and aspartic acid, which play a central role in the

catalytic process. Studies have shown that the activity of the NS3 serine protease not only depends on the presence of these amino acids but is also influenced by their spatial conformation. Furthermore, the interaction between NS3 and its cofactor NS2B is crucial for the activity of the serine protease, as NS2B can enhance the enzymatic activity of NS3, thereby increasing the efficiency of viral replication. Additionally, the serine protease function of NS3 may also play a role in regulating the host immune response, which in turn affects the pathogenicity and transmissibility of the virus [7].

The Nucleoside Triphosphatase (NTPase) Functional Domain of NS3 Protein

The NTPase functional domain of NS3 is responsible for hydrolyzing nucleoside triphosphates, providing energy for RNA synthesis and viral replication. The activity of this functional domain is closely related to the structure of NS3, particularly its ability to bind ATP. Studies have found that the NTPase activity of NS3 is influenced by conformational changes in its domains, which affect the binding and hydrolysis efficiency of ATP [8]. Additionally, the activity of NTPase may also be related to the intracellular localization of NS3 and its interactions with other viral proteins, thereby impacting the overall viral replication capacity.

The NTPase functional domain of the NS3 protein plays a crucial role in the viral replication process. Research by Wen et al.[9] revealed the NTPase activity of NS3 under specific polynucleotide stimulation and its reaction conditions, providing important clues for a deeper understanding of the replication mechanism of CSFV. The activity of NTPase not only affects the RNA synthesis of the virus but is also related to the metabolic activities of host cells. Therefore, studying the regulatory mechanisms of NTPase can help identify new antiviral targets, providing a theoretical basis for the development of effective antiviral drugs.

RNA Helicase Functional Region of NS3 Protein

The RNA helicase functional region of NS3 is also crucial in the viral RNA replication process. The NS3 protein possesses RNase activity, enabling it to unwind RNA strands during viral replication, facilitating RNA replication and transcription. Specifically, NS3 can unwind viral RNA through its helicase activity, maintaining a single-stranded state during replication, thereby promoting RNA synthesis and translation. This process is vital for the viral life cycle, as the unwinding of viral RNA is a prerequisite for replicating and expressing the viral genome.

Research shows that the helicase activity of NS3 is influenced by the spatial configuration of its domains, and specific amino acid residues are critical for the catalytic efficiency of the helicase [10]. Additionally, the helicase activity may be regulated by the host cell environment following viral infection, and NS3 can interact with other viral proteins and host cell factors to modulate the viral replication environment. For example, the NS3 of Zika virus supports the assembly of viral replication factories by utilizing the host's antiviral RNase L protein, thereby enhancing the virus's replication capacity [11]. This complex interaction network allows NS3 to play multiple roles in viral replication.

By analyzing the interaction between NS3 and NS5B, Wen et al.[12] discovered how the helicase and NTPase activities of NS3 are regulated differently, thereby enhancing the activity of CSFV's RNA-dependent RNA polymerase in viral replication. The studies by Wen et al.[12] and Sheng et al.[13] provided potential targets for future antiviral strategy development, indicating that interventions targeting the helicase activity of NS3 may effectively inhibit viral replication.

Studies of the Tertiary Structure of NS3

The three-dimensional structure analysis of the NS3 protein reveals its complex spatial conformation and functional domains, including the protease active site and helicase active site. NS3 typically forms a functionally complete protease complex with its cofactor NS2B, which plays a key role in the virus's life cycle.

Using high-resolution X-ray crystallography and nuclear magnetic resonance (NMR) techniques, scientists have conducted detailed structural analyses of the NS3 protein from CSFV. These studies reveal the spatial conformation of NS3 and how it binds to substrates, providing a structural basis for understanding NS3's function [14].

Additionally, researchers have successfully resolved the NS3 structures of various viruses. For example, the crystal structure of the NS3-like helicase from the Alongshan virus provides an in-depth understanding of the protein's function [15]. The tertiary structure of NS3 not only showcases the interactions between its functional regions but also reveals how its activity can be regulated by altering specific amino acid residues. These studies provide an important structural basis for the design and development of specific inhibitors targeting NS3, with significant clinical application prospects [16]. Research indicates that effective inhibitors can be developed to combat viral infections by targeting specific domains of NS3 [17].

Furthermore, studies have found that the conformation of NS3 may change during transmission, which could affect the efficiency of its enzymatic activity. These three-dimensional structural studies lay the groundwork for

further exploration of NS3's role in the virus life cycle and provide important information for the development of antiviral drugs targeting NS3 [10,15].

Factors Affecting the Subcellular Localization of NS3 Protein

The subcellular localization of the NS3 protein is crucial for its function, and the factors influencing its localization include the host cell environment, the viral infection status, and the structural characteristics of NS3 itself. Studies have shown that certain regions of NS3 can interact with the internal membrane structures of host cells, thereby affecting its distribution and localization within the cell [18]. Research indicates that different localizations of NS3 within the cell may influence its enzymatic activity and interactions with other viral proteins [19]. By regulating the subcellular localization of NS3, the virus can optimize its replication and assembly processes, thereby enhancing infection efficiency. Therefore, exploring the factors that affect NS3 subcellular localization can help in understanding the pathogenic mechanisms of CSFV and provide insights for developing new antiviral strategies [20].

Other Studies on NS3 Protein Function

In addition to the aforementioned functions, the NS3 protein is also involved in multiple biological processes, including regulating the immune response of host cells and affecting cell signaling pathways. Studies have found that NS3 can inhibit the host's antiviral response by interacting with host cell proteins, thereby promoting the survival and spread of the virus [18]. Furthermore, the function of NS3 is also related to the adaptive evolution of the virus, with research indicating that mutations in NS3 may affect the virulence and transmissibility of the virus [21]. Further studies suggest that NS3 may perform different functions in different cell types, providing new insights into the complex biological characteristics of CSFV. Through in-depth research on NS3 function, it is expected that more effective antiviral treatment strategies can be developed in the future [22].

The Role of NS3 in CSFV Replication

The NS3 protein plays a crucial role in the viral replication process, especially in the replication mechanisms of flaviviruses (e.g. hepatitis C virus, HCV). Research indicates that NS3 is not only one of the main enzymes of the virus but also participates in the synthesis and processing of viral RNA.

The Relationship Between NS3 and Viral RNA Synthesis

The non-structural protein NS3 of classical swine fever virus (CSFV) plays a vital role in viral replication. Research by Sheng et al.[13] shows that NS3 is closely related to viral RNA synthesis, particularly its interaction with the viral 3' non-coding region, which is considered crucial for the replication of the CSFV genome. Studies indicate that NS3 can promote RNA synthesis by interacting with NS5, which acts as an RNA polymerase and can efficiently synthesize viral RNA with the assistance of NS3. This interaction is essential for viral replication, as RNA synthesis is one of the core steps in the viral life cycle [23]. Additionally, the helicase activity of NS3 helps to unwind RNA secondary structures, providing a template for the RNA polymerase, further enhancing its importance in viral replication. Therefore, exploring the relationship between NS3 and viral RNA synthesis contributes to understanding the replication mechanism of CSFV and provides a theoretical basis for developing antiviral strategies against CSFV [13].

The Mechanism of NS3 in Viral Genome Replication

The role of NS3 in viral genome replication is relatively complex, involving multiple steps and interactions. First, NS3 interacts with NS5 (RNA polymerase) to form an effective replication complex, thereby enhancing the efficiency of RNA synthesis [23]. Studies have shown that structural changes in NS3 play a key role in RNA binding and catalytic reactions, effectively regulating the replication and transcription of viral RNA. In addition, NS3 alters the lipid environment within host cells by cleaving relevant host cell enzymes, providing the necessary conditions for viral RNA replication. During this process, NS3 forms a complex with other non-structural proteins (such as NS2B) to participate in RNA synthesis and processing, ensuring the integrity and replication efficiency of the viral genome. In summary, NS3 is not only an important enzyme in viral genome replication but also forms a complex regulatory network through interactions with other proteins to ensure effective viral replication and transmission.

The Role of NS3 in the Virus Assembly Process

NS3 plays an important role in the assembly process of CSFV. Research has found that NS3 participates in the formation and release of viral particles through interactions with other non-structural proteins. Specifically, the surface structure of NS3 can interact with viral membrane proteins, promoting the maturation and assembly of viral particles. This interaction not only affects the morphology of the virus but may also influence its infectivity. Therefore, the function of NS3 is indispensable in the virus's life cycle. Additionally, the endogenous self-cleavage characteristics of NS3 allow for functional separation. A study by Lamp et al.[7] revealed that the autocatalytic cleavage process in NS3 produces fragments with enzymatic activity, which are crucial for viral replication and assembly. This process

not only affects the structural integrity of the virus but may also regulate the efficiency of viral assembly. The biological significance of the self-cleavage event provides a new perspective on the multifunctional polymerase of the Flaviviridae family. These findings emphasize the multiple functions of NS3 in the viral life cycle, particularly its importance in the processes of viral assembly and maturation, making it an important target for studying viral assembly mechanisms and providing potential targets for future vaccine and antiviral drug development.

Interaction Between NS3 and NS5 During Viral Replication

There is a significant interaction between NS3 and NS5B during the replication of CSFV. A study by Xiao et al.[24] found that the full-length form of NS3 enhances IRES-mediated translation more effectively than the truncated form, demonstrating the key role of NS3 in viral proliferation. Furthermore, NS5B can significantly promote the stimulatory effect of NS3 on translation, providing new insights into the understanding of viral proliferation mechanisms. Sheng et al.[25] further explored the importance of NS3, NS5A, and NS5B in CSFV replication through mutation and complementation analysis, revealing the critical interaction between NS3 and NS5B. Wang et al.[26] indicated that NS3 binds to NS5B through its protease domain, enhancing the activity of the RNA-dependent RNA polymerase, highlighting the important function of NS3 in the CSFV replication life cycle. Wang et al.[27] identified two NS3 binding sites by deleting the terminal sequences of NS5B and explored how this interaction enhances the RNA-dependent RNA polymerase activity of NS5B. These studies provide direction for the development of antiviral drugs, revealing the complex interactions between NS3 and NS5 and their importance in viral replication.

The Function of NS3 in Viral Protein Translation

NS3 also plays an important role in viral translation. Research by Deng et al.[20] showed that NS3 can affect the translation efficiency of viral RNA through interactions with translation-related factors, thereby influencing the expression levels of viral proteins. This regulatory mechanism is crucial for the survival and reproduction of the virus. Additionally, a study by Zhu et al.[2] indicated that NS3 is an IRES-binding protein that enhances IRES-mediated translation by binding to the CSFV IRES. This mechanism suggests the importance of NS3 in viral replication, indicating that it not only participates in RNA synthesis but also directly affects the translation process of viral proteins. The IRES-binding characteristics of NS3 may provide the virus with an advantage for efficient translation within host cells, thereby promoting rapid viral proliferation. This finding offers new insights into the replication mechanism of CSFV and provides potential targets for future therapeutic strategies.

The NS3 Protein is Associated with Cytopathic Effects of CSFV *In vitro*

The NS3 protein plays a key role in the cytopathological characteristics of CSFV. A study by Aoki et al.[28] revealed that the accumulation of NS3 is closely related to the occurrence of cytopathic effects (CPE) in cell cultures, especially during infections in serum-free media. The degree of cytopathic effects is positively correlated with the expression level of NS3, indicating that NS3 may directly or indirectly act on important host cell proteins, leading to cell damage. Research by Xu et al.[29] further confirmed that the expression of NS3 is closely related to significant changes in cell morphology and increased apoptosis rates, providing important clues for understanding the pathogenic mechanism of CSFV and the formation of persistent infections. The study by Xu et al.[29] revealed the core role of NS3 in inducing apoptosis and cytopathic effects by transfecting the classical swine fever virus NS3 gene. The results showed that the expression of the NS3 protein is closely related to significant changes in cell morphology and increased apoptosis rates, affecting the cytopathic effects in PK-15 cells. This helps to understand the pathogenic mechanism of the swine fever virus and the formation mechanism of persistent infections. NS3 and NS2 can be detected in cells infected with cytopathic strains of the virus, while non-cytopathic strains only express the NS2-NS3 polyprotein in their infected host cells. Therefore, the non-structural protein NS3 can serve as a specific marker protein for cytopathic classical swine fever virus at the protein level. NS3 may directly or indirectly act on important host cell proteins, leading to cell damage. In cells infected with cytopathic CSFV, the more pronounced the cytopathic effects, the higher the detected NS3 content, indicating a close relationship between NS3 and the occurrence of CPE. As a specific marker protein for cytopathic classical swine fever virus at the protein level, the role of NS3 in the occurrence of CPE provides a new research direction for vaccine development.

Interaction of NS3 with Host Immune Response

Impact of NS3 on Host Immune Evasion Mechanisms

The NS3 protein plays a key role in *Flaviviridae* infections, interfering with the host's immune response through various pathways, allowing the virus to persist and spread within the host. For example, the HCV NS3/4A complex can cleave critical immune regulatory factors, disrupting cytokine signaling pathways. Relevant studies indicate that

NS3/4A can reduce the expression of pro-inflammatory cytokines by inhibiting the activation of NF-κB, thereby weakening the host's inflammatory response and antiviral immune response [30]. In the case of Zika virus (ZIKV), NS3 can also enhance the virus's immune evasion capability by interacting with intracellular adaptor proteins MAVS and MITA to inhibit the production of interferons [31]. Additionally, HCV NS3 has been found to promote T cell exhaustion, leading to a weakened immune response in chronic HCV infection patients, which in turn affects the ability to clear the virus [32]. Cao et al.[22] constructed a lentiviral vector expressing CSFV non-structural proteins and infected porcine monocyte-derived macrophages (pMDMs), finding that NS3 significantly downregulated the expression of Toll-like receptors (TLRs). This finding suggests that NS3 may help the virus escape host immune surveillance by inhibiting the activation of TLR signaling pathways, thereby interfering with the initiation of the innate immune response. Understanding these immune evasion mechanisms is crucial for developing new vaccines and therapeutic strategies, as it reveals how viruses exploit the host's immune system to promote their own replication and spread.

Researches on NS3-Induced Immune Response

The NS3 protein plays an important role in the immune response of pigs to classical swine fever. Research by Rau et al.[33] indicated that recombinant NS3 protein can effectively induce the production of CD8+ cytotoxic T cells, suggesting that NS3 may serve as an effective immunogen. A study by Vazquez et al.[34] found that NS3 enhances the activation of specific T cells by promoting antigen presentation and cytokine production through the activation of dendritic cells (DCs) and macrophages. Using recombinant vaccines and natural adjuvants, Hajikhezri et al.[35] observed that HCV NS3 antigen could effectively activate T cell responses, inducing a strong Th1-type cytokine response. Furthermore, Pouriayevali et al.[36] found that the immunogenicity of HCV NS3 is influenced by its conformation and modification state; optimizing the expression and purification conditions of HCV NS3 can further enhance the strength and durability of the induced immune response. These studies provide experimental evidence for further exploring the potential of NS3 as a vaccine component, particularly in how to enhance the immunogenicity of NS3 during vaccine development, which will be an important research direction.

Research by Voigt et al.[37] found that although vaccination with NS3 protein can stimulate the immune response in piglets, it does not provide effective protection against lethal CSFV challenge infection, indicating that further optimization of the delivery system is needed in vaccine design to enhance its protective effect. Nevertheless, in both *in vivo* and *in vitro* experiments, the combination of NS3 with E2 protein showed good immunogenic effects, potentially providing a breakthrough for the development of new vaccines [33]. These studies provide important experimental foundations for understanding the role of NS3 in immune responses and for vaccine development.

Interaction of NS3 with Host Proteins

The Interaction Between NS3 Protein and Host Cell Proteins Promotes Viral Replication and Immune Evasion

Research by Lv et al.[38] found that the interaction of CSFV NS3 protein with TRAF5 not only promotes viral replication but also affects the host's immune response by activating the p38 MAPK pathway. Additionally, TRAF6 has been identified as an inhibitor of CSFV replication; Lv et al.[39] found that TRAF6 inhibits CSFV replication by enhancing the activity of the NF-κB signaling pathway. These studies reveal the complex interplay between NS3 and host cell signaling pathways, providing new insights into the immune evasion mechanisms of CSFV.

NS3 Achieves Evasion Not Only by Suppressing Immune Responses but Also by Interacting with Host Cell Factors to Influence the Immune Environment

For example, the NS3 of Zika virus can inhibit the activation of the NLRP3 inflammasome, thereby reducing the release of pro-inflammatory cytokines, a mechanism that allows the virus to evade immune surveillance within the host [40]. Furthermore, NS3 may also affect the host's immune response by regulating the expression of cytokines. For instance, research by Latanova et al.[41] showed that HCV NS3 can increase the secretion of interleukin-1β, which may be related to its regulation of the host immune environment. This regulatory effect not only affects the host's immune response but may also create favorable conditions for the virus's persistent infection. Therefore, the role of NS3 in host immune evasion is multifaceted, highlighting its importance as a potential therapeutic target.

The NS3 Protein Regulates the Host's Signaling Pathways Through Interactions with Various Intracellular Proteins

For example, research by Cao et al.[42] demonstrated that Japanese Encephalitis Virus NS3 can interact with intracellular heat shock proteins (such as Hsp40), affecting viral replication and the host cell's stress response. Additionally, research by Abdullah et al.[30] found that HCV NS3 can interact with various kinases, regulating their activity and thereby influencing cell proliferation and apoptosis. These interactions not only support the survival of the virus but also interfere with the host's

immune response, forming a complex network of virus-host interactions.

The Interaction Between NS3 and Host Cell Proteins Also Aids in the Spread of the Virus Within the Host

The interaction of NS3 with host cell proteins not only affects the immune response but also plays a crucial role in viral transmission. Research by Silva et al.[18] indicates that Dengue virus NS3 can inhibit the enzymatic activity of host cell glycolytic enzymes (such as GAPDH) through interaction, thereby affecting cellular energy metabolism and promoting viral replication and spread. Additionally, the study by Palacios-Rápalo et al.[43] shows that the nuclear localization of Dengue virus NS3 may also influence the assembly and release of viral particles in human host cells (Huh7 cells), further enhancing its transmission capability within the host. Therefore, in-depth research on the interaction mechanism between NS3 and host proteins is of great significance for understanding the biological characteristics of the virus and developing new antiviral strategies.

The Binding of NS3 Protein to Host Cell Proteins May Negatively Regulate Viral Proliferation and Promote the Degradation of Viral Proteins

For example, Deng et al.[20] found that the host cell protein PSMB10 can interact with NS3 protein, inhibiting the proliferation of CSFV and mediating the degradation of NS3 through the ubiquitin-proteasome pathway. This finding not only reveals the complex interactions between the virus and the host but also provides new targets for the prevention and control of CSF. These studies emphasize the importance of NS3 in the viral transmission process, and further research will help uncover its specific mechanisms in host immune evasion and viral spread.

Research of NS3 as A Vaccine Target

CSFV has Three Genes That Can Induce the Production of CSF Antibodies, Namely E0, E2, and NS3

Among them, E0 and E2 proteins induce the body to produce protective antibodies, while NS2-3 antibodies do not have virus neutralization activity. CSF is an important viral disease that has severely impacted the pig farming industry. Studies have shown that the E0 and E2 proteins of CSFV can effectively induce the body to produce protective antibodies, thereby providing immune protection. However, as a non-structural protein, the antibodies produced by NS3 have a weaker ability to neutralize the virus, limiting its application in vaccine development. Nevertheless, NS3 remains an important research subject because it plays a key role in the virus's replication and infection processes. An effective vaccine needs to combine multiple antigens to maximize the immune system's response, so in-depth research on NS3 will help understand its potential role in vaccine design, providing new ideas and strategies for future vaccine development.

NS3-Based Vaccine Candidate Strategies

The NS3 protein is an important non-structural protein of various viruses, such as the hepatitis C virus and dengue virus, and has become a popular target for vaccine development due to its critical role in viral replication and pathophysiological processes. Studies have shown that NS3-based vaccine strategies can effectively induce specific immune responses. For example, using a necrotic dendritic cell vaccine expressing HCV NS3 can significantly enhance T cell responses, demonstrating good immunogenicity and protective effects. Additionally, DNA vaccines expressing HCV NS3 protein have also shown enhanced cellular immune responses in mouse models, providing strong support for NS3 as a vaccine target. Combining different immune adjuvants, such as the N-terminal heat shock protein gp96, can further improve the immune response of HCV NS3 vaccines, inducing strong Th1-type cytokine production [35]. These studies indicate that NS3-based vaccine candidate strategies have great potential in eliciting cellular immunity and antiviral protection.

Due to the importance of NS3, researchers have targeted it for vaccine development. In the development of existing QS vaccines and genetically engineered vaccines, NS3 is considered a potential protective antigen. Scientists hope to enhance the effectiveness of vaccines by improving the host's immune response to NS3. There have been several successful cases in the research of NS3 as a vaccine target. For instance, using modified vaccine vectors to express NS3 antigens has shown enhanced immune responses and better protective effects. Furthermore, researchers have explored combining different adjuvants and immune enhancers to improve the specific immune response to NS3. Through these strategies, researchers aim to develop more effective vaccines to combat CSFV and other related viruses [36].

Potential Applications of NS3 in Vaccine Design

The NS3 protein plays a crucial role in the immunogenicity of vaccines. Firstly, the Zika virus ZIKV NS3 can act as a potent T cell antigen, inducing the activation and proliferation of CD8+ T cells, which is essential for clearing viral infections. Secondly, HCV NS3 also exhibits good immunogenicity and antigen presentation capability, able to combine with various immune adjuvants to enhance the strength and durability of the immune response [36]. Thirdly, the use of a combined

vaccine strategy with modified NS1 and NS3 has shown significant immune enhancement effects, providing new ideas for the development of vaccines against dengue virus. In addition, the structural features and functional characteristics of NS3 make it an ideal candidate for designing multivalent vaccines that can target multiple viral strains simultaneously. In summary, NS3 is not only an important component of viral replication but also a significant target in vaccine development, and its role in immunogenicity ensures the effectiveness of vaccines.

Progress in NS3 Basic Vaccine Research

What is the potential of the NS3 protein in expression and surface display in insect host cells? Xu et al.[44] successfully displayed the CSFV NS3 protein on the viral envelope using a modified BACBd virus system, significantly enhancing antibody production in mouse models, providing a solid foundation for novel vaccine strategies. This study indicates that displaying CSFV NS3 through modified viral vectors can effectively stimulate the host's immune response. Additionally, researchers are exploring other expression systems and strategies to further enhance the immunogenicity and protective effects of NS3. For example, using natural adjuvants and cell-penetrating peptides as carriers simultaneously can improve the stability and biocompatibility of NS3, thereby enhancing the overall efficacy of the vaccine [45]. These studies provide new directions for the application of NS3 as a vaccine target and lay the groundwork for future vaccine development.

Future Research Directions for NS3

Development of Novel Inhibitors

NS3 protease is a key enzyme in the replication process of various viruses, such as the hepatitis C virus and dengue virus. Therefore, developing novel inhibitors targeting NS3 has significant clinical implications. In recent years, researchers have identified a series of promising new small molecule inhibitors through virtual screening and structural biology methods. For example, studies have shown that consensus scoring-based virtual screening can effectively discover inhibitors targeting the NS3 protein, which exhibit good inhibitory activity *in vitro* [46]. Additionally, the development of inhibitors against HCV NS3 is not limited to small molecules; biopharmaceuticals such as monoclonal antibodies have also shown potential applications in research [47]. Future research could focus on optimizing the pharmacokinetic properties of these inhibitors to enhance their efficacy and safety in clinical applications. Furthermore, combining computational drug design with high-throughput screening technology may accelerate the discovery and development of novel NS3 inhibitors.

Role of NS3 in Viral Mutation

The NS3 protein plays multiple roles in the viral life cycle, particularly in viral mutation and adaptation. Research indicates that NS3 is involved not only in viral protein processing and replication but may also influence the virus's ability to withstand adversity, such as the development of drug resistance [48]. In the case of the hepatitis C virus, mutations in NS3 can lead to resistance to protease inhibitors, posing challenges for antiviral therapy [49]. Moreover, mutations in NS3 may also affect its interactions with host cell factors, thereby altering the virus's pathogenicity and transmission capabilities [50]. Future research should focus on the mechanisms of NS3 mutations and their impact on viral adaptability, which will provide an important theoretical basis for developing new therapeutic strategies. Additionally, monitoring NS3 mutations can guide vaccine design and public health interventions to address the challenges posed by viral mutations.

There are Still Many Unresolved Mysteries Regarding CSFV NS3

Future research needs to further explore the specific mechanisms of NS3 in the interaction between the virus and the host, as well as the differences in immune responses among different pig breeds. Furthermore, developing novel vaccines based on NS3 and assessing their safety and efficacy in practical applications will be key focuses of future research.

Conclusion

Generally speaking, the non-structural protein 3 (NS3) of the CSFV plays a central role in viral biology, host immune response, and vaccine development *(Table 1)*. With a gradual deepening understanding of NS3's functions, we have made significant progress in the field of CSFV research, particularly in elucidating its role in viral replication, host immune evasion mechanisms, and vaccine design. However, despite numerous findings, there remain many unresolved mysteries related to NS3, presenting new challenges for future research.

Future research directions should focus on revealing the specific mechanisms of NS3 in the interactions between the virus and the host, as well as exploring the differences in immune responses among different pig breeds. This not only helps us understand how the virus affects the host's immune response but also provides a foundation for developing more targeted vaccines. Additionally, the development of novel vaccines based on NS3 will be an important approach to addressing CSFV outbreaks. Researchers need to strengthen the design of preclinical and clinical trials to ensure the safety and efficacy of new vaccines, thereby ensuring the sustainable development of the pig industry.

Table 1. *Summary of Classical Swine Fever Virus (CSFV) NS3 protein's functions*

Category of NS3 Function	Functional Description	References
Regulation of viral translation	NS3 regulates viral RNA translation, affecting viral protein expression levels	[2]
Protease activity	NS3 exhibits serine protease activity, involved in cleaving viral polyproteins, crucial for viral replication	[5]
Virus assembly	NS3 is involved in viral particle assembly, affecting virus morphology and infectivity	[7]
NTP enzyme activity	NS3 has NTPase activity, providing energy for RNA synthesis	[9]
Three-dimensional(3D) structural characteristics of the protein	NS3 contains catalytic triad (His-1663, Asp-1686, Ser-1694), and its resolved 3D structure aids drug design	[14,15]
RNA helicase activity	NS3 possesses RNA helicase activity, able to unwind secondary structures of viral RNA, facilitating RNA replication and transcription	[23]
Association of NS3 protein with the pathogenicity of CSFV	NS3 mutations may alter viral replication efficiency in host cells, correlating with virulence	[25,50]
Formation of replication complexes	NS3 interacts with proteins like NS5B to form replication complexes, enhancing RNA-dependent RNA polymerase activity.	[26]
Regulation of apoptosis	NS3 accumulation in infected cells may affect viral pathogenicity by modulating apoptosis	[29]
Immunogenicity differences	NS3 induces host antibody production, but its epitopes are less conserved than E2, affecting detection specificity	[32,36]
Immune evasion	NS3 suppresses host immune responses through various mechanisms, aiding viral immune evasion	[45]

When balancing different research perspectives and findings, we should fully consider factors such as experimental design, sample selection, and data analysis. In some studies, there may be differences in the interpretation of NS3 functions, especially under different experimental conditions or pig breed backgrounds. Therefore, focusing on multi-center, large-scale collaborative research will help integrate various research results and form more comprehensive and consistent conclusions.

In conclusion, research on NS3 not only provides us with an opportunity to gain a deeper understanding of CSFV biology but also offers new ideas for vaccine development and improvements in control strategies for the pig industry. We look forward to achieving greater breakthroughs in these areas to address the ever-changing viral challenges.

Highlight Keypoints

- CSFV NS3 has three conserved domains in biology, including serine protease, nucleoside triphosphatase (NTPase), and RNA helicase functional regions.
- NS3 plays a key role in the replication, pathogenicity, and immune evasion of CSFV.
- NS3 interacts with the host cells immune response.
- NS3 can serve as a vaccine target and has potential applications in vaccine design.

Declarations

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Review Article

Comprehensive Review of Fowl and Duck Adenovirus Vaccines Development: Innovations, Challenges, and Future Directions

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Abstract

Fowl adenovirus (FAdV) and Duck adenovirus are key poultry pathogens, causing inclusion body hepatitis, hydropericardium syndrome, Egg Drop Syndrome (EDS), and sudden mortality in broilers, layers, and ducks. These pathogens contribute significantly to economic losses in the global poultry industry. Consequently, measures such as vaccine development to control and prevent these agents have been extensively researched, with recent advancements showing promise. This review discusses recent advancements in vaccines for avian adenovirus species, challenges faced in studies, and future directions for developing effective vaccines against these viruses. Our study highlights that research has focused on 2nd (subunit) and 3rd (recombinant viral vector) generation vaccines, which combine multiple immunogenic proteins for single-shot protection against various avian diseases. Studies show that capsid proteins, particularly fiber, provide the highest protection rates, with reduced viral shedding and clinical signs in poultry. Significant discrepancies exist among studies evaluating vaccines for poultry due to variations in bird type, age, challenge strains, vaccine strains, dosage, administration frequency, small sample sizes, and unexamined immune responses or pathogenic mechanisms. These challenges hinder optimal vaccine identification, as many fail to protect chickens fully. Future studies should focus on real-life testing, FAdV infection mechanisms, and passive immunity transfer to progeny post-immunization.

Keywords: Duck adenovirus (DAdV); Fowl adenovirus (FAdV); immunity; poultry; vaccine

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Introduction

The Fowl Adenovirus (FAdV) belongs to the genus Aviadenovirus of the Adenoviridae family. FAdV is a non-enveloped DNA virus with icosahedral symmetry. Fowl adenovirus (FAdV) is a non-enveloped, double-stranded DNA virus composed of three major structural proteins: hexon, penton, and fiber [1]. Among 12 serotypes of fowl adenovirus (FAdV), FAdV-1, FAdV-4, and FAdV-10 both carry two fiber genes (i.e., fiber-1 and fiber-2), whereas other serotypes have only one [2].

High amino acid variability in the fiber protein, particularly in the head domain or knob region, leads to binding with different receptors [3]. The knob region of the fiber protein contains a significant portion of the antigenic site across all serotypes and includes a type-specific epitope for antibody neutralization [1,4].

Fowl adenoviruses (FAdVs) are classified into five species (A-E) and 12 serotypes. Serotypes 2, 11, 8a, and 8b are linked to inclusion body hepatitis, while serotype 4 is primarily associated with Hydropericardium syndrome. Species classifications follow ICTV nomenclature based on serotype groupings [5,6].

Hydropericardium syndrome (HPS), also known as hepatitis hydropericardium syndrome (HHS), affects chickens and was first reported in Pakistan 35 years ago [7]. Caused by virulent FAdV-4, HPS leads to amber-colored

fluid accumulation in the pericardial sac and an enlarged liver with hemorrhages or necrosis [8]. HPS outbreaks in Asia and Latin America cause major economic losses, including up to 80% mortality, reduced productivity, and the need for antibiotics due to adenovirus-induced immunosuppression [6,9-13].

Over the past two decades, the number of IBH outbreaks has risen across various geographic regions, highlighting the global spread of the disease. IBH affects broilers up to five weeks of age in the field, though sporadic cases have also been observed in layers and broiler breeders. Mortality during IBH outbreaks typically peaks within 3-4 days, reaching up to 10% and, in some instances, as high as 30% [6,14].

Vaccination is the most effective method for control and prevention of the disease, either by horizontal or vertical transmission in poultry farms [1,6,15]. Fowl adenovirus (FAdV) significantly impacts poultry due to mortality and treatment costs. Despite vaccines, poor cross-protection, incomplete efficacy, and emerging strains demand improved solutions. Technological advancements, including recombinant and vector-based platforms, offer innovation opportunities. A global approach is essential to address disparities in vaccine development, distribution, and accessibility, guiding future strategies.

First-Generation Vaccines (Conventional Vaccines)

1. Live Attenuated Vaccines

Fowl Adenovirus Species A

Adenoviral gizzard erosion (AGE), caused by FAdV-1, has been linked to significant economic losses in broiler flocks due to growth retardation and reduced slaughter weight [15]. AGE has also been reported in broilers infected with FAdV-8a and -8b [16,17]. Recent outbreaks in pullets and layers, especially in cages or alternative systems, have led to increased mortality and decreased egg production or weight [18-21]. These outbreaks in layer-type chickens are attributed to FAdV-1 infections, confirmed by virus detection and experimental reproduction in SPF birds [18,20,21].

Recently, the development of efficacious protection against the disease due to live vaccination with an apathogenic FAdV-1 was demonstrated in broilers [22]. However, the recent increase of reported AGE cases in layers and documented economic losses in natural outbreaks indicate the need for an efficacious protection strategy in older birds. Therefore, a study by Grafl et al.[18] produced a live-attenuated vaccine against AGE.

The study demonstrated that a live vaccine prevents symptoms and gross pathological changes in the gizzards. Additionally, no negative impacts on the development of the reproductive tract were observed in pullets and layers at 20 weeks of age. In vaccinated groups with single dose and double dose, homologous antibodies were detectable starting one week post-vaccination, with peak titers averaging 10.4±2.1 $\log_2$ at 0 days post-challenge (DPC) and 9.7±2.5 $\log_2$ at 7 DPC, indicating robust immune responses *(Table 1)* [18].

Fowl Adenovirus Species C

After identifying Fiber-2 as a critical factor in FAdV-4 pathogenicity, several studies explored the development of Fiber-2 recombinant vaccines [60,80,81]. Concerns over FAdV-4 vaccine efficacy and antibody detection led to development a live-attenuated vaccine. Serial passages of the virulent FAdV-4 KNU14016 strain in LMH cells reduced pathogenicity, evidenced by delayed and absent cytopathic effects after the 20th and 100th passages. Genetic analysis revealed a C-base insertion at 39,197 bp and a 26-bp sequence shortening in LMH80, supporting vaccine attenuation and improved delivery methods. Chickens injected with LMH10 had 89% mortality within five days and high viral shedding. LMH80 showed delayed mortality (starting at day 9) and minimal shedding. Different administration routes for LMH80 provided protection, with oral and intramuscular groups showing no mortality *(Table 1)* [40].

Recent research suggests that local immune responses triggered by vaccination play a crucial role rather than neutralizing antibodies [44]. However, the vaccine-induced cellular immune response and the involvement of various immune genes have not been fully explored. Thus, another study aimed to enhance understanding of the immune response elicited by live-attenuated viruses and the role of attenuated vaccines in immune defense during avian viral infections [69].

Most immunized animals were observed to survive and remain active, whereas LMH10 infection proved fatal in the absence of LMH80 pre-treatment. The immunization with LMH80 was shown to influence viral clearance, as the virus was detected less frequently among immunized animals, which corresponded with their higher survival rate *(Table 1)*. Moreover, the residual virus levels in the immunized group were determined to be lower than those in the control group.

LMH80 immunization boosted CD44+ expressing CD8+ T cells regardless of FAdV-4 infection. Helper T cells expanded significantly only after both immunization and viral challenge. Total T (CD3+) and γδ-T cell populations remained unchanged. Monocytes expressing MHCII and secreting cytokines increased upon FAdV-4 exposure, despite no pre-immunization monocyte expansion.

Table 1. Fowl adenovirus and duck adenovirus vaccines development in worldwide

No.	Country	The Type of Animal Studied	Syndrome	Serotype of FAdV	Type of Vaccine	Route of Administration and Dose	Protection Rate	Ref.
1	India	144-day-old Cobb broiler chicks	HHS (hepatitis-hydropericardium disease), IBH (Inclusion body hepatitis)	FAdV4 and FAdV11	Not mentioned	Subcutaneous (SC); 10^7 $TCID_{50}$ of FAdV serotype 4 and 11	Against FAdV serotype 4 and 11 was 100%	[5]
2	Austria	20-week-old specific-pathogen-free (SPF) layer-type chickens.	AGE (Adenoviral gizzard erosion)	FAdV1,	Live- attenuated	Oral; 0.5 mL of an apathogenic FAdV-1 (CELO strain)	Not mentioned 100% as no chicken died or revealed clinical signs.	[18]
3	Japan	2 weeks old SPF chickens	Hydropericardium syndrome (HPS)	FAdV-4	Recombinant subunit	SC; 100 µg of recombinant fiber-1 protein in 100 µL of PBS with incomplete Freund's adjuvant and boosted with the same preparation 14 days after the first immunization	Not mentioned, but all chickens showed no signs and gross anatomical changes (100%).	[12]
4	Malaysia	chicken embryo liver (CEL) cells adapted	-	Serotype 8b	-	-	-	[23]
5	Malaysia	SPF chickens	IBH	FAdV8b	FAdV8b attenuated vaccine	SC and oral; $10^{6.7}$ $TCID_{50}$/mL for SC and 0.1 mL of $10^{6.7}$ $TCID_{50}$/mL for oral immunization	100% of vaccinated chickens survived and did not develop any clinical symptoms.	[1]
6	Malaysia	day-old commercial broiler chicks, Cobb500	IBH	8b	Inactivated vaccine using binary ethyleneimine (BEI)	SC; vaccine formulated with Montanide ISA 71 VG at vaccine dose $10^{11.5}$ $TCID_{50}$/mL	100% of vaccinated chickens did not die or show signs and symptoms.	[24]
7	China	2-week-old specific pathogen-free (SPF) chickens	HHS	4	Recombinant vector vaccine (live attenuated)	Intramuscular inoculated with $2.5x10^4$ $TCID_{50}$ of the indicated virus in 200 mL of culture medium	100%	[25]
8	China	32- week-old SPF chickens	HHS, IBH	Recombinant	Recombinant vector vaccine from rR188I mutant strain	Intramuscular (IM); 10^5 PFU (plaque forming unit) of the indicated viruses and monitored daily for 1 week	100%	[26]
9	Iran	Chicken embryonated eggs	Egg drop syndrome (EDS)	*Duck Atadenovirus A*	-	-	-	[27]
10	China	Leghorn male Hepatocellular cells	IBH	FAdV- 11	-	-	-	[28]
11	Pakistan	Commercial broiler chickens	HPS (hydropericardium syndrome)	FAdV serotype 4	Subunit vaccine	SC; fiber-2 protein (25 µg/mL dissolved in PBS and emulsified in Freund's complete adjuvant (FCA))	80%	[29]
12	Austria	14-day old SPF chickens	IBH	FAdV serotype 8	Recombinant vaccine	IM; 50 µg of crecFib-8b/8a with 40% (wt/vol) antigen-oil-based adjuvant phase GERBU adjuvant	100%	[30]
13	Austria	Day-old SPF chickens	IBH	FAdV-7 and 8b	Recombinant subunit vaccine	IM; For the Pb-7 study, 50 µg of Pb-7 was combined in a 1:1 ratio with GERBU adjuvant P. In the Pb-8b study, a booster vaccination was administered at 25 days of age, consisting of 100 µg of Pb-8b mixed with Freund's incomplete adjuvant	64.3%	[31]
14	China	Babcock SPF chickens aged 2 weeks	HHS, IBH	FAdV4 and FAdV8a)	Recombinant chimeric vector virus vaccine	IM; $1x10^6$ $TCID_{50}$ of the recombinant virus FAdV4-F/8a-rF2	100% against both FAdV-4 and -8a	[32]

Table 1. *Fowl adenovirus and duck adenovirus vaccines development in worldwide*

No.	Country	The Type of Animal Studied	Syndrome	Serotype of FAdV	Type of Vaccine	Route of Administration and Dose	Protection Rate	Ref.
15	China	7 day-old chickens seronegative for FAdv-4	HHS	FAdV-4	rFiber2 subunit vaccine (which was mixed with an oil adjuvant) and Fiber2 DNA vaccine candidates	IM ($10^{6.75}$ $TCID_{50}$/0.1 mL)	rFiber2 subunit vaccine: 100% and Fiber2 DNA vaccine: 60%	[33]
16	India	7-day-old SFP chickens	IBH-HHS	Serotype 2/11	Recombinant fiber protein of FAdV-2/11	For dose-effect study: Subcutaneous; (10 µg/bird (group I), 25 µg/bird (group II), 50 µg/bird (group III), 75 µg/bird (group IV)) of recombinant fiber protein with Freund's complete adjuvant (FCA) (200 µL + 200 µL) For adjuvant effect study: 25 µg IM of (montanide (antigent to adjuvant ratio 3:7), resiquimod (1:2), and saponin (1:1)) and SC for FCA (1:1)	Dose effect study: 95% protection for all groups; adjuvant effect study: 100% protection for all groups	[34]
17	China	2-weeks old SPF chickens	HHS, swollen and hemorrhagic liver and kidney in ducks	FAdV-4 and DAdV-3	Recombinant viral vector vaccine rFAdV-4-Fiber-2/DAdV-3-RFP	The route of administration was not mentioned; $2x10^6$ $TCID_{50}$/200 µL	100%	[35]
18	Malaysia	1-day-old commercial broiler chickens	IBH	FAdV-8b	Inactivated vaccine	0.5 mL inactivated FAdV isolate of UPM08136P5B1 ($10^{7.5}$ $TCID_{50}$/mL)	100%	[36]
19	China	Two-week-old SPF chicken	HHS	FAdV-4	Recombinant vector vaccine live rHN20, rDL3-EGFP, or rHN20-EGFP, and rHN20-vvIBDV-VP2 (EGFP: Enhanced Green Fluorescent Protein) and Recombinant rHN20-vvIBDV-VP2	IM; 200 µL	100 %	[37]
20	Malaysia	Day-old broiler chickens	IBH	FAdV-8b	Attenuated	SC; 0.5 mL of UPM08136CEL20B1 $10^{6.5}$ $TCID_{50}$	100%	[38]
21	China	one-day-old SPF chickens	HHS	FAdV-4	Recombinant vector live-attenuated vaccine virus FA4-EGFP expressing EGFP-Fiber-2 fusion protein	IM 10^6 $TCID_{50}$ of the indicated virus in 200 µL of 1% culture medium	100%	[39]
22	South Korea	3-days old SPF chickens	HHS	FAdV-4	Live attenuated FAdV-4 after 80 passages (LMH80)	IM; 10^5 $TCID_{50}$	100%	[40]
23	China	6-day old specific pathogen-free (SPF) chicken eggs were	HHS	FAdV-4	Recombinant vector vaccine	yolk sac route; purified virus of $2x10^8$ vp (virus particle) in 100 µL PBS from two recombinant viruses: FAdV4-GFP, FAdV4-GX4C, and FAdV4-CX19A	0%, as all the embryos died at different time points.	[41]
24	China	6-day-old SPF chicken eggs	HHS	FadV-4	Recombinant vector vaccine XHE-CX19A, or XGAM1-CX19A	Egg yolk; $1x10^8$ Vp in 100 µL PBS	100% only for the XGAM1-CX19A virus group	[42]
25	China	21-day-old SPF chickens were	HHS and Avian influenza (AI) signs and symptoms (Sudden death, respiratory distress, swelling, discoloration, diarrhea, decreased egg production, lethargy, loss of appetite, nervous signs)	FadV-4	Triple vaccine (FAdV-4 fiber 2 as a recombinant subunit vaccine) and inactivated vaccines against NDV and AI	Subcutaneous injection with 4 µg of fiber-2 protein, $10^{8.5}EID_{50}$ of H9N2 AI SZ virus and$10^{8.2}$ EID_{50} of ND N7a virus	100%	[43]
26	Austria	One-day-old SPF chicks	IBH, HHS	FAdV-4 and FAdV-11	Chimeric recombinant subunit vaccine	0.5 mL intramuscular injection of the vaccine containing 50 µg of crecFib-4/11 homogenized in a 40% (wt/vol) antigen oil-based adjuvant	100%	[44]

Table 1. Fowl adenovirus and duck adenovirus vaccines development in worldwide (continued)

No.	Country	The Type of Animal Studied	Syndrome	Serotype of FAdV	Type of Vaccine	Route of Administration and Dose	Protection Rate	Ref.
27	Austria	One-day-old SPF chicks	IBH, HHS	FAdV-4 and FAdV-11	Chimeric recombinant subunit vaccine named crecFib-4/11	IM; 50 µg of crecFib-4/11 homogenized in an oil-based adjuvant	100%	[45]
28	China	7-day-old SPF chickens	IBH, HHS	FadH-4,8b and 11	Recombinant vector trivalent vaccine Oil-Adjuvant Inactivated rFAdV-4- Fiber/8b + 11	IM; a dose of rFAdV-4-fber/8b + 11 in the oil- emulsion vaccine was 10^6 $TCID_{50}$ in 200 µL per bird	100%	[46]
29	China	SPF chickens	HHS	FAdV-4	Probiotic recombinant subunit bacteria vaccine	PO; *L. lactis* NZ9000/pTX8048, $1.0x10^{10}$CFU; *E. faecalis* MDXEF-1/ pTX8048, $5.0x10^9$ CFU; *L. lactis* NZ9000/pTX8048-Fiber2-CWA, $1.0x10^{10}$ CFU; *L. lactis* NZ9000/ pTX8048-DCpep-Fiber2-CWA, $1.0x10^{10}$CFU; *E. faecalis* MDXEF-1/ pTX8048-Fiber2-CWA, $5.0x10^9$ CFU; *E. faecalis* MDXEF-1/pTX8048-DCpep-Fiber2-CWA, $5.0x1.0x10^9$ CFU	Survival rates of 60%, 80%, 90%, and 100% in groups *L. lactis*/pTX8048-Fiber2-CWA, *L. lactis*/pTX8048-DCpep-Fiber2-CWA, *E. faecalis*/ pTX8048-Fiber2-CWA, and *E. faecalis*/ pTX8048-DCpep-Fiber2-CWA, respectively	[47]
30	Saudi Arabia	Not- applicable	IBH, HHS, AGE	All serotypes	Multi-epitope	Not- applicable	Not mentioned *in-vivo* and *in-vitro*	[48]
31	Pakistan	One-day-old broiler chickens	HHS	FAdV-4	Virus-like-particle (VLP) subunit vaccine	SC; 100 µg of recombinant HBc-fused hexon epitopes at the neck region	Up to 90% using HBc-hexon (Asp348-Phe369)	[49]
32	China	21-day-old SPF chickens	IBH	FAdV-8a	Inactivated oil emulsion vaccine has	Group A received 0.3 mL of the vaccine with a concentration of $10^{6.5}$ $TCID_{50}$ per 0.1 mL through IM injection. Groups B and C were administered 0.3 mL of the vaccine with concentrations of $10^{5.5}$ $TCID_{50}$ per 0.1 mL and $10^{4.5}$ $TCID_{50}$ per 0.1 mL, respectively	100%	[50]
33	China	2-weeks old SPF chickens	HHS	FAdV-4	Recombinant subunit vaccine	A total of 46 chickens were randomly assigned to six groups: four immunization groups (A-D), a negative control group (E), and a challenge control group (F). Groups A, B, and D received IM injections of rFH protein at doses of 2.5 µg, 5 µg, and 7.5 µg per bird, respectively. Group C was given an initial dose of 5 µg rFH protein per bird, followed by a booster of 5 µg per bird 14 days later. Groups E and F served as controls. Three weeks post-immunization, Groups A-D and F were exposed to the FAdV-4 HB1505 strain, with each bird receiving 0.2 mL (106.0 $TCID_{50}$) of the virus intramuscularly and 0.1 mL orally	100%	[51]
34	Pakistan	14-day-old broiler chickens	HHS	FAdV-4	Inactivated	IM; LD_{50} infectivity titer $1x10^{5.6}$, LD_{50} of $1x10^{4.6}$, and LD_{50} of $1x10^{3.6}$	Not mentioned	[52]
35	China	SPF chickens and Layer hens	EDS	DAdV-1	Subunit fiber vaccine	HA (haemagglutination) titer of 11 log_2 through IM route	90-100%	[53]

Table 1. *Fowl adenovirus and duck adenovirus vaccines development in worldwide (continued)*

No.	Country	The Type of Animal Studied	Syndrome	Serotype of FAdV	Type of Vaccine	Route of Administration and Dose	Protection Rate	Ref.
36	China	Chicken	HHS	FAdV-4	Recombinant yeast expressing Fiber-2 of FAdV-4	The yeast culture mixture was prepared with feed and administered to chickens via oral immunization, followed by two booster doses at five-day intervals. Each immunization included an oral dose of $1x10^9$ CFU of ST1814G/Fiber-2 per chicken	100%	[54]
37	China	20-day-age Muscovy ducks	yellowish livers with hemorrhagic spots, kidney enlargement, and bleeding	DadV-3	Recombinant DadV-3 expressing VP1 protein (rDAdV3-VP1-188)	The rDAdV3-VP1-188 and DAdV3 were inactivated with 2% formaldehyde and mixed with Freund's adjuvant in equal volumes. The resulting mixture, along with PBS and DAdV3 at a concentration of $10^{3.00}$ $TCID_{50}$, was injected subcutaneously in the neck of the subjects every two weeks. This approach was used to assess the vaccine's immunogenicity and efficacy	100%	[55]
38	China	2-week-old specific-pathogen-free (SPF) chickens	HHS	FAdV-4	Subunit vaccine (fusion protein containing His-knob)	IM in the pectoral muscle. 150 µg of the purified His-knob-containing fusion protein mixed with a polymeric adjuvant	100% with purified His-knob	[2]
39	China	2-week-old White Leghorn SPF chicken	HHS	FAdV-4	Recombinant subunit fiber-2 vaccine	IM; 2.5 µg, 5 µg, 10 µg, 30 µg of protein per 0.3 mLof vaccine	100 % when at least 10 µg of subunit vaccine (F2-knob protein) is used	[56]
40	China	seven-day-old SPF chickens	HHS	FAdV-4	Subunit Fiber2 and penton vaccine with oil adjuvant	SC; purified recombinant proteins were emulsified in a ratio of 1:2 (v:v) with Marcol™ 52 white oil. 300 µL of 40 µg, 20 µg, 10 µg, and 5 µg of protein at one site on the nape	100%	[57]
41	China	2-week-old SPF chickens	HHS	FAdV-4	Recombinant Subunit vaccine from Fiber-2	Five groups of chickens received IM injections: Group A received 0.3 mL of vaccine with 2.0 µg of WZ-Fiber-2 protein, Group B with 2.0 µg of ON1-Fiber-2 protein, and Group C with 0.3 mL of inactivated WZ strain virus ($\geq 1x10^{6.0}$ $TCID_{50}$). Group D was given 0.3 mL of PBS-emulsified vaccine as a negative control, while Group E remained unvaccinated as a blank control	100 %	[58]
42	China	SPF) chickens aged 2 weeks	HHS	FAdV-4, H9N2 influenza virus	Recombinant vector bivalent FAdV4-HA(H9) vaccine	Chickens were injected with $1x10^5$ $TCID_{50}$ of attenuated virus in 200 µLof culture medium intramuscularly	75% against FAdV-4	[59]
43	South Korea	10 weeks-old SPF chickens	HHS	FAdV-4	Recombinant subunit yeast vaccine (inactivated yeast)	IM; The chickens were allocated into five groups, each consisting of five birds, including three groups for vaccine candidates: IBH-Fiber1-His (Fiber-1), IBH-Fiber2-His (Fiber-2), and IBH-hexonL1-His (Hexon-L1), along with a negative control group and a positive control group. The *S. cerevisiae* cells, containing their respective recombinant proteins, were inactivated by heating at 60°C for 1 h. A volume of 0.5 mL, containing 10^6 cells, was combined with incomplete Freund's adjuvant (Sigma-Aldrich, MO, USA) in a 1:1 ratio and administered into the thigh muscle	100% protection using fiber-1 and fiber-2	[60]

Table 1. Fowl adenovirus and duck adenovirus vaccines development in worldwide (continued)

No.	Country	The Type of Animal Studied	Syndrome	Serotype of FAdV	Type of Vaccine	Route of Administration and Dose	Protection Rate	Ref.
44	Austria	(SPF) broiler chicks	IBH	FAdV-8a and 8b	Recombinant subunit vaccine	On the first day of life, chickens in groups I, II, and III were vaccinated intramuscularly with 50 µg of recombinant Fib-8a protein, combined in a 1:1 ratio with GERBU Adjuvant P (GERBU Biotechnik GmbH, Heidelberg, Germany). Chickens in groups IV and V (challenge controls) received a mixture of phosphate-buffered saline (PBS) and adjuvant, while group VI (negative control) received PBS alone	100% when challenged with homologous serotype	[61]
45	China	2-week-old specific SPF chickens	HHS	FAdV-4	Recombinant live-attenuated vaccine	IM; $2x10^5$ TCID50 in 200 µL of 1% culture medium	100%	[62]
46	China	SPF chicken embryonated eggs	HHS	FAdV-4	Recombinant virus	Yold sack route; purified FAdV4- GFP or FAdV4XF2- GFP of $2x10^8$ vp in 100 µL PBS	40% of embryos survived when infected with FAdV4XF2- GFP	[63]
47	China	SPF White Leghorn chickens	HHS	FAdV-4	Recombinant bacterial vector vaccine expressing hexone protein on their cell wall.	Oral; *L. lactis* NZ9000/pTX8048, $1x10^{10}$ CFU; *E. faecalis* MDXEF-1/pTX8048, $5x10^9$CFU; *L. lactis* NZ9000/pTX8048-SP-ΔHexon-CWA, $1x10^{10}$CFU; *L. lactis* NZ9000/pTX8048-SP-DC-ΔHexon-CWA, $1x10^{10}$ CFU; *E. faecalis* MDXEF-1/pTX8048-SP-ΔHexon-CWA, $5x10^9$CFU; *E. faecalis* MDXEF-1/pTX8048-SP-DC-ΔHexon-CWA, $5x10^9$CFU	90% for MDXEF-1/DC- 1Hexon-CWA	[64]
48	China	LMH cells	lethargy, drooping neck, closed eyes, huddling, decreased appetite, and respiratory distress	DAdV-3	Monoclonal antibody against fiber-2 of DAdV-3	Serial dilutions of mAb (1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, and 1: 3200) were mixed with 200 TCID50 of DAdV-3, incubated for 1 h at 37°C, and then inoculated into fresh LMH cells in a 96-well plate	CPE was barely observed in infected LMH cells treated with mAb 3E7 The mAb effectively neutralized DAdV-3 at 1:50-1:400 dilutions, while mAb 3C2 (control antibody) showed no neutralizing activity	[65]
49	India	Day-old SPF chickens	IBH-HHS	FAdV-2/11	Recombinant subunit vaccine	SC; Group-1 (penton base): 100 µg; Group-2 (fibre): 100 µg; Group-3 (penton + fibre): 50 µg + 50 µg	Up to 80% protection from fiber-immunized group	[66]
50	China	7-d-old SPF chickens	HHS	FAdV-4	Subunit vaccine	IM; Subunit vaccines (Fiber1/2 knob subunit vaccine and fiber-2 subunit vaccine) were prepared by emulsifying recombinant proteins with Freund's complete adjuvant (1:1) at a protein concentration of 100 mg/mL	100% for vaccinated groups	[67]
51	China	one-day-old SPF chickens	HHS	FAdV-4	Recombinant live attenuated vaccine candidate	IM; $2.5x10^4$ $TCID_{50}$ of indicated virus in 200 µL 1% culture medium	100%	[68]
52	Korea	One-day-old SPF chickens were	HHS	FAdV-4	Live-attenuated vaccine	Chickens were injected intramuscularly with 0.1 mL of 80-times passaged FAdV-4 (LMH80)	Over 80%	[69]

Table 1. *Fowl adenovirus and duck adenovirus vaccines development in worldwide (continued)*

No.	Country	The Type of Animal Studied	Syndrome	Serotype of FAdV	Type of Vaccine	Route of Administration and Dose	Protection Rate	Ref.
53	China	2-week-old SPF White Leghorn chickens	HHS and Newcastle disease signs and symptoms (gasping, coughing, sneezing; nervous signs such as tremors, twisted necks, paralysis, and inability to stand; digestive issues like greenish diarrhea; a drop in egg production)	LaSota strain of NDV and FAdV-4	Attenuated recombinant virus	Groups one and two of chickens were immunized intramuscularly (IM) with 100 µL (10^7 EID_{50}) of live rLaSota-fiber2 and 100 µL (10^7 EID_{50}) of inactivated rLaSota-fiber2, respectively	100%	[70]
54	China	SPF Chickens	HHS, and infectious bursal disease signs and symptoms (Ruffled feathers, depression and reluctance to move dehydration diarrhea (watery, white, or greenish) vent picking and swelling around the vent)	FAdV-4 and very virulent IBDV vvIBDV HLJ0504 strain (vvIBDV)	Recombinant- viral vector inactivated bivalent FAdV-4/IBDV vaccine	IM; 0.3 mL of vaccine (10^7 PFU) inactivated with 0.1% formaldehyde at 2 weeks of age	100% protection against IBVD and FAdV-4	[71]
55	China	2-week-old specific-pathogen-free (SPF) chickens were	HHS	FAdV-4	Recombinant inactivated vaccine	IM or SC; the virus was inactivated with 0.1% formaldehyde and mixed with oil adjuvant at a ratio 1:2; chickens were immunized with 0.3 mL (10^7 PFU) of inactivated rHN20 vaccine at 2 weeks of age	100%	[72]
56	China	day-old chickens	HHS	FaDV-4	Recombinant vaccine	100 µg of the purified MLFRPs-FAdV4:F1-P-H-F2, FAdV4:F1-P-F2-H, FAdV4:F1-H-F2-P, FAdV4:F1-F2-P-H, and FAdV4:F1-F2-H-P-were emulsified with an equal volume of Freund's complete adjuvant (FCA) and utilized as immunogens for intramuscular injection	100% for FAdV4:F1-P-F2-H with a single shot	[73]
57	China	day-old chickens	HHS and IBH	FAdV-4 and -8b	Inactivated recombinant vaccine	IM; The inactivated FA4-F8b vaccine was prepared by mixing with oil adjuvant (1:3), yielding a final dose of 10^6 $TCID_{50}$ in 0.4 mL per chicken	100% against serotypes 4 and 8b	[74]
58	Malaysia	day-old broiler chickens	IBH	FAdV-8b	Attenuated UPM11142CEL20B1 strain	Chicks in groups B, C, and D were subcutaneously inoculated on day 0 with 0.5 ml of attenuated FAdV isolate UPM11142CEL20B1 ($10^{5.8}$ $TCID_{50}$/mL), while group A remained uninoculated. On day 14 post-inoculation (pi), group C received an attenuated booster, and group D received an inactivated booster ($10^{8.3}$ $TCID_{50}$/ml) via the same route	100%	[75]

Table 1. Fowl adenovirus and duck adenovirus vaccines development in worldwide (continued)

No.	Country	The Type of Animal Studied	Syndrome	Serotype of FAdV	Type of Vaccine	Route of Administration and Dose	Protection Rate	Ref.
59	China	3-week-old SPF chickens	HHS and IBH	FAdV-4 and 8b	Inactivated recombinant rFAdV-4-fiber/8b	Fifty 3-week-old SPF chickens were divided into five groups: vaccinated with the FAdV-4 challenge, vaccinated with the FAdV-8b challenge, unvaccinated with the FAdV-4 challenge, unvaccinated, and a sham control group. The vaccinated groups received 200 µL of the inactivated rFAdV-4-fiber/8b ($2x10^5$ $TCID_{50}$) vaccine intramuscularly	100% protection against both serotypes 4 and 8b	[76]
60	China	SPF white Leghorn chickens	HHS	FAdV-4	Live recombinant viral vector rHN20 vaccine	Different routes: Two groups of chickens served as non-inoculated and challenged controls with no immunization. Three groups were immunized with 10^6 PFU of rHN20 in 200 µL DMEM/F12 via different routes (intranasal, subcutaneous, and intramuscular) at 2 weeks of age. Additionally, two groups were intramuscularly immunized with varying doses of rHN20 (10^5 or 10^4 PFU per bird) in 200 µL DMEM/F12 at 2 weeks of age	100%	[77]
61	Pakistan	Non-vaccinated broiler chickens	HHS	FAdV-4	Subunit	Seven-day-old birds were subcutaneously injected with the specified amount of freshly purified or stored protein (100 µg of the protein per dose), which was adjuvanted with Montanide™ ISA71 VG at a 1:1 (w/v) ratio of adjuvant to protein	100%	[78]
62	China	14-day postbirth (dpb) SPF chickens	HHS	FAdV-4	Recombinant subunit vaccine	The purified protein samples (0.5 mg/mL) were emulsified with a white oil adjuvant at 1:2 to create a water-in-oil vaccine preparation. Chickens in Group I and Group II were administered 50 µg of fiber2 and FliBc-fiber2-SP vaccines per chicken, respectively. Groups III and IV were injected with an equivalent volume of sterile PBS	100% protection from FliBc-fiber2-SP+FAdV-4	[79]

FAdV-4 infection caused a threefold reduction in splenic B cells, prevented by LMH80 pre-treatment. LMH80 immunization regulated T cells, macrophages, and B cells, enhanced memory T cell subsets and CD44+, CD4+, CD8+ cells, and maintained MHCII+ macrophages. Activated CD4+ and CD8+ T cell infiltration increased in the liver [69].

Fowl Adenovirus Species E

Vaccine development faces manufacturing scale-up challenges [82]. Cell culture-based production offers a more efficient alternative to traditional embryonated egg culture, enhancing vaccine availability to meet the demands of the expanding production animal population [23].

Anchorage-dependent cells require attachment surfaces for proliferation [83]. Tissue culture flasks improved contamination control but limited large-volume vaccine production. Microcarriers, particularly spherical bead-type ones like Cytodex™ 1, enable industrial-scale mammalian cell proliferation, supporting ADC growth in stirred tank bioreactors for vaccine production [84]. It is a multipurpose microcarrier that can be used to grow various cells. While culturing the influenza virus vaccine in a stirred tank bioreactor on Vero cells, Cytodex™ 1 was used and optimized to the production capacity of 6000 L [85].

For virus propagation, cells are usually needed, and they are necessary in higher volumes for vaccine production. This is because cell concentration is of significant importance in determining viral titer [86], and viral titer has control on the efficacy of vaccines, which makes the need for a high volume of cells for volume production of vaccine an ongoing requirement. Therefore, a study aimed to propagate fowl adenovirus serotype 8b (FAdV-8b) in chicken embryo liver (CEL) cells adapted to Cytodex™ 1 microcarriers using a bioreactor *(Table 1)*.

FAdV isolate (UPM08136) was successfully propagated in chicken embryo liver (CEL) cells using Cytodex™ 1 microcarriers in a stirred tank bioreactor (STB), enabling large-scale virus production for vaccine development. No molecular changes were observed in the hexon and fiber proteins. This method was used to create an attenuated vaccine against FAdV-8b [23].

In another study by the same author [38], an inactivated and attenuated vaccine was inoculated in chickens to see the difference between each and the booster dose effect *(Table 1)*.

No clinical symptoms or histopathological changes were observed in unchallenged chickens. Inoculated groups (B, C, D) had higher liver weights at 14 and 21 dpi and lower liver-to-BW ratios at 35 dpi, indicating protection. Group B had the highest antibody titers at 42 dpi (>2000 ELISA units), while Group D exceeded 4000 units at 35 dpi.

Inoculated chickens showed significantly higher CD3+, CD4+, and CD8+ T lymphocytes than controls, with elevated CD3+ cells in the liver, spleen, and thymus at multiple intervals. CD4+ and CD8+ cells increased significantly, especially in the thymus at 42 dpi. Inoculation reduced FAdV viral load and shedding while confirming attenuated isolate stability in a stirred tank bioreactor, enhancing virus production and cost-efficiency. The study provided novel insights into chickens' cell-mediated immune response to FAdV8b vaccines, showing booster doses significantly accelerate higher antibody levels by 35 dpi [38].

A study using subcutaneous FAdV vaccination showed no clinical signs or histological changes in vaccinated chickens, unlike controls with liver discoloration and splenomegaly. Vaccinated groups had higher body weights and FAdV antibody titers at 35 and 42 dpi. CD3+ and CD4+ T lymphocytes increased in vaccinated chickens, with reduced viral genome copies in the liver and cloaca, indicating lower viral shedding and better clearance [75].

2. Inactivated Vaccines

Fowl Adenovirus Species C

With the rise of viral resistance to numerous antiviral drugs, controlling viral issues becomes increasingly challenging and can lead to significant economic losses [87]. Few commercial HPS vaccines use traditional liver homogenates, potentially causing allergic reactions due to non-specific proteins. A study explored developing a cell-free vaccine to address these concerns [52].

The study showed that a 20-dose HPS-infected liver vaccine induced a higher serum anti-HPS ELISA antibody titer (1110.4) at 40 days than 25 doses (1071.9) or 30 doses. The infectivity titer of $1x10^{5.6}$/mL BLD50 produced stronger antibody responses (1052.5±18.04) than $1x10^{4.6}$/mL (772.6±133.1) and $1x10^{3.6}$/mL (588.00±61.97). The liver homogenate vaccine achieved higher antibody titers (2009.3) than primary hepatocyte culture vaccines. Oil-based tissue culture vaccines (1148.45) outperformed gel-based ones (1137.2). Findings support liver homogenate and Montanide-adjuvanted cell culture vaccines for inducing robust anti-HPS responses [52].

Fowl Adenovirus Species E

Inactivated vaccines are easier to administer and distribute globally without specialized storage, benefiting regions with limited medical resources or infrastructure [50]. Findings of the literature reveal that several inactivated vaccines have been developed. For instance, one study used Fowl adenovirus 8b (UPM08136) isolated from an IBH outbreak in Malaysia.

The study used Montanide adjuvant with an inactivated virus and compared booster and non-booster groups. No clinical signs or lesions were observed in vaccinated groups, while challenged controls showed pale livers and symptoms. Antibody titers in the non-booster group reached nearly 4000 ELISA units by 35 DPI. CD4+ T-lymphocytes in the spleen and CD8+ T-lymphocytes in the liver were significantly higher ($P<0.05$) in vaccinated groups. Viral copy numbers in the liver were markedly lower in booster and non-booster groups, demonstrating the vaccine's effectiveness in reducing viral replication and shedding [36].

Selecting the right chemical for virus inactivation is essential in vaccine development, preserving the virus's structural integrity and entry-associated domains to effectively trigger virus-neutralizing antibody responses [88]. Alkylating agents like β-propiolactone (BPL) and binary ethyleneimine (BEI), as well as gamma and ultraviolet radiation, target the viral genome while preserving neutralizing epitopes. Cross-linking agents like formaldehyde and glutaraldehyde, or denaturing methods involving pH and temperature changes, modify viral proteins, risking epitope degradation and reduced immunogenicity [89].

Among these agents, BEI stands out for its mechanism of inactivating non-enveloped viruses by targeting their RNA/DNA genome. The active component of BEI, ethylenimine, reacts explicitly with nucleic acids, leaving other viral proteins unaltered [90]. This selective interaction makes BEI a promising agent for preserving viral-neutralizing epitopes, which are critical for vaccine efficacy. Building on this understanding, a study was conducted to evaluate the inactivation of FAdV-8b using BEI. The methodology and findings of this study contribute valuable insights into the development of vaccines targeting FAdV-8b while ensuring the preservation of its immunogenic properties.

The vaccine inactivated with BEI for 32 hours was safe and immunogenic in broiler chickens. At 28 DPI, antibody titers were 1±0 for the control group, 321±189 for Group A1, and 690±484 for Group B1, with Groups A1 and B1 showing significantly higher titers ($P<0.05$). Booster groups A2 (602±367) and B2 (874±317) also had significantly higher titers ($P<0.05$) than the control. No significant difference ($P>0.05$) in antibody titers was found between booster and non-booster groups, regardless of inactivation time [24].

Commercial FAdV vaccines exist for FAdV-4 and FAdV-8b but not for FAdV-8a. An inactivated FAdV-8a vaccine using the CY21 strain (15 LMH cell passages) was tested. Group A ($10^{6.5}$ $TCID_{50}$/0.1 mL) had antibody titers exceeding 6000 ($P<0.0001$), significantly higher than the control. Group B ($10^{5.5}$ $TCID_{50}$/0.1 mL) showed titers below 2000 ($P<0.05$). Group C ($10^{4.5}$ $TCID_{50}$/0.1 mL) displayed clinical symptoms, liver lesions, and viral DNA in organs. Viral shedding was fully inhibited in Group A, partially (40%) in Group B, and not prevented in Group C. Group A demonstrated complete protection with no viral DNA in organs *(Table 1)* [50].

Second Generation Vaccines

1. Subunit Vaccine

Fowl Adenovirus Species C

Subunit vaccines are highly immunogenic and eliminate the risk of incomplete inactivation associated with whole-virus vaccines, making them a promising option for controlling HHS. Several studies have shown that subunit vaccines can effectively protect against virulent FAdV-4 challenges [91,33]. However, most current research on subunit vaccines has concentrated on the Fiber-2 protein of FAdV-4, with the protective potential of vaccines targeting the knob domains of Fiber-1 and Fiber-2 yet to be explored. Therefore, subunit vaccines from the Fiber-1/2 knob and Fiber-2 proteins of FAdV-4 was produced [67].

AST and ALT levels were significantly higher ($P<0.05$) in unvaccinated challenged chickens at 3, 5, and 7 dpc compared to controls and vaccinated groups. Necropsies revealed severe HHS lesions in unvaccinated challenged chickens, while vaccinated groups showed healthy organs. The Fiber-1/2 knob vaccine offered superior protection against FAdV-4, with no lesions, compared to minor lesions in the Fiber-2 vaccine group.

qPCR revealed significantly lower viral DNA copy numbers in the heart, liver, spleen, lungs, and kidneys of vaccinated groups compared to unvaccinated controls. The Fiber-1/2 knob vaccine group showed significantly reduced viral loads at 3 and 5 dpc ($P<0.05$) and earlier antibody detection (7 dpv) compared to 14 dpv in the Fiber-2 group. Neutralizing antibody titers in the Fiber-1/2 group increased to 4.1, 5.6, and 6.5 at 14, 21, and 28 dpv, respectively, compared to 3.3, 4.1, and 4.3 in Fiber-2. Body weight loss was also minimized with Fiber-1/2 vaccination [67].

Similarly, another study [73] explored the development of subunit vaccines by combining multiple capsid protein-derived epitopes into multilinked fusion recombinant proteins (MLFRPs), which were recombinantly expressed in *E. coli*. Unlike previous research that primarily assessed the immunogenicity of individual capsid proteins by expressing their complete amino acid sequences [33,51], this approach focused on leveraging the combined immunogenic potential of multiple epitopes within a single recombinant construct.

The study designed multiantigen epitope tandem proteins (MAETPs) from four FAdV-4 capsid proteins (hexon,

penton, fiber1, and fiber2), selecting efficient antigenic epitopes using bioinformatics tools. The epitopes were linked with GGGGS linkers, and DNA sequences encoding MAETPs were chemically synthesized and assembled into multilinked fusion recombinant proteins (MLFRPs) using T4 ligases. The resulting constructs were cloned into pET-28a vectors and expressed in *E. coli*. Five MLFRPs (FAdV4:F1-P-F2-H, FAdV4:F1-F2-P-H, FAdV4:F1-F2-H-P, FAdV4:F1-P-H-F2, FAdV4:F1-H-F2-P) were produced and evaluated for their potential as protective antigens through chicken immunization.

Microneutralization assays showed that MLFRP-immunized chicken sera effectively neutralized FAdV-4, with FAdV4:F1-P-F2-H (over 1200) and FAdV4:F1-F2-P-H (nearly 1200) eliciting the highest neutralizing antibody titers. FAdV4:F1-P-F2-H provided full protection with a single immunization, while other proteins required two doses. After one immunization, protection rates were 83.33% for FAdV4:F1-F2-P-H and the inactivated vaccine, 66.67% for FAdV4:F1-F2-H-P, and under 50% for FAdV4:F1-P-H-F2 and FAdV4:F1-H-F2-P. Hepatic lobules in the FAdV4:F1-P-F2-H group appeared intact, while other groups displayed vacuolated or blurred cells. Further studies could investigate alternative adjuvants to enhance immune responses [73].

Bioinformatics, especially immunoinformatics, aids in designing multi-epitope subunit vaccines efficiently. A study by Mugunthan et al.[48] used these techniques to create a cost-effective FAdV vaccine, activating B and T cells with sustained activity over 50 days, promoting long-term immune memory.

2. Recombinant Subunit Vaccines

Fowl Adenovirus Species A and Duck Adenovirus Serotype 1 Vaccine

Duck Atadenovirus A (DAdV-1), responsible for egg-drop syndrome '76 (EDS '76) in laying hens, belongs to the Atadenovirus genus within the Adenoviridae family, with a linear double-stranded DNA genome of 30-35 kb [92]. First identified in 1976, EDS '76 has one serotype [93]. DAdV-1 typically infects waterfowl, such as ducks and geese [94]. In laying hens, it reduces egg production and quality. Vaccination is a control measure [95,96]. DAdV-1's capsid protein contains neutralizing epitopes, aiding vaccine development [97,98]. The fiber protein, which includes the N-terminal tail, shaft, and C-terminal knob domain, is crucial for inducing virus-neutralizing antibodies and serves as a target for subunit vaccines [99-102].

Although the production of subunit vaccine from the fiber was successful and induced with the fiber protein also induced lymphocyte proliferation response, cytokine secretion, and reduced viral load in SPF chickens [103], there is no data regarding its efficacy in layer hens. Thus, a study [53] was conducted to fill this gap.

The study [53], the first to evaluate the subunit fiber vaccine of DAdV-1 in layer hens, demonstrated superior efficacy over the inactivated vaccine. HI titers at 21 and 28 dpi reached 11.1±1.0 $\log_2$ and 12.5±1.4 $\log_2$, surpassing the inactivated vaccine. Egg production rates remained at 90-100% for vaccinated hens, while unvaccinated hens dropped to 12%. A critical HI titer of at least 7 $\log_2$ was essential to prevent production losses, protecting against the virus's effects for up to 180 days post-challenge [43].

Fowl Adenovirus Species C Vaccines

Evaluation of the fiber gene of FAdV has become necessary because, through the interaction of the fiber knob with host cells, the fiber gene is responsible for tissue tropism, which is also very important in the virulence of FAdV [23]. As one of the capsid proteins of FAdV-4, Fiber2 has been identified as an efficient protective immunogen for subunit vaccine candidates [16]. In one study [12], immunization with rFiber-1 was evaluated.

The study showed that neutralizing antibody levels increased significantly one week after the second immunization and remained significant for up to 10 weeks. All chickens stayed healthy, with viral genome content in tissues below detection limits. The rFiber-1 protein provided protective efficacy, with an average antibody titer of $\log_2$ 7.8 *(Table 1)* [11].

Similarly, A subunit vaccine candidate was developed using the recombinant Fiber2 protein (the rFiber2 subunit vaccine) expressed in bacteria from the hypervirulent FAdV-4 GZ-QL strain, isolated in Guizhou province. Additionally, a DNA vaccine candidate, the Fiber2 DNA vaccine, was created using the recombinant plasmid pVAX1-Fiber2 [33].

Both vaccine candidates induced significant Fiber2-specific antibody levels (rFiber2 subunit 50μg: 24 pg/mL; 100 μg: nearly 26 pg/mL; 150μg: 22 pg/mL; Fiber2 DNA vaccine: 20-22 pg/mL). The rFiber2 subunit vaccine showed superior efficacy (80-100%) compared to the Fiber2 DNA vaccine (50-60%) and commercial inactivated vaccine (80%). Higher dosages, especially 100 μg, produced significantly higher antibody titers. Both vaccines elicited robust cellular and humoral immune responses without significant histopathological changes [33].

Building on the development of the fiber-2 recombinant subunit vaccine, a triple vaccine was formulated by combining the fiber-2 protein antigen with inactivated H9N2 AI and NDV antigens, offering a multivalent approach to protect against multiple avian pathogens [43].

No significant difference in ELISA antibody titers against FAdV-4 was observed between the triple vaccine group (19 log_2) and the monovalent vaccine group (over 19 log_2) containing 4 mg of fiber-2 protein (P>0.05), both showing higher levels than unvaccinated controls (P<0.0001). Fiber-2 did not interfere with other antigens. Immunization with fiber-2 protein induced stronger IFN-γ secretion and FAdV-4-specific cellular immunity (P<0.05). The triple vaccine provided complete protection, with no viral shedding or histopathological changes observed [43].

Expanding on recombinant fiber-based proteins in vaccine development, a chimeric fiber vaccine, crecFib-4/11, was engineered to combine epitopes from FAdV-4 and FAdV-11, highlighting another innovative approach to enhance immunogenicity.

Vaccination with crecFib-4/11 led to elevated systemic antibody levels against the vaccine antigen (over 3 OD at 27 days post-challenge), as measured by ELISA, though neutralizing antibodies against FAdV-4 were not produced. Nonetheless, vaccinated birds challenged with virulent FAdV-4 exhibited significantly reduced clinical symptoms and pathological lesions.

Vaccinated birds showed increased B lymphocytes in the liver throughout the post-challenge period, correlating with hepatic lymphoid infiltration. The vaccine primed a rapid rise of these cells in the blood, followed by their presence in the liver. Monocyte/macrophage levels increased in the blood, liver, and spleen of vaccinated+challenged and control groups, while thymus levels decreased in challenge controls. CD4+ T lymphocyte levels remained stable, except for a late decrease in the bursa fabricius in challenge controls, which vaccination prevented. Cytotoxic CD8α+ T cells were maintained across groups, with a vaccine-induced rise in target organs. Vaccination reduced viral loads in the liver, spleen, and bursa fabricius [44].

In line with the development of chimeric fiber vaccines, another study [51] explored a different strategy by focusing on a subunit vaccine using the combination of fiber-2 protein from FAdV-4 HB1505 and hexon, aiming to evaluate its immunogenic potential and efficacy through various dosage levels.

The study assessed rFH protein vaccine doses: 2.5 μg (75% survival, 6/8), 5 μg (100%, 8/8), double 5 μg (100%, 8/8), and 7.5 μg (100%, 8/8), compared to 0% (0/7) in challenge controls. Vaccinated groups maintained body weight and showed significantly higher OD ELISA titers (~2 for 2.5 μg; nearly 3 for higher doses), indicating strong humoral responses. Viral loads in vaccinated livers were significantly reduced. Truncated fiber two proteins (Gly275 to Pro479) expressed in *E. coli* improved solubility, while adding a hexon epitope (Met21 to Val55) with a G3S linker enhanced immunogenicity [51].

Expanding on the investigation of mixed subunit vaccines comprising hexon and fiber proteins to identify the optimal protective dose, the subsequent study concentrated on assessing fiber and penton proteins, individually and in combination, to optimize further dosing for adequate protection [57].

Complete protection (10/10) was achieved 21 dpi with fiber-2 doses of 20 μg/bird and 200 μg/bird, while penton base protein provided complete protection only at 200 μg/bird. At seven dpi, vaccinated chickens had significantly elevated OD450 values (P<0.001): 1.006 (Fiber-20 μg), 1.458 (Fiber-200 μg), 2.059 (Penton-20 μg), and 2.576 (Penton-200 μg). Viral loads in tissues were reduced compared to the challenge control group, though cloacal swab loads remained higher. Fiber-2 provided full protection at 10 μg/bird, with 5 μg offering 90% protection. Penton base required 200 μg for complete protection, with lower doses showing 70%-60% protection. No adverse effects on body weight gain or inflammation were observed [57].

The prokaryotic expression system was used for its high yield, low cost, and ease of management in subunit vaccine production. To address inclusion body formation during fiber-2 expression, the culture temperature was lowered to 16°C, and Rosetta (DE3) cells were used, yielding 1.5 mg/mL of soluble protein. Fiber-2 and penton base proteins were successfully expressed with strong immunogenicity. The use of Marcol™ 52 white oil as an adjuvant enabled cost-effective, scalable subunit vaccines for FAdV-4 [57].

Studies have shown that the trimeric knob domain of the EDS virus from fowl adenovirus group III, when used as a subunit vaccine, can induce hemagglutination inhibition titers and serum-neutralizing activity comparable to those of the full-length fiber protein [98,103,104]. However, the immune efficacy of the knob protein in FAdV-4 has not yet been reported. Subunit vaccines are effective for controlling FAdVs due to their safety, ease of mass antigen production, and low cost. Therefore another study focused on producing a subunit vaccine from the knob region of the FAdV-4 and a minimum dose for complete protection [46]. The study evaluated F2-knob subunit vaccine doses (2.5 μg, 5 μg, 10 μg, and 30 μg) combined with ISA 71 VG adjuvant. Antibody levels exceeded the OD 0.125 cut-off by day 14, with Groups C (10 μg) and D (30 μg) surpassing 0.4 OD and exceeding 0.8 by day 21. Groups C and D had significantly higher antibody titers than Group E (inactivated whole virus) at 14 days post-immunization (P<0.0001).

All chickens immunized with 5 μg, 10 μg, or 30 μg of F2-knob protein and the inactivated whole virus vaccine were fully protected against FAdV-4, showing no clinical symptoms. FAdV-4 virus shedding remained negative in

Groups C, D, and E. Symptoms like lethargy and green feces appeared in Groups F (challenge control) and A (2.5 μg) but not in Groups B (5 μg), C (10 μg), D (30 μg), and E. Group E had significantly higher neutralizing antibody titers than Groups A ($P<0.05$), B ($P<0.001$), C ($P<0.001$), and D ($P<0.01$) after the challenge [46].

Genome sequence alignment of all FAdV-4 strains revealed that pathogenic and non-pathogenic strains are classified into two genotypes. Recent Chinese isolates exhibit a natural 1966 bp deletion and other genomic differences compared to the classical non-pathogenic strain ON1. However, the biological properties of Fiber-2 proteins in FAdV-4 strains with varying virulence remain unexplored. In a study, the Fiber-2 proteins of the highly virulent WZ strain and the non-virulent ON1 strain of FAdV-4 were successfully expressed and purified [58].

Chickens immunized with 2 μg of WZ-Fiber-2 protein or the inactivated vaccine achieved complete protection (10/10) with no morbidity, mortality, or histopathological changes. FAdV-4 DNA shedding remained at background levels, contrasting with the ON1-Fiber-2 and PBS groups, where viral shedding persisted until death. Viral DNA levels in the WZ-Fiber-2 group were significantly lower than in the ON1-Fiber-2 group, comparable to the inactivated vaccine group. Blood titers in the WZ-Fiber-2 group rose from 0.2 OD at two weeks post-priming to over 0.4 OD at three weeks, significantly surpassing the ON1-Fiber-2 and PBS groups ($P<0.0001$) [58].

In recent years, lactic acid bacteria (LAB) have been extensively used as delivery systems for key pathogen antigens, including the circumsporozoite protein of *Plasmodium falciparum* [105], the spike protein of SARS-CoV-2 [106], and the heavy-chain antigen of *Clostridium botulinum* serotype A neurotoxin [107]. Research has demonstrated that FAdV-4 structural proteins, such as Hexon, Penton, Fiber 1, and Fiber 2, can be expressed in *Escherichia coli* and other systems to develop subunit vaccines [70,91,108]. However, the effectiveness of live recombinant LAB in delivering FAdV-4 structural proteins to protect against homologous challenges has not yet been evaluated. Building on previous research, it was hypothesized that oral immunization, capable of inducing strong mucosal and humoral immune responses, could serve as an effective strategy to prevent HPS caused by FAdV.

Chickens immunized with recombinant strains expressing 1Hexon-CWA or DC-1Hexon-CWA exhibited significantly higher Hexon-specific IgG (nearly 1.5 to over 1.5 OD, 14 days post-immunization) and sIgA levels (over 1 to over 1.5 OD) compared to control groups ($P<0.01$). DC-1Hexon-CWA strains, incorporating the DCpep, induced higher antibody levels ($P<0.01$). Elevated mRNA levels of ChIL-2, ChIFN-γ, ChIL-4, and ChIL-10 were observed, with MDXEF-1/DC-1Hexon-CWA achieving the highest cytokine expression ($P<0.01$). Peripheral blood lymphocytes (PBLs) showed enhanced proliferation in the MDXEF-1/DC-1Hexon-CWA group ($P<0.01$). *E. faecalis* strains provided superior protection, with the highest survival rates and delayed mortality after FAdV challenge. Mild HPS symptoms occurred [64].

Intramuscular injections of inactivated or attenuated vaccines have not successfully triggered intestinal mucosal immunity. Recently, subunit vaccines, which share similarities with inactivated vaccines, have been shown to possess stable and safe properties [109,110]. In theory, vaccination methods that can stimulate effective immune responses in the intestinal mucosa offer a promising approach to preventing pathogens transmitted orally [110]. There remain significant risks of active infection when using live, attenuated, and even inactivated vaccines. Additionally, commercially available vaccines that effectively stimulate intestinal mucosal immunity are lacking. Therefore, exploring new types of vaccines is essential. Henceforth, a recombinant bacteria for the vaccine (probiotics surface-delivering Fiber2 protein) was created.

Two weeks after primary, secondary, and third immunizations, IgG and sIgA levels steadily increased in groups immunized with Fiber2-expressing probiotics (IgG: nearly 1 to over 1.5 OD; sIgA: over 1 to over 1.5 OD) and remained higher than controls. DCpep-fused groups (*L. lactis*/pTX8048-DCpep-Fiber2-CWA and *E. faecalis*/pTX8048-DCpep-Fiber2-CWA) showed significantly higher antibody levels ($P<0.01$). Elevated mRNA levels of IL-2, IFN-γ, IL-4, IL-10, IL-6, and IL-17 were observed in all groups except *L. lactis*/pTX8048-Fiber2-CWA. Peripheral blood lymphocytes (PBLs) exhibited significant responses to rFiber2 protein. Viral loads and clinical symptoms were significantly reduced in vaccinated groups. Elevated serum IgG and sIgA confirmed the vaccine's success in inducing humoral and mucosal immunity, contributing to immune protection in all vaccinated chickens [47].

Building on the development of recombinant bacteria for probiotic surface delivery of the Fiber-2 protein, the focus has also been shifted to dendritic cells (DCs) as key antigen-presenting targets and the exploration of fusion proteins, such as flagellin-antigen constructs, to enhance immune responses and advance vaccination strategies. Dendritic cells (DCs), known for their strong antigen-presenting ability, are crucial targets for vaccines, enabling precise and effective antigen delivery to enhance immunogenicity [111-113]. Short peptides targeting DCs can be fused with antigens for vaccine preparation [114]. *Salmonella* flagellin, a Toll-like receptor five agonist, stimulates immune responses and is an adjuvant via oral

or injection routes. Fusion proteins combining antigens with flagellin retain activity, facilitating new vaccination strategies against infections and cancers [115,116].

Recombinant FAdV-4-fiber2 and FliBc-fiber2-SP proteins were produced by fusion PCR, inserted into the pET-SUMO-His vector, and expressed in *E. coli*. Purified via His-tag kits, proteins were verified by SDS-PAGE and Western blot using anti-FAdV-4-fiber2 serum.

ELISA results showed anti-fusion protein IgG antibodies in Groups I (fiber2) and II (FliBc-fiber2-SP) at 7 dpv, significantly increasing by 21 dpv ($P<0.001$). FliBc-fiber2-SP induced higher IgG levels (nearly OD2) than fiber2 (over 1.5 OD) ($P<0.05$). Serum IL-4 and IL-2 levels were significantly elevated in immunized groups ($P<0.01$), with FliBc-fiber2-SP showing higher IL-2 levels ($P < 0.05$). Post-challenge, Group II had lower viral loads ($P<0.05$) and a 100% survival rate, while Group I had 80% protection. Group III exhibited severe pathological changes. RT-qPCR confirmed significantly reduced viral loads in vaccinated groups ($P<0.001$), confirming the superior efficacy of FliBc-fiber2-SP [117].

In addition, recent efforts to control FAdV-4 have focused on developing subunit vaccines using viral capsid proteins like hexon, fiber, and penton, as well as non-structural proteins such as the 100k protein. *Saccharomyces cerevisiae*, the first fully sequenced eukaryote, is a cost-effective eukaryotic expression system widely used for producing pharmaceutical products, including subunit vaccines. *S. cerevisiae* and other yeast species trigger immune responses by promoting the maturation of dendritic cells, facilitating the presentation of yeast-expressed antigens through MHC class II and MHC class I proteins [118]. Consequently, *S. cerevisiae* has been utilized as a vaccine carrier in various studies.

Recombinant yeast expressing Hexon-L1, Fiber-1, and Fiber-2 proteins effectively stimulated immune responses against FAdV-4 in chickens. Antibodies were undetectable at zero and one wpi but appeared at two wpi, with Fiber-1 showing the highest titer (2.4 $\log_2$), followed by Fiber-2 (1.8 $\log_2$) and Hexon-L1. By three wpi, Fiber-1 (4 $\log_2$) and Fiber-2 (3.4 $\log_2$) titers were significantly higher than Hexon-L1 (3 $\log_2$) ($P<0.05$). Protection was observed in Fiber-1 and Fiber-2 groups, with no gross or histopathological lesions post-challenge. The Fiber-2 group exhibited superior serum neutralization, highlighting the potential of yeast-expressed Fiber-1 and Fiber-2 proteins as protective FAdV-4 vaccine candidates [60].

Fowl Adenovirus Species D Vaccine

FAdV-2/11 has become the most commonly isolated type from cases of Inclusion Body Hepatitis (IBH) and Hepatitis-Hydropericardium Syndrome (HHS) in chickens, leading to significant economic losses globally. FAdV-2 and FAdV-11 are closely related both serologically and molecularly. As a result, isolates from either serotype are collectively referred to as FAdV-2/11 [79,119].

E. coli-produced recombinant proteins fiber-1, fiber-2, hexon loop-1, and penton base were evaluated for FAdV-4 protection, with fiber-2 showing superior efficacy. However, no comparative studies exist for FAdV-2/11, highlighting the need to assess these proteins individually and in combination for developing suitable recombinant subunit vaccines.

The study validated the successful expression and characterization of recombinant penton base and fiber proteins, which are critical for advancing vaccine development [66]. This study addresses a research gap by comparing the immune potency of penton base, fiber, and their combinations for fowl adenoviruses beyond FAdV-4. The fiber group demonstrated the highest survivability rate at 80%, compared to 68% mortality in the challenged control group, 44% in the penton base group, and 34% in the penton + fiber group. Antibody titers peaked at seven days post-immunization (1960.09±87.06) and increased sharply post-challenge (28 dpc: 6536.23±227.06). Viral shedding in feces ceased by day 8 in the fiber group, earlier than other groups, underscoring the fiber protein's superior protective efficacy [66].

Fowl Adenovirus Species E Vaccines

Despite uncertainties surrounding the immune mechanisms behind fiber-induced protection and the variability of fiber types across species, a recombinant fiber from F AdV-8a was tested for protective efficacy against homologous (-8a) and heterologous (-8b) IBH strains [61]. This study is the first to extend the immune response profile beyond the challenge time, comparing pre-stimulated and naïve responses. Cellular immune subpopulations, including CD4+ T lymphocytes, were stimulated by FAdV fiber subunits, with an increase in CD8α+ T cells after a booster immunization with FAdV-8b fiber [120].

The recombinant fiber vaccine provided significant protection against FAdV-8a but limited cross-protection for FAdV-8b. Vaccinated birds showed normal liver-to-body weight ratios and reduced viral loads. Neutralizing antibodies were detected in 73.5% for FAdV-8a/TR59 (4.4 $\log_2$±3.1), 38.8% for FAdV-8a/11-16629 (1.7 $\log_2$±2.3), and minimally for FAdV-8b/764. Cellular immunity revealed elevated B cells and TCR+ T cells [61].

In another study, information on multiple linear epitopes predicted in the Fowl Aviadenovirus E (FAdV-E) fiber head (knob) was utilized to develop chimeric fibers by exchanging sequences between two serotypes, each

containing the proposed epitopes [30]. Two consecutive segments of amino acid positions 1 to 441 and 442 to 525/523 in the fibers of FAdV-8a and -8b, types of Fowl Aviadenovirus E that cause inclusion body hepatitis, were swapped reciprocally to result in novel chimeras, crecFib-8a/8b and crecFib-8b/8a.

The bivalent crecFib-8b/8a vaccine protected against FAdV serotypes 8a and 8b, with viral loads undetectable in vaccinated groups. Antibody titers exceeded 3 OD by 2 wpv, peaking at nearly 3.5 OD at four wpv. VV8b/8a achieved higher peak titers (3.14±0.73 OD) compared to VV8a/8b (0.51±0.65 OD) [30].

Cross-protection is essential for IBH, which involves different serotypes from two FAdV species, but it holds less significance for HHS, which is linked to a single serotype. The penton base protein gene is relatively conserved within serotypes of the same FAdV species, indicating its potential for broad protection against IBH. Thus, another study marks the first evaluation of the recombinant penton base protein as a subunit antigen for IBH, aiming to determine its effectiveness as a standalone vaccine [31]. Recombinant subunit vaccines for FAdV were produced by cloning and expressing penton base proteins Pb-7 and Pb-8b from FAdV-7 and FAdV-8b strains, respectively. Full genome sequencing confirmed strain identities. Each recombinant antigen was tested separately *in vivo* to assess immunogenicity and antigenic differences between serotypes.

The Pb-7 study reported mortality rates of 35.7% in vaccinated and 40% in control groups, with clinical signs by 5 dpc. In the Pb-8b study, only one control bird showed mild signs. Hepatic lesions indicative of IBH were common post-infection. Pre-challenge antibody levels were low in both studies, with OD means at 20 dpv of 0.11±0.17 (Pb-7) and 0.09±0.14 (Pb-8b). A Pb-8b booster increased antibody titers to 1.23±0.70 OD at 6 dpb, compared to 0.08±0.04 in controls. Despite boosted titers, no pre-challenge sera showed *in vitro* neutralizing activity against FAdV-8b [31].

3. Virus-Like Particle (VLP) Vaccines

Fowl Adenovirus Species C

Hexon, a key capsid protein of adenoviruses, is highly immunogenic [121]. It contains conserved pedestal regions (P1 and P2), shared across adenovirus types, and seven hypervariable regions (HVR1-7) that vary among adenoviruses and are found in three loops (L1, L2, and L4) [122,123]. Due to its immunogenic properties, hexon has been used as an antigen in vaccine development against adenoviral infections. However, vaccines that are both readily producible and capable of using hexon to provide complete protection against adenoviral infections are still unavailable [100,124]. Recently, virus-like particles (VLPs) based on the hepatitis B virus core protein (HBc) have gained significant attention as vaccine carriers. HBc can be efficiently produced as VLPs across various expression systems, can hold large foreign antigens at its central immunodominant region (MIR), and can stimulate a humoral response when in a properly folded, particulate form [125]. Hence, a study focused on the production of VLP vaccine [49].

The HBc-hexon (Asp348-Phe369) construct provided 90% protection against pathogenic FAdV-4, outperforming HBc-hexon (Ser19-Pro82) and HBc-hexon (Gly932-Phe956) constructs, which offered 70% and 40% protection, respectively. The control groups vaccinated with a commercial inactivated vaccine or PBS had 50% survival rates. Histopathological analysis showed no inclusion bodies in the HBc-hexon (Asp348-Phe369) group, though mild vacuolar degeneration was observed. HBc-hexon (Ser19-Pro82) and HBc-hexon (Gly932-Phe956) groups displayed moderate degeneration and necrosis with cellular infiltration. The superior protection from HBc-hexon (Asp348-Phe369) likely stems from the immune response elicited by this conserved epitope displayed at HBc's major immunodominant region. Serum antibody titers exceeded 0.4 OD for HBc-hexon (Asp348-Phe369) and HBc-hexon (Ser19-Pro82).

Moreover, the sequence and structure conservation of the epitope region among adenovirus hexon proteins suggests potential broader applications of this vaccine strategy against infections caused by other adenovirus strains [49].

4. Recombinant Virus Vaccines

Fowl Adenovirus Species C Vaccines

Recent studies found that Fiber-1, not Fiber-2, directly triggered the viral infection of FAdV-4 via its shaft and knob domains [2,126]. However, the molecular basis of Fiber-2 in the pathogenesis of the highly pathogenic FAdV-4 needs to be further elucidated. A study found that fiber-2 interacts with karyopherin alpha 3/4 (KPNA3/4) through its N-terminal 1-40 amino acids, with KPNA3/4 shown to promote the replication of FAdV-4. The study used CRISPR-Cas technology to omit the fiber-2.

Co-IP and western blot analyses identified the N-terminal 1-40 amino acids of Fiber-2 as crucial for KPNA3/4 interaction. FAV4_Del replicated significantly slower than wild-type FAdV-4, with viral titers 100 times lower (10^5 $TCID_{50}$/mL vs. $6x10^7$ $TCID_{50}$/mL) at 96 hpi. Immunofluorescence and western blot analyses confirmed these results. FAdV-4-infected chickens exhibited symptoms at 2 dpi, with mortality rates of 10%, 90%, and 100% at 3, 4, and 5 dpi, respectively, along with severe necropsy findings. Conversely, FAV4_Del-infected

chickens showed no symptoms or organ damage. Viral titers from cloacal swabs in FAdV-4-infected chickens ranged from 10^3-10^4 $TCID_{50}$/ml, but no virus was detected in FAV4_Del-infected chickens from 2 to 8 dpi. Liver viral titers in FAdV-4-infected chickens reached 10^5–10^7 $TCID_{50}$/mL, whereas FAV4_Del-infected chickens had titers below 10^3 $TCID_{50}$/mL, with similar findings in kidney and spleen tissues. Chickens previously infected with FAV4_Del displayed complete protection upon challenge, with no symptoms or virus detection, unlike the control group, where high viral titers (10^3-10^4 $TCID_{50}$/mL) were found in cloacal swabs and liver, spleen, and kidney tissues at 2 to 4 dpc. These results highlight FAV4_Del's potential as an attenuated vaccine candidate [127].

Ensuring the safety of vaccine production and administration is a critical priority in preventing unintended exposure to virulent pathogens. To reduce the potential biosafety risks of the virulent strain-inactivated vaccine during production or clinical immunization, the development of inactivated vaccines comprising non-pathogenic strains should be pursued. Therefore, an inactivated recombinant vaccine was produced [72].

Neutralizing antibodies exceeding 4 log_2 at seven dpv and 8 log_2 at 14 dpv were detected in IM and SC vaccinated chickens. By two dpi, 40% of challenge control chickens died, while immunized groups remained symptom-free throughout.

Anatomical and histopathological analysis revealed no abnormalities in the immunized and healthy control groups. In contrast, the control group showed yellow-brown pericardial effusion, liver enlargement, and extensive necrosis. High FAdV-4 DNA levels were detected in their liver, kidney, and spleen, while viral loads were negligible in the immunized and healthy controls. Given FAdV-4's ability to infect various avian hosts, the vaccine could be applied to commercial chickens, ducks, and wild birds, reducing environmental biosafety risks [68,72,128-131].

Third Generation Vaccines

1. Recombinant Vector Vaccines

Fowl Adenovirus Species C Vaccines

Hepatitis-hydropericardium syndrome (HHS) caused by the highly pathogenic fowl adenovirus serotype 4 (FAdV-4) has resulted in substantial economic losses to the poultry industry globally [48]. The fiber-2 gene, a significant virulence determinant, is also a vital vaccine target against FAdV-4. Therefore, the CRISPR/Cas9-based homology-dependent recombinant technique was used to replace the fiber-2 gene with EGFP (enhanced green fluorescent protein) and generate a novel recombinant virus, designated FAdV4-EGFP-rF2. Although FAdV4-EGFP- rF2 showed low replication ability compared to the wild-type FAdV-4 in LMH cells, FAdV4-EGFP-rF2 could effectively replicate in LMH-F2 cells with the expression of Fiber-2 [25]. FAdV4-EGFP-rF2 was highly attenuated in chickens and protected FAdV-4. Without fiber-2, it induced neutralizing antibodies comparable to those with fiber-2. Fiber-1 triggers infection, while fiber-2 determines virulence and serves as a protective immunogen. FAdV-1, FAdV-4, and FAdV-10 uniquely possess fiber-1 and fiber-2, highlighting fiber-2's significance in pathogenesis and vaccine development [2,25,126].

Although the hexon and fiber-2 genes are associated with the pathogenic CH/HNJZ/2015 strain's virulence [132], the roles of these two genes in other virulent strains and their exact locations remain unknown. A single amino acid at position 188 of the hexon protein was further identified as the determinant for FAdV-4 pathogenicity. Virulence based on a single amino acid often appears in RNA viruses but is rarely reported in DNA viruses. Amino acid 367 of the Tembusu virus E protein plays a critical role in pathogenesis [133], and amino acid 431 of the H1N1 swine influenza virus (SIV) PB2 protein determines its virulence in mice [134]. Recently, there was a report that the single amino acid R188 of the hexon protein is responsible for novel FAdV-4 pathogenicity. Thus, the hexon gene, but not fiber-2, was identified as the critical virulence gene for FAdV-4 [26].

All chickens survived and showed no symptoms when inoculated with the rR188I mutant strain, and their serum neutralized the non-pathogenic E188I mutant strain. FAdV-4 hexon sequences from natural non-pathogenic strains (ON1, KR5, B1-7) showed a conserved isoleucine at position 188, whereas pathogenic strains had arginine. The R188I mutant may activate the innate immune or complement systems to neutralize non-pathogenic FAdV-4 [26].

The infection of FAdV-8a alone cannot cause severe disease [135,136]; however, the outcome may become complicated when co-infected with other pathogens or other serotypes of fowl adenoviruses [135,137], which raises concerns for the prevention of FAdV-8a. Previously, De Luca et al.[61] and Schachner et al.[30] demonstrated that either wild-type fiber or chimeric fiber derived from FAdV-8a could protect against homologous challenge. So far, only subunit chimeric Fiber proteins (crecFib-4/11 and crecFib-8a/8b) and inactivated chimeric FAdV-4 with FAdV-8b Fiber were developed [30,44,74]. However, the chimeric FAdV-4 with a fiber of FAdV-8a has not yet been developed. Thus, in a study, A new recombinant virus, FAdV4-F/8a-rF2, which expresses the Fiber protein of FAdV-8a, was created using the CRISPR-Cas9 and Cre-LoxP systems, with FA4-EGFP serving as the template virus.

FAdV4-F/8a-rF2 showed vaccine potential, with chickens displaying no symptoms, lesions, or virus shedding. Histopathology matched negative controls, indicating attenuation. High neutralizing antibody titers were observed for FAdV-4 (938.7) and FAdV-8a at 21 days post-infection. This first bivalent vaccine candidate against FAdV-4 and FAdV-8a in China offers promising protection [32].

Additionally, while various inactivated and subunit vaccines have been developed for FAdV-4 or FAdV-8 individually [61,120,138], no inactivated or recombinant genetically engineered vaccine targeting both FAdV-4 and FAdV-8 simultaneously has been documented.

The recombinant FA4-F8b expressed FAdV-8b fiber and FAdV-4 Fiber-1/Fiber-2. The inactivated vaccine induced neutralizing antibodies against FAdV-4 and FAdV-8b, with NT averages at 7, 14, 21, and 28 dpv reaching 0, 1.3, 3.5, 7.8 (FAdV-8b) and 0, 1.8, 3.8, 9.8 (FAdV-4). No antibodies formed for FAdV-8a. All vaccinated chickens survived; challenge controls died at 3 dpc with HHS lesions. Histopathology showed no symptoms in vaccinated groups. Elevated viral titers were detected in the liver, spleen, kidney, and cloacal swabs of challenge control groups (3, 4, 5, and 7) at 1-9 dpc, while Groups 2 and 6 showed minimal to no viral presence. FA4-F8b demonstrates robust protection, effectively preventing infection and HHS symptoms [74].

Due to FAdV-4's emergence and high pathogenicity, vaccine development is limited. Live FAdV-4 vector bivalent or multiple vaccines could reduce production costs and workload. Therefore, deleting 10 left-end and 13 right-end ORFs identified non-essential regions for replication using an EGFP-indicator virus, marking the first systematic identification. This provides insertion sites for exogenous genes and valuable information for gene function studies, supporting the development of live FAdV-4 vector bivalent or multiple vaccines to reduce production costs and workload.

The FAdV-4 vector expressed vvIBDV VP2 protein, and rHN20-vvIBDV-VP2 conferred complete protection against FAdV-4 and vvIBDV, with nearly $\log_2 8$ antibody titers at 21 dpv. Seven combinations (rDL1-EGFP to rDR3-EGFP) enable multivalent vaccine development, demonstrating successful exogenous gene delivery and protection against HHS and related diseases [37].

Although several inactivated or subunit vaccines have been developed against FAdV-4, live-attenuated vaccines for FAdV-4 are rarely reported. Hence, a recombinant virus FA4-EGFP, expressing the EGFP-Fiber-2 fusion protein, was generated by the CRISPR/Cas9 technique.

Necropsy and histopathology showed no lesions in FA4-EGFP-inoculated chickens, unlike severe hepatic damage in wild-type FAdV-4 infections. Viral loads were significantly lower, with no spleen detection and shedding ceasing by three dpi. Neutralizing titers at 14 dpi for 10^6, 10^5, and 10^4 $TCID_{50}$ were 2.8, 3.0, and 2.3, rising to 7.5, 5.2, and 3.5 at 21 dpi, outperforming the inactivated vaccine (0.8, 3.9) [127].

Adenoviral genes are genus-common or genus-specific. Genus-common genes, conserved in the genome's central region, are crucial for structural proteins, replication, and encapsidation. Genus-specific genes at genome ends encode non-structural proteins for virus-host interactions. While HAdV-C genus-specific genes are well-studied, FAdV genes remain unexplored. Understanding these functions supports attenuated vaccine development, offering high efficacy, easy inoculation, and low costs [81,91,139]. A reverse genetics system was developed to modify the FAdV-4 genome using cell-free restriction digestion and Gibson assembly. Three recombinant viruses were created: FAdV4-GFP (replacing ORF1, ORF1b, and ORF2 with GFP), FAdV4-GX4C (replacing ORF4 with mCherry), and FAdV4-CX19A (deleting ORF19A). Inoculation of chicken embryos with FAdV4-GX4C resulted in 100% mortality between days 5-10, while FAdV4-GFP caused embryo deaths starting on day 8. FAdV4-CX19A had delayed mortality on day 11, suggesting that ORF19A is linked to virulence but is not essential for replication. Enhanced replication was observed in FAdV4-CX19A, though not statistically significant [41].

Understanding viral gene roles is crucial for vaccine development and adenoviral vector optimization. Identifying essential genes aids vector construction, while deleting nonessential genes increases cloning capacity. Essential gene deletion creates replication-defective vectors, and trans-expressed essential genes enable virus-packaging cell lines. Genus-specific essential genes in FAdV remain unidentified. An adenoviral plasmid carrying deletions spanning all 22 genus-specific ORFs of FAdV-4 was constructed to investigate this. Four out of 14 embryos died in the XHE-CX19A group, while all embryos survived in the XGAM1-CX19A group, highlighting differences in virulence among mutants. Using reverse genetics, 21 FAdV-4 mutants with deletions across the genome's ends were constructed. No genus-specific gene was essential for replication in LMH cells or primary chicken hepatocytes, providing a foundation for FAdV-4 vector development. Growth differences in mutants between LMH cells and chicken embryos indicate potential for attenuated FAdV-4 vaccine construction [42].

Identifying and manipulating viral essential and non-essential genes supports adenoviral vector construction and recombinant vaccine development, exemplified by a trivalent vaccine targeting serotypes 4, 8, and 11.

A recombinant FAdV-4 virus containing fiber genes of FAdV-8b and FAdV-11 was constructed using an amp-ccdB cassette and a p15A-cm-HNJZ-fber/8b plasmid [46]. The chimeric virus rFAdV-4-fber/8b + 11, inactivated with formaldehyde and formulated into an oil-emulsion vaccine, induced detectable antibodies in SPF chickens by one week post-immunization. Anti-FAdV-4 Fiber-2 and FAdV-11 antibody levels reached nearly 1 OD (450 nm), and anti-FAdV-8b levels approached 2 OD by the second week. All vaccinated chickens survived FAdV-4, FAdV-8b, and FAdV-11 challenges without clinical signs.

Vaccinated chickens showed no gross or histopathological lesions, unlike the challenge control groups. Viral DNA copy numbers in the liver, heart, spleen, kidney, lung, cecal tonsil, pancreas, bursa fabricius, proventriculus, and duodenum were significantly lower in vaccinated chickens. FAdV-4, FAdV-8b, and FAdV-11 excretion ceased by 3, 4, and 5 days post-challenge, respectively.

A novel recombinant virus, rFAdV-4-fber/8b + 11, was developed in another study to address the common clinical co-infections with different FAdV serotypes. This recombinant virus co-expresses the Fibers of FAdV-8b and FAdV-11 by inserting the FAdV-11 fiber gene into the 1966-bp deletion region of the rFAdV-4-fber/8b genome, providing a potential trivalent vaccine to prevent and control HHS and IBH [46].

To address challenges in co-infections and enhance antiviral strategies, novel recombinant FAdV-4 viruses have been developed, employing advanced genetic editing techniques to efficiently express foreign genes.

Traditional reverse genetics for recombinant fowl adenovirus is inefficient. Using FA4-EGFP as a template, the fiber-2 gene was edited via CRISPR/Cas9 and Cre-Loxp technologies, creating an efficient double-fluorescence system. This method successfully developed recombinant virus FAdV4-HA(H9), expressing the HA gene of H9N2 influenza. A rapid and efficient method for generating fiber-2-edited attenuated recombinant FAdV-4 was demonstrated for the first time in this study [59] utilizing CRISPR-Cas9 and Cre-LoxP systems. The recombinant virus FAdV4-HA(H9) showed vaccine potential against FAdV-4 and H9N2 AIV. Chickens challenged with XZ491 exhibited significantly lower oropharyngeal viral titers at 5 dpc when inoculated with FAdV4-HA(H9) compared to controls, as confirmed by qRT-PCR. The recombinant virus demonstrated attenuation and protective efficacy against H9N2 AIV [59].

The Fiber protein of FAdV is crucial for infection and pathogenicity. Unlike most FAdV serotypes, FAdV-4 has two Fiber proteins: Fiber-1 and Fiber-2 [140]. Fiber-2 is closely linked to virulence, with fiber-2-edited recombinant viruses showing significant attenuation in SPF chickens [132]. Fiber-1, however, directly facilitates viral infection via its knob and shaft domains interacting with the CAR homology receptor [126]. Building on these advancements, further efforts have focused on targeting additional structural proteins to develop novel recombinant FAdV-4 viruses with enhanced functionality.

In a study by Mu et al.[62], another structural protein, Fiber-1 of FAdV-4, was targeted to rescue a novel recombinant virus, FAdV4-RFP_F1, which expresses a fusion protein of RFP and Fiber-1. To explore the potential for editing Fiber-1 and to create a live-attenuated FAdV-4 vaccine or vector, researchers used CRISPR/Cas9 to modify the region between the tail and shaft at the 87th amino acid, generating the recombinant virus FAdV4-RFP_F1 expressing a fusion protein of Fiber-1 and Red Fluorescent Protein (RFP). FAdV4-RFP_F1 was successfully generated.

In vivo evaluation showed FAdV4-RFP_F1 caused no clinical symptoms or mortality in SPF chickens, unlike WT FAdV-4, which caused 100% mortality by four dpi and severe hepatitis-hydropericardium syndrome. WT FAdV-4 reached 10^8 $TCID_{50}$/mL in organs, while FAdV4-RFP_F1 showed no detectable virus by $TCID_{50}$. PCR confirmed early tissue presence, indicating high attenuation and reduced pathogenicity.

Chickens infected with FAdV4-RFP_F1 produced high neutralizing antibody levels (mean titer ~27.4) by 21 dpi, unlike controls. Upon WT FAdV-4 challenge, 82% of control chickens died with severe lesions and high viral titers, whereas FAdV-4-RFP_F1-infected chickens showed no symptoms, mortality, or detectable viral titers, confirming effective protection. The N-terminal domain of fiber-1 was identified as a potential insertion site for foreign gene expression [62].

FAdV-C is another species of fowl adenovirus that contains two fiber genes. Recently, a novel FAdV-C4 strain has been identified as the cause of hepatitis-hydropericardium syndrome (HHS) outbreaks in chickens in China, leading to substantial economic losses in the poultry industry [141, 142]. Despite its impact, the virology of FAdV-4 remains poorly understood. Another study aimed to investigate the distinct roles of FAdV-4 fibers in viral infection using reverse genetics techniques.

Recombinant FAdV-4 viruses expressing Fiber-1 and Fiber-2 were developed. Fiber-1 was essential for rescuing FAdV-4, as no GFP+ foci formed in pKFAV4XF1-GFP-transfected LMH cells, whereas Fiber-2 was dispensable, enabling replication in pKFAV4XF2-GFP cells. Cytopathic effects and GFP signals confirmed virus growth, and sequencing validated the Fiber-2 mutation. Fiber-1 knob protein (F1H6) inhibited FAdV-4 infection in LMH cells by up to 75% at 1-4 µg/mL, while Fiber-2 knob (F2H6)

had no effect, confirming Fiber-1 as the primary binding ligand. In chicken embryos, FAdV4-GFP caused 100% lethality by day 12, while 40% of FAdV4XF2-GFP-infected embryos survived up to 14 dpi. FAdV4-GFP exhibited 2-3 orders of magnitude higher viral levels in the liver than FAdV4XF2-GFP [63].

Vaccination with live attenuated and inactivated vaccines continues to be the most practical approach to controlling Newcastle Disease (ND). The naturally avirulent NDV strain LaSota has been widely used as a live vaccine globally for over 60 years, demonstrating excellent safety and stability [143]. Advances in reverse genetics have enabled the development of the LaSota strain and other NDV strains as vectors for expressing foreign antigens, offering applications in vaccine development and gene therapy [144,145]. The NDV LaSota strain was used as a vector to generate a recombinant NDV virus expressing the full-length fiber-2 gene from a novel FAdV-4 genotype isolated in China [146]. Since unpublished data indicated that live rLaSota-fiber2, delivered via drinking water or ocular administration, did not protect against hypervirulent FAdV-4 challenge, despite offering full protection against NDV, the efficacy of rLaSota-fiber2 as a bivalent vaccine candidate against FAdV-4 and NDV was assessed through intramuscular administration in another study [70].

The NDV LaSota strain expressing the fiber-2 gene of hypervirulent FAdV-4 was developed as an attenuated recombinant vaccine. Single-dose vaccination of 2-week-old SPF White Leghorn chicks with live or inactivated rLaSota-fiber2 induced strong antibody responses against NDV (over 6 $\log_2$) and FAdV-4 (over 1.5 OD). The live vaccine generated higher and earlier titers (over 0.5 OD for FAdV-4 and over 6 $\log_2$ for NDV) compared to the inactivated vaccine (less than 0.5 OD for FAdV-4 and over 2 $\log_2$ for NDV).

Complete protection was observed with the live vaccine, while the inactivated formulation conferred 70% protection against FAdV-4. NDV shedding ceased by day 3 post-challenge in the live vaccine group and by day 6 in the inactivated group. Both groups showed reduced FAdV-4 shedding compared to non-vaccinated birds, which continued shedding until death. The live vaccine induced higher HI antibody titers and better overall protection [70].

Given the need for vaccines offering dual protection against FAdV-4 and NDV, other viral pathogens have also been recombined with FAdV to confer dual immunogenicity. Co-infections of novel FAdV-4 and vvIBDV have been observed in farms due to the overlapping susceptible ages of chickens, resulting in more severe diseases and posing challenges to the poultry industry [146]. Developing a vaccine providing simultaneous protection against both viruses is considered essential. In previous research, an artificial non-pathogenic FAdV-4 strain expressing vvIBDV VP2 was constructed [26]. In a recent study, the immunogenicity of this recombinant virus as an inactivated vaccine was evaluated.

An inactivated vaccine was developed using the recombinant FAdV-4 rHN20-vvIBDV-VP2 strain, created by inserting the vvIBDV VP2 gene into a non-pathogenic FAdV-4 backbone. The inactivated bivalent vaccine, containing 10^7 PFU/mL of virus and stored at −80°C, induced 100% neutralizing antibody positivity against FAdV-4 and vvIBDV three weeks post-immunization. All vaccinated chickens survived FAdV-4 and IBDV challenges without clinical signs. Histopathology and viral load analysis at four dpi showed no liver or bursal lesions in immunized groups, unlike non-immunized chickens, which exhibited severe hepatic damage and lymphocyte depletion.

The vaccine effectively inhibited FAdV-4 and vvIBDV replication, prevented pathological damage, and reduced environmental shedding. High viral loads were detected only in non-immunized chickens. Strong neutralizing antibody responses were observed, with titers exceeding 8 $\log_2$ for FAdV-4 and nearly 8 $\log_2$ for IBDV. Derived from a non-pathogenic strain, the vaccine minimized risks associated with incomplete inactivation or contamination. It was suitable as a standalone bivalent vaccine and a replacement for monovalent and VP2 subunit vaccines. Identified FAdV-4 genome regions offer potential for future vaccine development [26].

Although several inactivated or subunit vaccines have been developed against FAdV-4 and DAdV-3, such as the recombinant viral vector FAdV-4 and inactivated IBDV vaccine [26], there remains an urgent need to develop a novel bivalent vaccine candidate targeting both FAdV-4 and DAdV-3.

The Fiber-2 protein of DAdV-3 can induce neutralizing antibodies and be used as an efficient protective immunogen to offer complete protection against DAdV-3 infection [65,147]. Based on the previous study, fiber-2-edited or fiber-2-deleted FAdV-4 is a highly attenuated and protective vaccine candidate [25,39]. However, a bivalent vaccine against both FAdV-4 and DAdV-3 is not available. Previous studies revealed that Fiber-1 of FAdV-4 directly triggered the viral infection via its shaft and knob domains, and Fiber-2 of FAdV-4 was identified as a significant virulent determiner [2]. More recently, it was found that Fiber-2 of FAdV-4 was not necessary for viral replication and induction of neutralizing antibody, and fiber-2-edited or fiber-2-deleted FAdV-4 was a highly attenuated and protective vaccine candidate [25,39], highlighting that fiber-2 can be as an editable or inserting site for generating live-attenuated recombinant FAdV-4 vaccines against both

FAdV-4 and other pathogens. Therefore, a recombinant FAdV-4 expressing Fiber-2 protein of DAdV-3 using CRISPR/Cas9 and Cre-LoxP systems were generated [35].

The recombinant virus rFAdV-4-Fiber-2/DAdV-3 replicated efficiently in LMH cells, reaching a peak titer of $10^{8.5}$ TCID50/mL. In SPF chickens, it induced high antibody levels and neutralizing titers against FAdV-4 and DAdV-3 without clinical symptoms. This study is the first to generate rFAdV-4-Fiber-2/DAdV-3 using CRISPR/Cas9 and Cre-LoxP. However, its efficacy in ducks and potential to induce cellular immunity were not tested [35].

In addition to recombinant vector vaccines from other viral families, vaccines derived from the same genus, but different serotypes have also been introduced.

Isolation of multiple FAdV serotypes from the same diseased bird is common, highlighting the lack of cross-protection between different serotypes [45,148]. Mixed infections involving HHS and IBH have also been observed in field cases [149]. However, there is currently no commercially available vaccine that targets both FAdV-4 and FAdV-8b infections. FAdVs from different species exhibit significant structural and genomic differences. Previous research has shown that the virulence of FAdV-4 is not dependent on fiber-1, although fiber-1 plays a direct role in mediating infection by pathogenic FAdV-4 [2, 150]. In a recent study, a novel hypothesis was proposed for the first time: replacing the fiber-1 of FAdV-4 with the fiber of FAdV-8b. To test this, a chimeric FAdV-4 virus containing the fiber of FAdV-8b, named rFAdV-4-fiber/8b, was successfully constructed.

Chickens vaccinated with the inactivated rFAdV-4-fiber/8b vaccine developed antibodies against FAdV-4 fiber-2 and FAdV-8b fiber, reaching over 1.5 OD and nearly 1.5 OD by the third week, respectively. All vaccinated chickens survived, while the control group showed 50% mortality 102 h post-infection. Necropsy confirmed protection, with vaccinated chickens displaying healthy organs, unlike unvaccinated ones challenged with FAdV-4 or FAdV-8b, which exhibited severe lesions, including liver necrosis and hemorrhages.

qRT-PCR revealed significantly lower FAdV-4 and FAdV-8b loads in vaccinated chickens, while unvaccinated ones showed high viral copies (10^4-10^{12}). Vaccinated and control groups exhibited no lesions, unlike unvaccinated chickens with severe organ damage, confirming the robust protective efficacy of the inactivated rFAdV-4-fiber/8b vaccine.

PCR analysis revealed that viral shedding was minimal in vaccinated chickens. In the FAdV-8b challenge group, only 2 of 10 vaccinated chickens shed the virus on day 1 but shedding ceased entirely by day 2. Vaccinated chickens in the FAdV-4 challenge group showed no viral shedding throughout the experiment. A single dose provided full protection against both serotypes [76].

Recent studies on FAdV-4 vaccines have mainly focused on inactivated, subunit, and genetically engineered vaccines, while live FAdV-4 vaccines have not been extensively studied [70,138]. Live vaccines are typically based on low-pathogenic or non-pathogenic strains. It has been reported that three naturally non-pathogenic FAdV-4 strains have been isolated: ON1 (Canada), KR5 (Japan), and B1-7 (India). However, the protective effectiveness of these strains against FAdV-4 remains uncertain [77].

The FAdV-4 virulent strain HLJFAd15 (GenBank No. KU991797) from Heilongjiang, China, caused 100% mortality in SPF chickens. Sequencing revealed a 1966-base pair deletion at the genome's right end, identifying it as a novel FAdV-4 genotype [151]. The hexon of the HLJFAd15 strain was replaced with that of the non-pathogenic ON1 strain, creating the recombinant FAdV-4 strain rHN20, which maintained similar viral titers to the wild-type virus but lacked pathogenicity. Chickens immunized with 10^6 PFU of rHN20 showed strong neutralizing activity against FAdV-4 at 7 and 14 days post-vaccination. The intramuscular group exhibited the highest activity (over $6\log_2$) compared to intranasal ($6\log_2$) and subcutaneous (over $4\log_2$) groups. At 7 days, only the 10^6 and 10^5 PFU groups showed neutralizing activity. Post-challenge with 2000 PFU of FAdV-4, vaccinated chickens showed no clinical symptoms and achieved 100% protection, unlike the control group, which experienced mortality within 4 days.

High FAdV-4 copy numbers were detected only in the viscera of dead chickens from the unimmunized challenge control group. Immunized groups, regardless of route or dose, showed background levels similar to non-inoculated controls. The rHN20-based live vaccine provided effective protection, eliminating the need for antigen purification and adjuvant addition. Severe hydropericardium and liver lesions were only seen in challenge controls. Histopathological analysis confirmed healthy liver structures in immunized groups, demonstrating the vaccine's efficacy against HHS [77].

Fowl Adenovirus Species D Vaccines

FAdV-11-associated IBH is increasingly reported worldwide [152,153]. However, its pathogenesis remains poorly understood due to limited genome sequences and technical challenges in manipulating its large genome. Only 14 complete FAdV-11 genome sequences exist, with just one being non-pathogenic. A recent study [28] introduced the first reverse genetics platform for FAdV-11, offering an efficient tool to study its virulence genes and develop multivalent recombinant vaccines.

The FAdV-11 reverse genetics platform enables identifying virulence-associated genes and developing multivalent recombinant vaccines. ORF11 was found non-essential for *in vitro* replication, making it a suitable site for foreign gene insertion, facilitating future vaccine development [28].

2. Duck Adenovirus Vaccines

Duck adenovirus includes DAdV-1 and DAdV-2. In 2014, strain CH-GD-12-2014 was isolated in Guangdong Province, potentially representing DAdV-3 due to low genetic similarity with DAdV-2. Infected ducks exhibited yellowish livers with hemorrhagic spots, kidney enlargement, and bleeding [154]. To address the absence of an effective DAdV-3 vaccine, the VP1 protein of DHAV-1 was recombined into the DAdV-3 genome, creating a recombinant virus for dual prevention against DHAV-1 and DAdV-3 [55].

The antibody response to DAdV-3 in rDAdV3-VP1-188 and DAdV3 groups peaked at 4 weeks post-vaccination (over 3 OD values) and declined by 7 weeks. No antibodies were detected in the negative control group. Ducks in DAdV3 and rDAdV3-VP1-188 groups showed no significant histopathological damage [55].

3. DNA Vaccines

Fowl Adenovirus Species C Vaccine

A subunit vaccine using recombinant Fiber2 protein from the hypervirulent FAdV-4 GZ-QL strain and a Fiber2 DNA vaccine were developed. Both induced significant Fiber2-specific antibody levels (rFiber2 subunit: 50 µg at 24 pg/mL, 100 µg at nearly 26 pg/mL, 150 µg at 22 pg/mL; Fiber2 DNA vaccine: 20-22 pg/mL). The rFiber2 subunit vaccine achieved higher efficacy (80-100%) compared to the Fiber2 DNA vaccine (50-60%) and a commercial inactivated vaccine (80%). No significant histopathological changes were observed [33].

Challenges to FAdV Vaccine Development

Live-attenuated fowl adenovirus (FAdV) vaccines face limitations in efficacy, safety, and production. Long-term effects on layers remain unassessed, with concerns about potential viral shedding through cloaca and gizzard routes, raising questions about their safety and effectiveness in pullets [18]. Small sample sizes in studies limit generalizability, highlighting the need for further exploration of alternative vaccination routes and dosages to optimize immunization protocols [75].

The lack of vaccine production from propagated virus strains, as noted by Ugwu et al., raises concerns about genetic stability, including point substitutions in key viral genes. The absence of details on cell requirements and bioreactor conditions complicates large-scale vaccine production. The lack of vaccine production from propagated virus strains, as noted by Ugwu et al.[23], raises concerns about genetic stability, including point substitutions in key viral genes. The absence of details on cell requirements and bioreactor conditions complicates large-scale vaccine production. Some studies reported reduced pathogenicity in vaccinated chickens, but liver and tissue abnormalities persisted, along with viral shedding and suboptimal antibody responses, especially following intramuscular administration [40].

Undefined vaccine formulations and unspecified commercial vaccine types in studies hinder the comparison and assessment of vaccine effectiveness [5]. Further research is needed to enhance vaccine development by improving immunogenicity, understanding cellular immune responses, and refining production methods.

Live-attenuated and inactivated FAdV vaccines face limitations, including insufficient cellular immunogenicity research and the need to explore alternative vaccination routes, adjuvants, and doses. For example, Wu et al.[50] suggest further research on cross-protection against different FAdV serotypes, while Ugwu et al.[36] emphasize the need for alternative adjuvants beyond Montanide. Similarly, Mohamed Sohaimi et al.[24] note that the potential of adjuvants in enhancing cellular and humoral immunity has yet to be fully explored, particularly for inactivated FAdV-8b vaccines.

Concerns about the limited duration of vaccine efficacy studies, such as those monitoring efficacy for only four weeks [24] and the failure to assess the impact of booster doses on immune responses [36], also remain prominent. While some studies show improvement in viral shedding and body weight with boosters, more comprehensive investigations are needed to determine their long-term impact.

The lack of research on various adjuvants and formulations is another key limitation. Mehmood et al. [52] emphasize the need to compare adjuvants and determine optimal vaccine doses based on age and regimen. They highlight the importance of exploring alternative cell sources for vaccine production, assessing cross-protection against multiple serotypes, and developing more effective inactivation methods [72].

Many studies stress the need for field trials to confirm vaccine efficacy, highlighting the importance of optimizing formulations, dosages, and including cellular immunity, varied adjuvants, and diverse regimens for effective FAdV vaccine development [36,52].

Developing recombinant subunit vaccines for FAdV faces challenges, notably the insufficient evaluation of cellular immune responses, including T-cell activation

and cytokine production, limiting insights into vaccine-induced immunity [12,21,53,60,66,67]. Additionally, several studies did not assess the potential impact of different adjuvants, highlighting the need to explore alternative adjuvant formulations to enhance vaccine efficacy [30,49,67].

Several studies observed adverse clinical signs such as dullness, ruffled feathers, liver changes, and gastrointestinal issues post-vaccination, suggesting potential safety concerns that should be addressed in future research [34,51,57,60,66]. Mortality rates, although low, were reported in some groups, raising concerns about vaccine-associated risks [30,34]. Moreover, viral shedding and histopathological lesions were still detected in vaccinated birds, indicating that vaccine formulations and delivery methods might require refinement to eliminate these issues [47,73].

Vaccine efficacy concerns exist between chicken breeds, such as layer SPF and broilers, due to immune response variations impacting effectiveness [44]. Many studies focused on short-term effects, lacking data on long-term immunity or humoral response durability [46,49]. Some vaccines require multiple doses for optimal protection, posing challenges for large-scale vaccination programs [47,73].

Alternative vaccination routes, such as oral, spray, or intramuscular, require further exploration alongside optimal dosing strategies and dose comparisons [12,43]. Cross-protection against different FAdV serotypes remains underexplored, necessitating further research for broader vaccine coverage [61,117]. Identifying specific mutations in virulent FAdV-4 strain domains linked to unique immunogenic properties also warrants investigation [58].

Finally, the small sample sizes used in some studies limit the generalizability of the findings and call for larger-scale trials and extended monitoring to understand vaccine efficacy and safety under real-world conditions [30,57].

The limitations of recombinant vector vaccines against various FAdV serotypes, as discussed in multiple studies, highlight several common challenges and areas for further investigation.

1. **Inactivation and Antigenic Alterations:** The use of 2% formaldehyde for inactivating recombinant viruses, such as FAdV-1 and DAdV-3, may alter viral antigens and reduce immune response efficiency, raising concerns about the impact of inactivation methods on vaccine integrity and protective efficacy [55].
2. **Unexplored Immune Responses:** Many studies did not assess humoral or cellular immunity, which is crucial for a comprehensive understanding of vaccine efficacy [42]. The lack of investigation into cellular immune responses, especially in live vaccine studies, is a common limitation across various serotypes, including FAdV-4 and DAdV-3 [37,39]. Additionally, the long-term protection rate in chickens was not consistently evaluated [76], and more studies are needed to measure the effectiveness of these vaccines in poultry.
3. **Lack of *In Vivo* Testing and Protection in Chickens:** Many recombinant vector vaccines have not been tested in chickens [63], with some studies lacking data on protection rates or immune responses [28,41,42]. The ability of FAdV-4 Fiber-1 to induce neutralizing antibodies requires further investigation, along with the roles of Fiber-1 and Fiber-2 in protection against FAdV-1 [25]. The absence of *in vivo* data emphasizes the need for additional research to confirm vaccine and antibody efficacy in chickens [65].
4. **Cost and Production Challenges:** The preparation of recombinant vaccines, mainly inactivated ones, is costly [55], which raises concerns about the scalability and cost-effectiveness of these vaccines for widespread use, suggesting that production methods need optimization to reduce costs and improve feasibility.
5. **Optimization of Administration Route and Dose:** variations in vaccination doses and routes, such as oral or subcutaneous delivery, have not been sufficiently tested in many studies, limiting the potential for improving vaccine administration and effectiveness [25,46,62,68].
6. **Viral Replication and Protection Levels:** While some vaccines have demonstrated replication *in vitro,* their performance *in vivo* has been inconsistent. For example, FAdV-4 vaccines have shown slow replication in cell cultures, which may hinder their protective capabilities *in vivo* [39]. The effectiveness of vaccines in inducing robust immune responses and achieving full protection against FAdV infections in chickens remains a key area for further exploration since some studies reported incomplete protection [33,59,64].
7. **Co-infection and Cross-Protective Efficacy:** The impact of co-infections, such as FAdV-4 combined with IBDV or other poultry pathogens like NDV, has not been thoroughly evaluated, despite the potential for co-infection to alter vaccine efficacy [35,37,70]. Furthermore, vaccines developed for one serotype may not offer cross-protection against others, as shown in studies of FAdV-8a and FAdV-4 vaccines [74].
8. **Safety Concerns:** Some recombinant vaccines have raised safety concerns, including minor liver inflammation and potential viral replication in tissues [25,77]. Additionally, the risk of reversion to virulence in recombinant live attenuated vaccines [32] necessitates further studies to assess long-term safety and stability.

9. **Lack of Molecular Investigation Towards Pathogenicity and Mechanism of Neutralization:** For instance, the molecular basis of pathogenicity and the mechanism of virus neutralization FAdV-4 and 8b remain poorly understood, highlighting the need for further investigation [26,76].
10. **Geographical and Environmental Considerations:** Most studies often focused on specific strains or geographical regions, limiting the generalizability of findings. Broader investigations are necessary to account for diverse environmental factors and interactions with other pathogens that influence vaccine efficacy.
11. **Maternal Antibodies and Field Conditions:** The impact of maternal antibodies and field conditions on vaccine performance has not been thoroughly addressed. These factors can significantly influence the immune response and effectiveness of vaccines in commercial poultry operations.

Recombinant vector vaccines for FAdV show promise but have challenges, such as preserving antigen integrity, testing adjuvants and vaccination methods, and evaluating immune responses. *In vivo* testing is crucial for safety and efficacy. Future research should focus on improving production, cross-protection, and addressing safety concerns to enhance vaccine effectiveness for poultry.

Future Directions Towards Fowl Adenovirus Vaccine Development

Future FAdV vaccine development focuses on targeting multiple serotypes, viral components, and innovative methods. Genomic studies on FAdV-9 suggest non-essential genes like ORF1 and ORF19 could be used for foreign gene expression, enhancing FAdV-based vaccines or gene therapy tools [155,156]. Fiber gene variations in FAdV-8b indicate potential for fiber-based vaccines [42], while FAdV-4's unique use of shorter fiber and CAR receptor provides opportunities for targeted vaccine development [126].

Multi-epitope vaccine strategies, incorporating T and B cell-activating peptides, offer cost-effective, faster production than traditional vaccines [48,157,158]. For FAdV-4, studies on fiber-1 and penton show they are vital for replication and immune response, with complex roles in pathogenicity, suggesting future vaccine potential [80,81,150]. Host-virus interaction studies highlight Hsp70 and DnaJC7 as modulators of FAdV-4 replication [159]. miRNA-based strategies, like gga-miR-181a-5p, show promise in antiviral responses and vaccine development [160].

A study showed FAdV-4 Fiber-1, particularly its shaft and knob domains, conferred superinfection resistance against FAdV-8b in LMH cells, unlike FAdV-8b's hexon, penton, or Fiber proteins. Knocking out the CAR receptor suppressed FAdV-8b replication, but CAR is not its primary receptor. These findings suggest targets for controlling FAdV-4 and FAdV-8b infections [74]. In duck adenovirus (DAdV), Fiber-2 of DAdV-3 shows potential as a subunit vaccine, with epitope 108LALGDGLE115 identified [65].

Conclusion and Recommendation

Future vaccination strategies should account for genotype, bird age, and microbiota diversity. Large-scale application of developed vaccines is essential to assess their effectiveness or need for optimization. The transfer of passive immunity to progeny warrants further investigation. Integrated research between vaccinologists and immunologists is crucial to better understand cell-mediated immune responses and improve vaccine efficacy [161].

Highlight Keypoints

- FAdV and duck adenovirus are major pathogens in poultry and necessitates an effective vaccine strategy against the disease outbreak in poultry farms.
- Recent advances in the development of various vaccines against numerous avian adenovirus species, such as FAdV species A-E and Duck adenovirus 1 and 3 were discovered, in addition to the challenges that the conducted studies faced and the future aspects that must be focused on towards the production of effective vaccines.
- Multiple studies show that capsid proteins, especially fiber, provide the highest protection rates and the least viral shedding and clinical signs in poultry.
- Significant discrepancies exist among studies evaluating vaccines for poultry due to variations in bird type, age, challenge strains, vaccine strains, dosage, administration frequency, small sample sizes, and unexamined immune responses or pathogenic mechanisms.
- Future studies should prioritize testing vaccine candidates under real-life conditions, exploring FAdV infection mechanisms, and assessing passive immunity transfer to progeny post-immunization.

Declarations

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Review Article

Associations Between FASN Gene Polymorphism and Milk Production Traits in the Dairy Cattle: A Systematic Review and Meta-Analysis

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Abstract

In this study, the association between FASN gene polymorphism and milk production traits in dairy cattle was examined through a systematic review and meta-analysis. The Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines were followed for the analysis. The following databases were utilized: Google Scholar, Web of Science, PubMed, Taylor & Francis, Wiley Online Library, ResearchGate, Springer, and NCBI. The analysis was executed using the co-dominant model. Standardized mean differences (SMDs) and 95% confidence intervals were calculated using both random and fixed effect models to determine the effect size of the FASN gene polymorphism on milk production traits. All data were analysed using Stata 11.2 software. The results of the meta-analysis demonstrated statistically significant associations between FASN gene polymorphism and yield traits ($P<0.05$). The AA genotype exhibited a notable advantage over other genotypes with respect to milk and protein yield ($P<0.05$), and all genotype mean differences were statistically significant for fat yield ($P<0.05$). A subsequent analysis revealed no statistically significant differences between the genotypes concerning fat percentage or protein percentage ($P>0.05$). The results of the meta-analysis indicate that, in the context of marker-assisted selection in the field of dairy cattle breeding, the utilization of AA genotyped individuals can be advantageous in enhancing milk and protein yield.

Keywords: Meta-analysis, FASN, Polymorphism, SMD, Milk production traits

Introduction

In recent years, dairy farmers have increased their focus on milk quality, in addition to milk yield. This shift is driven by the understanding that quality has a direct impact on the selling price within the dairy industry [1]. The enhancement of the genetic quality of dairy cattle through selection constitutes a method employed to improve milk quality [2-4]. Furthermore, single-nucleotide polymorphism (SNP) at the DNA level can be utilized for marker-assisted selection to select superior cattle [5,6]. Milk production is a quantitative trait that is governed by the cumulative additive effect of a limited number of candidate genes. Consequently, genome-wide association studies (GWAS) targeting candidate genes associated with milk production traits are imperative for identifying statistically significant single-nucleotide polymorphisms (SNPs) that may be crucial in marker identification efforts [2,3,6].

SNPs have been utilized in numerous studies to enhance production performance and to understand the genetic background of quantitative traits. Genomic research studies focusing on the identification of markers for bovine milk production parameters have been reported from many parts of the world [2,3,6]. The employment of functional genetic markers has been demonstrated to be a remarkably efficacious strategy across various breeds, enabling precise characterization and evaluation of marker-assisted selection (MAS) methodologies within the framework of the domestic dairy sector. The integration of genomic and quantitative data facilitates the refinement of systems, thereby enhancing production efficiency. The identification of SNPs in candidate genes responsible for milk parameters is of great importance, considering the benefits of genomic selection [7].

The present article engages with the FASN gene, a candidate gene that plays a pivotal role in the synthesis of fatty acids. This gene is situated in a critical linkage region that has been demonstrated to be associated with milk production and the composition of milk fat. FASN gene polymorphisms represent a significant genetic factor influencing milk production and fat composition

in dairy and beef cattle [8-14]. It is a homodimeric enzyme that plays an important role in the general metabolism of animals [15] and during embryonic growth. It also plays a critical role in fat synthesis, especially in adult mammals [16,17]. The expression of the FASN gene in dairy cows has been demonstrated to influence milk fat composition by modulating fatty acid synthesis.

Genomic selection studies related to the FASN gene have been shown to facilitate genetic enhancement by increasing milk fat productivity and enabling genetic improvement, thereby enhancing the economic performance of cattle breeding and milk quality. In cattle, the FASN gene is located in chromosomal regions where there are multiple quantitative trait loci (QTL) affecting traits such as milk fat and fatty acid composition and adipose tissue [18]. The bovine FASN gene is located on the long arm of chromosome 19 (BTA19)-19q22 [19]. The complete sequence of the gene is 19,770 base pairs (bp) in length and contains 42 exons and 41 introns [20].

The A/G polymorphism in exon 34 of the bovine FASN gene is the FASN-16024G>A single-nucleotide substitution, which was identified by Roy et al.[21] in their study as 16009A>G. This significant polymorphism results in an alteration of the THR amino acid for ALA in a region where ketoacyl reductase and enol reductase activity are present. Of the 13 single-nucleotide polymorphisms (SNPs) identified in the FASN gene, two non-synonymous SNPs have been reported to directly relate to lactation parameters in exon 34 [22]. A/G at position 5848 was predicted to result in an amino acid change from threonine to alanine (T1950A), and T/C at position 5863 was predicted to result in an amino acid change from tryptophan to arginine. T1950A genotypes were found to express W1955R genotypes [22,23]. Consequently, it has been asserted that these SNPs are associated with yield traits in Holstein and Japanese Black cattle [24].

Meta-analysis is a statistical methodology designed for the estimation and evaluation of a common effect size, achieved by combining the results of several independent studies on a specific topic. Furthermore, it facilitates a re-evaluation of research findings, enabling the formulation of more robust and reliable conclusions [25]. This approach is employed in instances of duplicate sample size, that is, when independent studies are repeated in response to a comparable research question [26]. In the context of genetic studies targeting livestock, genes that influence traits of economic significance are of particular interest in the identification of genes. In the context of dairy cattle, the focus is on genes that contribute to variation in milk production. In the field of animal husbandry, dairy genes and hormones are regarded as significant genes due to their association with quantitative traits and biological significance [27].

The present meta-analysis was conducted to derive a comprehensive conclusion and consolidate the findings of numerous independent studies on the association between the FASN gene polymorphism and milk production traits in dairy cattle.

Materials and Methods

The Search Strategy for Sources

The selection of articles for the meta-analysis was conducted by the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist. A review of the extant literature on the effects of FASN gene polymorphisms and milk production traits (milk yield, fat yield, protein yield, fat content, protein content) in dairy cattle reveals a plethora of studies published in various journals, including Science Direct, Web of Science, PubMed, Taylor & Francis, Google Scholar, Wiley Online Library, ResearchGate, Springer, and NCBI, up to the year 2025. To identify additional studies not included in the previous search, a cross-check was performed on all references in these studies.

Inclusion and Exclusion Criteria

The following criteria have been established to determine inclusion and exclusion:

All authors of the article were conducted independently, and the articles selected for meta-analysis met the following criteria: Polymorphism of the FASN gene and association with milk yield, fat yield, protein yield, fat content, and protein content. The present study exclusively focused on the breeding of cattle of the Bos taurus species. The third item is a sample size, or the number of animals used for each genotype. The fourth point of analysis focuses on the standard deviation and errors associated with the relevant trait, as well as the mean average of that trait for each genotype.

The exclusion criteria were as follows:

-Reports submitted in abstract form.

-Absence of related indicators (e.g., milk yield, fat yield, protein yield, fat content, protein content).

-Summarized and presented publications.

-Absence of a number of cattle for each genotype.

-Inadequate indication of average means and standard deviation/errors per genotype.

-Repeating studies.

Data Extraction

The data was entered into a standard extraction form created in Microsoft Excel 2021 (Microsoft Corp.) to compute the least squares means and standard deviations

(SD) necessary for meta-analysis. A comprehensive review of the extant literature yielded the following information: The following variables were collected for each publication: the year of publication, the country of publication, the first author, the number of cattle, the genotype distribution, the milk yield, the fat yield, the protein yield, the fat content, the protein content, and the mean value and the standard deviation. The following formula was used to convert the standard error (SE) to the standard deviation $(SD = SE\sqrt{N})$.

Statistical Analysis

In the meta-analysis, the fixed effect model was employed for homogeneous data, and the random effect model was used for heterogeneous data, in order to determine the differences between means and proportions. The heterogeneity assumption was calculated based on I^2 with α=0.10 in the heterogeneity analysis. Standard mean differences (SMDs) and standard deviations with 95% confidence intervals (CIs) were calculated to estimate the difference between mean values (α=0.05 was accepted for the effect size test of the traits included). The standardised mean difference (SMD) was calculated using the Cohen method when the number of studies exceeded 10, and the Hedges method when the number of studies was less than 10. The PRISMA flow diagram illustrates the inclusion and exclusion criteria utilized for the selection of studies, with the relevant studies undergoing assessment through a forest plot. The statistical analyses were conducted using Stata 11.2 software (StataCorp 2001; Stata Statistical Software). The significance level was set at α=0.05 for the effect size test of the traits included in the study.

Results

Study Selection

In this study, a total of eighty-nine studies with a theoretical connection were identified through a systematic search of the database and a subsequent screening of the reference list. A total of 89 studies were included in the meta-analysis. However, after the preliminary evaluation, 46 of these studies were identified as duplicates and were thus removed. Following a comprehensive evaluation of the 43 papers, 35 reports were determined to be unsuitable for additional consideration *(Fig. 1)*. The rationale for

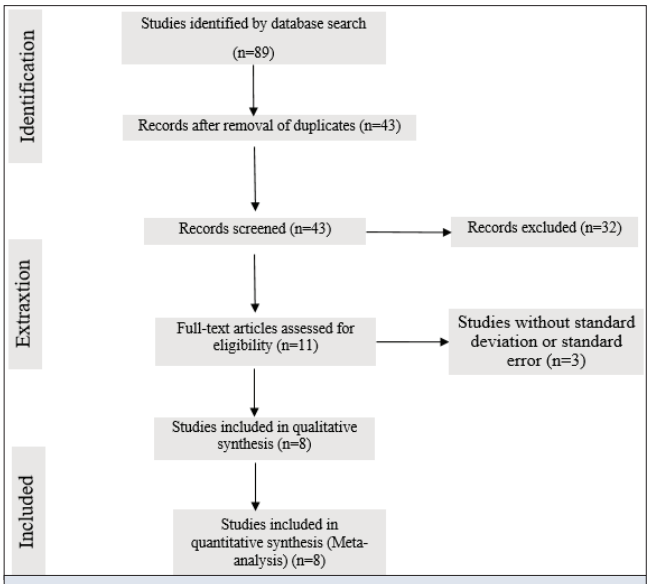

Fig 1. Flow chart summarizing literature search and article selection procedure

Table 1. *Characteristics of studies included in the meta-analysis of FASN gene polymorp-hisms and milk production traits*

Trait	Reference No	Breed	Genotype Frequency			Mean±SD		
			AA	AG	GG	AA	AG	GG
Milk yield	7	Holstein	0	63	37	0±0.0	1247.45±387.34	1189.5±445.26
	11	Simmental	2	15	32	6462.95±1294.01	5773.65±1535.64	5627.25±1483.79
	11	Crossbred Holstein	0	9	25	0± 0.0	6850.3±1390.8	6048.15±1570.75
	22	Holstein	0	58	137	0±0.0	11864.5±3577.13	11672.35±2498.95
	29	Simmental	1	20	51	8352.0±0.0	7430.95±1138.09	7404.86±1073.26
	29	Hols×Sim	0	9	24	0±0.0	7794.48±835.8	7501.45±1100.22
	30	Holstein	73	14	4	8080.82±2787.91	7812.57±1220.9	6995.75±652.6
	31	Holstein and Simmental	0	378	1018	0±0.0	8527.0±3071.87	8277.0±3094.89
	32	Holstein (1)[&]	12	57	40	9060.0±1118.9	8187.0±1509.97	8708.0±1650.71
	32	Holstein (2)	12	57	40	12566.0±1895.01	11018.0±2017.53	11386.0±2042.83
	32	Holstein (3)	12	57	40	12599.0±1389.1	11919.0±1864.81	12191.0±2042.83

Table 1. *Characteristics of studies included in the meta-analysis of FASN gene polymorp-hisms and milk production traits (continued)*

Trait	Reference No	Breed	Genotype Frequency			Mean±SD		
			AA	AG	GG	AA	AG	GG
Fat yield	7	Holstein	0	63	37	0±0.0	168.61±58.5	158.71±54.01
	29	Simmental	1	20	51	305±0.0	296.24±40.2	304.22±48.71
	29	Hols×Sim	0	9	24	0±0.0	347.33±46.95	308.85±53.47
	31	Holstein and Simmental	0	378	1018	0±0.0	349.8±124.43	339.6±124.43
	32	Holstein (1) &	12	57	40	387±51.96	351.0±67.95	363.0±56.92
	32	Holstein (2)	12	57	40	517±41.57	471.0±90.6	463.0±82.22
	32	Holstein (3)	12	57	40	491±48.5	489.0±90.6	480.0±88.54
Fat content	7	Holstein	0	63	37	0±0.0	4.05±0.71	4.22±0.61
	11	Simmental	2	15	32	3.43±0.72	3.93±0.85	3.99±0.85
	11	Crossbred Holstein	0	9	25	0±0.0	4.68±0.78	3.98±0.9
	22	Holstein	0	58	137	0 ± 0.0	3.77±1.14	3.87±0.06
	29	Simmental	1	20	51	3.65±0.0	4.01±0.4	4.11±0.43
	29	Hols×Sim	0	9	24	0±0.0	4.47±0.54	4.12±0.43
	30	Holstein	73	14	4	3.68±0.26	3.69±0.11	3.87±0.07
	31	Holstein and Simmental	0	378	1018	0±0.0	4.13±0.39	4.11±0.32
	32	Holstein (1) &	12	57	40	4.28±0.28	4.31±0.45	4.22±0.57
	32	Holstein (2)	12	57	40	4.42±0.42	4.29±0.45	4.12±0.57
	32	Holstein (3)	12	57	40	3.93±0.52	4.12±0.6	3.97±0.6
	33	Holstein × Friesian	26	19	0	2.48±0.18	2.79±0.21	0.0±0.0
Protein yield	7	Holstein	0	63	37	0±0.0	118.35±37.46	115.54±41.73
	29	Simmental	1	20	51	270±0.0	254.54±33.93	258.22±36.1
	29	Hols×Sim	0	9	24	0±0.0	282.13±22.07	268.41±35.39
	31	Holstein and Simmental	0	378	1018	0±0.0	290.5±95.27	285.4±95.72
	32	Holstein (1) &	12	57	40	306±34.64	279.0±45.3	290.0±44.27
	32	Holstein (2)	12	57	40	416±48.5	364.0±60.4	373.0±56.92
	32	Holstein (3)	12	57	40	413±45.03	388.0±60.4	387.0±56.92
Protein content	7	Holstein	0	63	37	0.0±0.0	2.9±0.24	3.08±0.18
	11	Simmental	2	15	32	3.49±0.37	3.61±0.43	3.64±0.4
	11	Crossbred Holstein	0	9	25	0.0±0.0	3.83±0.39	3.79±0.45
	22	Holstein	0	58	137	0.0±0.0	3.33±0.38	3.27±0.23
	29	Simmental	1	20	51	3.23±0.0	3.44±0.24	3.49±0.19
	29	Hols×Sim	0	9	24	0.0±0.0	3.63±0.2	3.59±0.24
	30	Holstein	73	14	4	3.08±0.26	3.07±0.04	3.1±0.02
	31	Holstein and Simmental	0	378	1018	0.0±0.0	3.43±0.39	3.48±0.32
	32	Holstein (1) &	12	57	40	3.39±0.17	3.41±0.15	3.35±0.19
	32	Holstein (2)	12	57	40	3.42±0.24	3.3±0.15	3.33±0.19
	32	Holstein (3)	12	57	40	3.28±0.17	3.26±0.23	3.19±0.19

SD: standard deviation, & (1,2,3): Lactation period

exclusion is outlined below: The present study was not designed to investigate the association between FASN gene polymorphisms and fatty acid traits in muscle. Furthermore, the data provided was inadequate for the objectives of the study. The study revealed a paucity of essential information, including genotype frequencies, standard deviation, and the necessary single-nucleotide polymorphisms (SNPs). The emphasis was placed on other traits, rather than on milk yield.

Table 1 provides information about the studies that are the focus of the research. It presents the means and standard deviations of the phenotypic characteristics of the examined breeds and genotypes in each study.

Association Analysis

The present study incorporated a total of five performance traits derived from eight articles. To facilitate a more profound comprehension of the subject, the collective results of the meta-analysis were illustrated in the forest plot displayed in *Fig. 2*, *Fig. 3*, *Fig. 4*, *Fig. 5*, and *Fig. 6*. The study presented the significant results of a systematic review and meta-analysis of FASN gene polymorphisms and their association with milk production traits (milk yield, fat yield, fat content, protein yield, and protein content) in dairy cattle.

In the context of association analysis, forest plots derived from these meta-analyses facilitate enhanced comprehension of the outcomes. These plots offer a visual representation of the means of the compared groups, the position of the overall mean differences across all studies, and the position of the mean differences of each study in the forest plots. To interpret the results of the meta-analysis, the black squares in the Forest plot graph represent the standard mean difference for the relevant feature, and the horizontal lines represent the 95% confidence interval. The results of the meta-analysis demonstrated a significant discrepancy between the comparison groups (P<0.05). This discrepancy was indicated by the presence of a diamond shape and a vertical dashed line, as well as by the presence of a continuous line when the confidence interval of the effect size was not altered. A similar evaluation was made within the confidence interval line of each study: If the confidence interval intersected with the vertical line, the difference was deemed to be non-statistically significant (P>0.05). Conversely, if the confidence interval did not intersect with the vertical line, the difference was considered to be statistically significant (P<0.05).

The analysis encompassed a total of five performance traits derived from eight studies, with each genotype subjected to separate analysis within the framework of co-dominant models. The results of this analysis are presented in *Table 2*. Furthermore, the results of the meta-analysis of the differences between allelic groups of the examined yield traits are presented in *Table 2* and *Fig. 2*, *Fig. 3*, *Fig. 4*, *Fig. 5*.

The random effect model was applied due to the heterogeneity regarding fat content exhibited by the AA-AG and AG-GG genotypes, and protein content exhibited by the AG-GG genotype. The fixed effect model was employed for other traits because they were found to be homogeneous.

Table 2. *Meta-analysis results of the associations between FASN gene polymorphisms and milk production traits*

Traits		N	SMD	95%CI	I^2	Model	P-Value (Meta-analysis)
AA vs AG	Milk yield	5	0.45	0.15;0.75	0.0	F	**0.003****
	Fat yield	3	0.37	0.00;0.73	0.0	F	**0.048***
	Fat content	6	-0.46	-0.94;0.02	**64.7**	R	0.060
	Protein yield	3	0.63	0.27;1.00	0.0	F	**0.001****
	Protein content	6	0.04	-0.26;0.34	0.0	F	0.798
AA vs GG	Milk yield	5	0.36	0.02;0.70	0.0	F	**0.040***
	Fat yield	3	0.42	0.04;0.79	0.0	F	**0.030***
	Fat content	5	-0.09	-0.43;0.25	0.0	F	0.610
	Protein yield	3	0.53	0.16;0.91	0.0	F	**0.006****
	Protein content	5	0.18	-0.16;0.52	0.0	F	0.291
AG vs GG	Milk yield	11	0.05	-0.04;0.14	0.0	F	0.309
	Fat yield	7	0.07	-0.03;0.17	0.0	F	0.144
	Fat content	11	0.02	-0.20;0.24	**67.0**	R	0.831
	Protein yield	7	0.02	-0.07;0.12	0.0	F	0.659
	Protein content	11	-0.04	-0.25;0.17	**64.0**	R	0.728

**P<0.05; **P<0.01; F: Fixed; R: Random; n: number of publications; Variation in SMD attributable to heterogeneity*

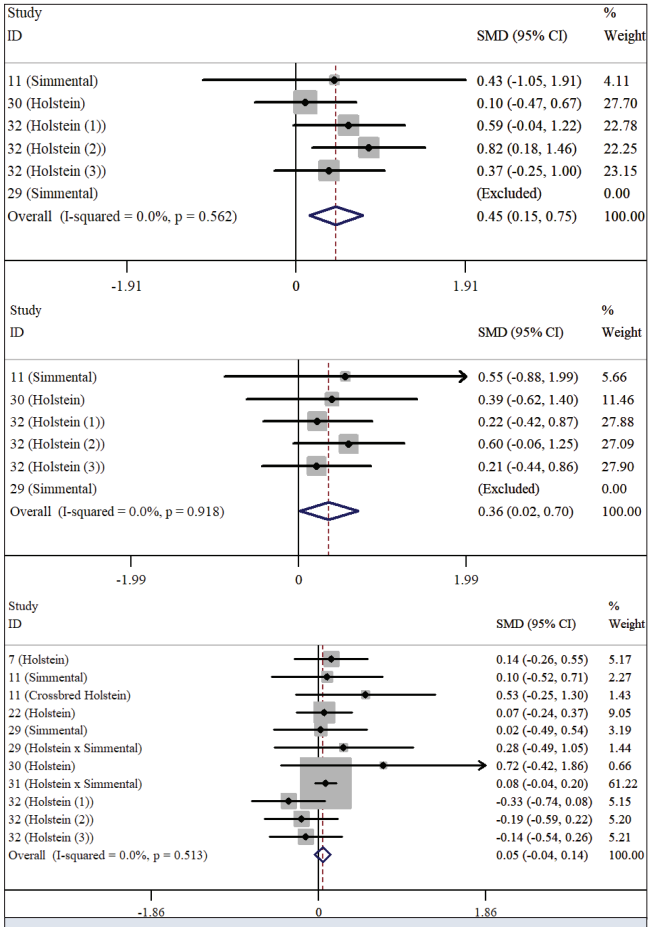

Fig 2. Forest plots of the studies examining the effect of *FASN* gene polymorphism on milk yield trait (Co-dominant model, from top to bottom; AA-AG, AA-GG, AG-GG, respectively)

The weight of the studies is represented by a square in each of the figures below. The horizontal line in the graph indicates the confidence interval for each study, with the overall result represented by a diamond at the bottom of the graph.

A subsequent meta-analysis of the available literature yielded results that were statistically significant (P<0.05) for the association between AA-AG, AA-GG, and milk yield, while AG-GG indicated non-significant (P>0.05) results *(Fig. 2)*. In a similar vein, the polymorphisms of the FASN gene with fat yield were found to be statistically significant (P<0.05), with the AA-AG and AA-GG polymorphisms demonstrating a statistically significant association with fat yield, and the AG-GG polymorphism indicating a non-significant association (P>0.05) *(Fig. 3)*. However, the relationship between fat content and the AA-AG, AA-GG, and AG-GG genotypes was found to be insignificant (P>0.05) *(Fig. 4)*. As demonstrated in Figure 5, the mean differences in protein yield for AA-AG and AA-GG genotypes were found to be statistically significant (P<0.01). However, no statistically significant differences were observed between AG-AG genotypes (P>0.05). In

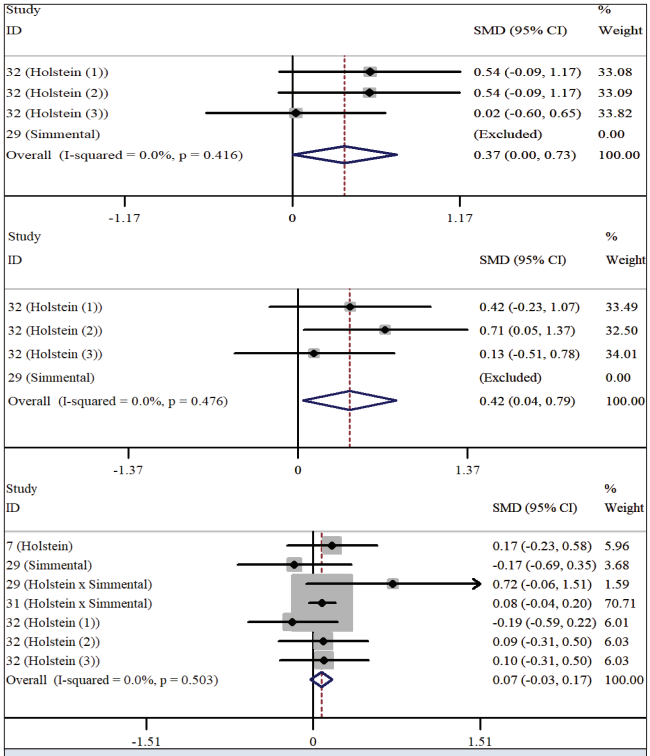

Fig 3. Forest plots of the studies examining the effect of *FASN* gene polymorphism on fat yield trait (Co-dominant model, from top to bottom; AA-AG, AA-GG, AG-GG, respectively)

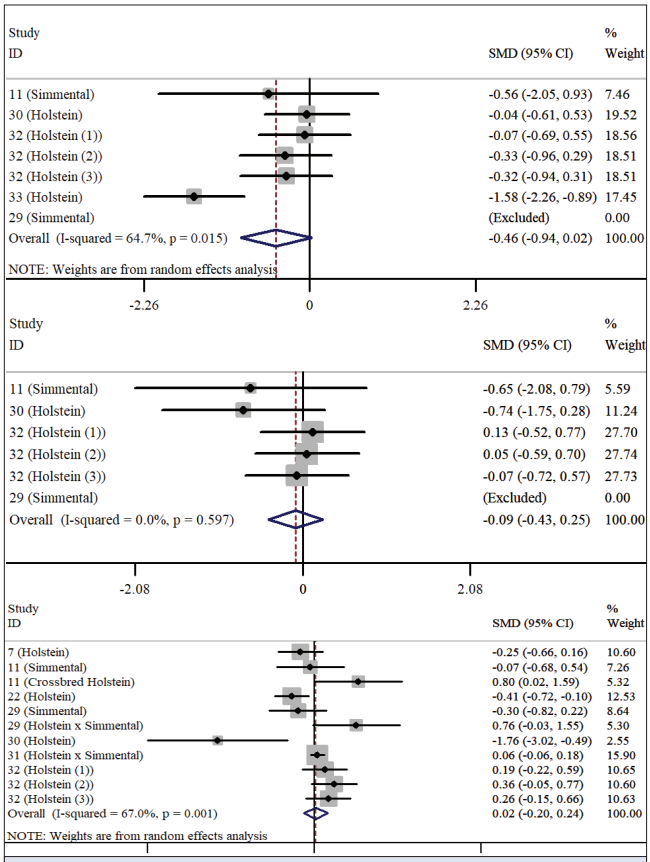

Fig 4. Forest plots of the studies examining the effect of *FASN* gene polymorphism on fat content (Co-dominant model, from top to bottom; AA-AG, AA-GG, AG-GG, respectively)

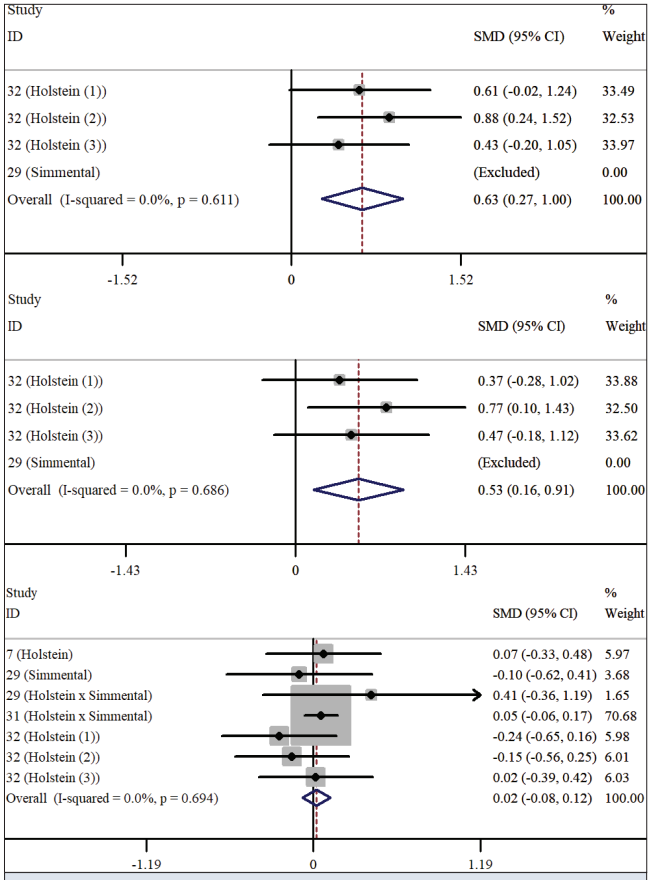

Fig 5. Forest plots of the studies examining the effect of FASN gene polymorphism on protein yield trait (Co-dominant model, from top to bottom; AA-AG, AA-GG, AG-GG, respectively)

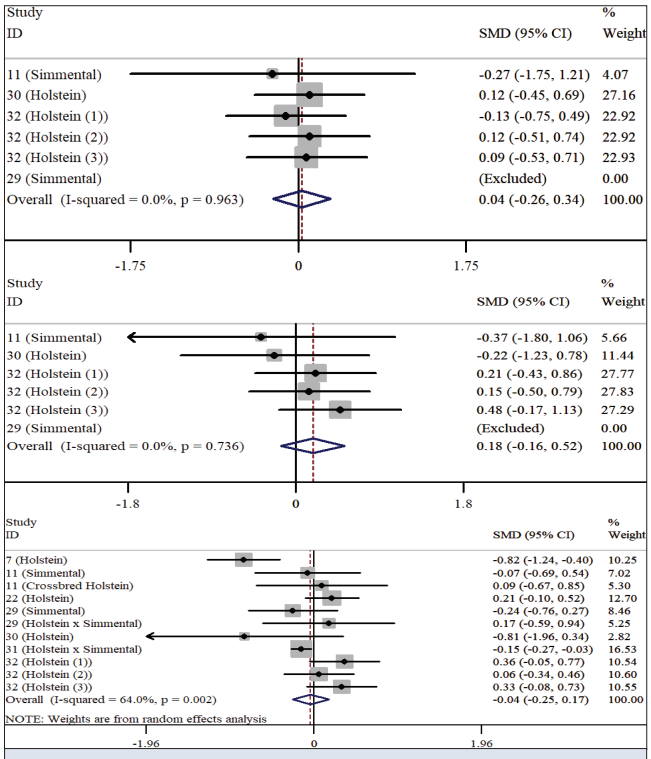

Fig 6. Forest plots of the studies examining the effect of FASN gene polymorphism on fat content (Co-dominant model, from top to bottom; AA-AG, AA-GG, AG-GG, respectively)

the protein content analysis, the mean differences of all genotypes were not significant (P>0.05) (*Fig. 6*).

Discussion

Regarding the milk yield trait, the P values of the studies incorporating FASN gene polymorphism are predominantly above the significance level, indicating that no statistically significant relationship was observed (P>0.05) [22,28-32]. In a particular study, the relationship between milk yield and FASN gene polymorphism was examined, and it was found to have a significant effect on polymorphism (P<0.05) [28]. Among the five studies that examined the relationship between FASN gene polymorphism and fat yield, a significant difference (P<0.05) was identified in the Ciecierska et al., 2013 study; however, no significant difference was found in the other studies (P>0.05) [7,27,31,32]. Among the nine studies that examined the relationship between fat content and FASN gene polymorphism, Matsumoto et al.[22] and Mauric et al.[28] reported a significant difference (P<0.05). However, no significant difference was found in other studies (P>0.05) [7,28,29,31-33]. A single study has identified a significant relationship (P<0.05) between protein yield and FASN gene polymorphism [15], while the remaining studies have not detected a significant difference (P>0.05) [7,29,31,32]. The majority of studies examining the relationship between FASN gene polymorphisms and protein content found no significant relationship (P>0.05) [21,27-30], but four studies found a significant relationship (P<0.05) [7,28,29,31].

The results of the meta-analysis conducted in this study indicate a significant association between the FASN gene polymorphism and milk, fat, and protein yields. The results indicate that the genotype comparisons that demonstrated a significant relationship with milk, protein, and fat yield were AA-GG and AA-AG, while the genotypes that did not demonstrate a significant relationship were determined as AG-GG. No significant correlation was identified between the protein and fat content and the FASN genotypes. A meta-analysis of the extant literature yielded a finding that was consistent across studies: individuals carrying the AA genotype exhibited higher yields of milk, fat, and protein. This finding suggests that the FASN locus is an important candidate gene for marker-assisted selection.

Conclusion

In this learning study, it was observed that the FASN gene was effective on milk components. It has been posited that this particular gene warrants consideration, particularly in the context of selection applications focused on fat, protein, and milk yield. An analysis of the fat and protein content characteristics revealed no

statistically significant differences. Consequently, it is hypothesized that these traits may be more influenced by other environmental and genetic factors. Future studies should include genotype × environment interactions. The utilization of substantial data sets is instrumental in enhancing reliability and attaining more generalizable outcomes. The study suggested the inclusion of FASN gene polymorphisms in cattle breeding programs and thus demonstrated its potential application as a genetic marker to enhance milk production efficiency. It is hypothesized that further consideration of the FASN gene may result in enhanced milk yield and quality.

Declarations

Availability of Data and Materials: The data and materials of this study are available from the corresponding author (M. Özdemir).

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

Artificial Intelligence: AI and AI-assisted technologies have not been used during the writing process of this study.

Author Contributions: Idea/Concept: DG, IA, ES; Design: MO, DG, IA, ES; Data Collection and/or Processing: DG, IA, ES; Analysis and/or Interpretation: MO, DG; Writing of the Manuscript: MO, DG, IA, ES; Critical Review: MO.

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Review Article

Understanding One Health and Zoonosis: A Systematic Review with a Bibliometric Analysis of Turkish Research and Global Perspectives (1974-2023)

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Abstract

A bibliometric analysis explored the understanding of the "One Health" and "zoonosis" concepts among Turkish researchers and identified existing gaps. The analysis examined trends in research on "One Health," "zoonosis," and "zoonoses," focusing on publications from Türkiye and globally. Data from Scopus were analyzed using VOSviewer and RStudio software. The results showed that research interest in these concepts has grown in Türkiye since the 2000s. However, the number of articles from Türkiye remains low compared to developed countries. The interdisciplinary nature of "One Health" and "zoonosis" research in Türkiye reflects contributions from various academic fields. Medical sciences lead in research, followed by immunology and veterinary sciences. In Türkiye, "One Health" is predominantly associated with animals, especially felines, and zoonotic pathogens such as *Toxoplasma*, *Anthrax*, and *Echinococcus*. It also relates to epidemiology, infectious diseases, and antibiotic resistance. Globally, "One Health" has a broader perspective, encompassing environmental health, education, veterinary medicine, and food safety. Regarding "zoonosis," Türkiye focuses on pathogens such as *Anthrax*, *Echinococcus*, *Brucella*, *Leishmania*, and Hantavirus. Globally, additional pathogens such as *Rickettsia*, *Taenia solium*, and *Giardia* are included. "Zoonosis" is also linked to climate change, animal welfare, and vector- and food-borne diseases. Despite increased interest, Türkiye's scientific production remains limited. Emphasis on interdisciplinary collaboration and broader conceptual frameworks could enhance understanding and research impact.

Keywords: Bibliometric analysis, One Health, Pathogens, Türkiye, Zoonosis

Introduction

Pathogens (viruses, bacteria, fungi, parasites, and prions) in the environment pose challenges for humans, animals, and plants. Disease transmission between animals and humans is a concern because human-animal health and the ecosystem are closely linked [1]. This tight link has given birth to many concepts, such as "One Health" and "zoonosis". Although the concept of "One Health" was formally introduced by the WHO in the early 2000s [2], some may argue that its principles existed since the earliest days of life on Earth. The One Health High-Level Expert Panel (OHHLEP) defined One Health as "…an integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals, and ecosystems. It recognizes that the health of humans, domestic and wild animals, plants, and the wider environment (including ecosystems) are closely linked and interdependent. The approach mobilizes multiple sectors, disciplines, and communities at various levels of society to work together to foster well-being and tackle threats to health and ecosystems while addressing the collective need for clean water, energy, and air, safe and nutritious food, taking action on climate change, and contributing to sustainable development" [3].

The zoonosis concept refers to a disease that spreads between vertebrate animals and humans, a definition established in 1951 by the World Health Organization's Expert Committee on Zoonoses. The term "zoonosis" (plural: zoonoses) is composed of two words originating from Greek, "zoon" meaning animal and "noson" meaning disease. Rudolph Virchow invented this term in the late 19th century to define illnesses in humans caused by animals [4]. One of the earliest documented outbreaks of a zoonotic disease is the plague in Athens in 430 BC. This outbreak led to the death of nearly 100.000 people. Subsequent pandemics, such as the Justinian plague in 541 AD and the "Black Death" in 1345, were also caused by *Yersinia pestis*. Another notable zoonotic epidemic termed the "American Plague" occurred from 1793 to 1798, claiming the lives of approximately 25.000 individuals [5].

Since the onset of the 20th century, numerous zoonotic diseases have emerged, posing significant challenges to public health worldwide. For the past two decades, there has been a renewed acknowledgment of the necessity for a holistic approach to this health concern [6]. This led to a tripartite FAO-OIE-WHO collaboration in 2010 [7]. Since then, the amount of research on "One Health" topics has grown worldwide.

Within this scenario, Türkiye's singular geographic and ecological diversity brings forth an appropriate illustration of the call for a One Health approach. Türkiye serves as a natural bridge between Asia, Africa, and Europe, with a subtropical climate. This positioning gives Türkiye a global importance and role in the transmission of emerging and re-emerging diseases including zoonotic diseases. In addition to its geography, which allows it to benefit from diverse climatic conditions with different flora and fauna, there are many marshes and sanctuaries for immigrant birds in Türkiye, such as the "Sultan Marshes" in the Kayseri region in Central Anatolia, the "Manyas Bird Paradise" in the Marmara region, the "Kizilirmak Delta" or the "Çernek Ringing Station" in Bafra near Samsun in the Black Sea region, the "Hevsel Bird Paradise" in Diyarbakir in the southeast, and the "Aras Bird Paradise" in the northeast. This diversity provides habitats for various arthropod vectors throughout the year, giving these sites significant epidemiological importance. Türkiye is also a touristic country welcoming millions of tourists from all over the world each year, and it experiences waves of migrants from different countries, who find Türkiye a country of passage to Europe. From an epidemiological point of view, this migratory flow constitutes one of the pathways for the introduction and dissemination of zoonotic diseases.

Indeed, interest in the "One Health" concept has improved in Türkiye, reaching a significant milestone with the country's first One Health congress in 2015. However, despite this growing interest, studies evaluating scientific knowledge and research output in this field have revealed room for improvement [8-10].

We aim to assess the understanding of the concepts One Health and zoonosis among professionals in the health sector, scientists, and policymakers. Through a bibliometric investigation, we compare Türkiye's expertise with global knowledge, identifying both research gaps and emerging challenges. The goal is to help researchers identify emerging challenges and potential research directions in the field of One Health on a global scale.

Material and Methods

Bibliometric analysis enables a quantitative analysis of documentary sources in a specific field. A bibliometric analysis can highlight key themes, notable developments, emerging trends, and gaps in a study, an approach also referred to as "scientific mapping." Thus, performance analysis and scientific mapping constitute the two fundamental pillars of bibliometric methodology.

We used the bibliometric approach to analyze the research trends for "One Health," "zoonosis," and "zoonoses" by the number of publications. We used only the bibliographic database Scopus for several key reasons. Firstly, it gives one of the most comprehensive collections of articles covering a wide range of disciplines relevant to the One Health and zoonosis framework, including medicine, veterinary science, environmental science, and social sciences. Secondly, only data collected from Scopus allowed us to do the analyses on RStudio and VOSviewer. Data collected from other databases were not compatible with the software package. Thus, Scopus was chosen for its comprehensive citation metrics, multidisciplinary scope, and broad coverage across a wide range of academic fields. Additionally, it is the preferred platform for bibliometric analyses, as most scientific documents and citation data used in such studies are indexed in Scopus.

The search formula in Scopus was set as: (TITLE-ABS-KEY ("One Health") OR TITLE-ABS-KEY ("one-health") OR TITLE-ABS-KEY ("one health") OR TITLE-ABS-KEY ("One health") OR TITLE-ABS-KEY ("zoono*") OR TITLE-ABS-KEY ("Zoono*")) AND (LIMIT-TO (DOCTYPE, "re") OR LIMIT-TO (DOCTYPE, "ar")) AND (LIMIT-TO (AFFILCOUNTRY, "Turkey")) for Türkiye and (TITLE-ABS-KEY ("One Health") OR TITLE-ABS-KEY ("one-health") OR TITLE-ABS-KEY ("one health") OR TITLE-ABS-KEY ("One health") OR TITLE-ABS-KEY ("zoono*") OR TITLE-ABS-KEY ("Zoono*")) AND (LIMIT-TO (DOCTYPE, "re") OR LIMIT-TO (DOCTYPE, "ar")) AND (EXCLUDE (AFFILCOUNTRY, "Turkey")) for "Worldwide" situation. For the global situation, only Türkiye was excluded, and a total of 67.455 research and review articles were found. The research was limited to articles published up to 2023. Scopus limits the export of the bibliometric data to a maximum of 20.000.

We used the software VOSviewer [11] for author keyword maps and R version 4.3.1 (2023-06-16) with the packages bibliometrix. The R software was used to generate a collaboration map, and Excel tables automatically used the function "biblioshiny()" [12] and exported data in Excel format. With VOSviewer, a co-occurrence analysis was performed with authors' keywords, with a minimum occurrence of five. At this phase, a modification occurred in the dataset where "zoonoses" was substituted with "zoonosis".

Results

Number of Publications

A comparison was made by using the combination of three concept words: "zoonosis", "zoonoses", and "One Health"

for Türkiye and the world. The number of published articles is important because it shows the level of interest. A total of 1300 articles have been found, and among them, only 123 were review articles; the rest were research articles *(Table 1)*. The oldest article on this topic was published in 1974, long before WHO officially recognized the importance of the "One Health" concept. Meanwhile, the rise of interest among Turkish scientists started in the 2000s *(Fig. 1)*, with an increase in the number of publications and the number of citations. The number of publications dropped in 2014 and 2018 and began to rise again in 2019. In other parts of the world, scientists have been interested in the "One Health" concept since World War II and that interest continues to grow today *(Fig. 1)*. An analysis of the number of published documents per country shows that most of the publications have been from developed countries such as the USA, UK, China, Brazil, Germany, France, Italy, Australia, India, Canada, Spain, Iran, Japan, Netherlands, Switzerland, and Belgium. Even though the developed countries' scientific production is far greater than Turkish scientific production, many countries have not produced as much as Turkish scientists *(Fig. 4;* countries depicted in red signify those with fewer documents relative to Türkiye).

The aforementioned concepts have been referenced across diverse academic domains. In Türkiye, they are used 50% of the time in medicine while globally the figure is 34%. In microbiology and immunology, the concepts have been given more importance in the rest of the world, with 21% of the publications in this field versus 18% in Türkiye. Nevertheless, the concepts are mostly used in medicine, immunology, microbiology, veterinary medicine, agriculture, and biological science *(Fig. 2)*. In *Fig. 3*, the presentation highlights the preeminent ten authors who have demonstrated significant contributions to the field. Among the notable contributors to the field, with the most publications, are Inci A, Simsek S, Ahmed H, Kilic S, Celebi B, Yildirim A, Duzlu O, Pekmezci GZ, Ozkul A, and Ertabaklar H *(Fig. 3)*.

Authors' Keywords Analysis

A comparative examination of the "One Health" paradigm reveals disparities in its conceptualization in Turkey in juxtaposition with the global perspective *(Fig. 5)*. The associations in Türkiye underscore the confluence of the term "One Health" with animals, emphasizing feline species and diverse pathogens such as *Toxoplasma, Borrelia*, coronavirus, and various viruses. *Fig. 5* suggests that in Türkiye the "One Health" theme focuses on zoonotic diseases, epidemiology, infectious diseases, and antibiotic resistance. An inconsistency becomes apparent when examining the worldwide discussion of the "One Health" concept. Notable divergences include environmental health, education, veterinary medicine,

Table 1. *Numbers and types of documents published in Türkiye*

Type of Document	Numbers
Article	1177
Review	123
Total	**1300**

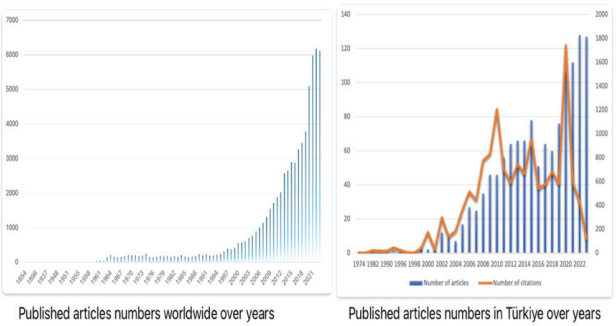

Fig 1. Number of publications per year worldwide vs in Türkiye. The figure shows how scientific production on "One Health" and "zoonotic" topics has evolved over the years

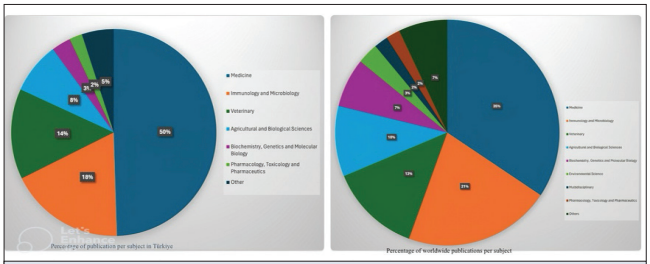

Fig 2. Percentage of publications per subject in Türkiye and worldwide. The figure illustrates how scientific production is distributed across different subjects

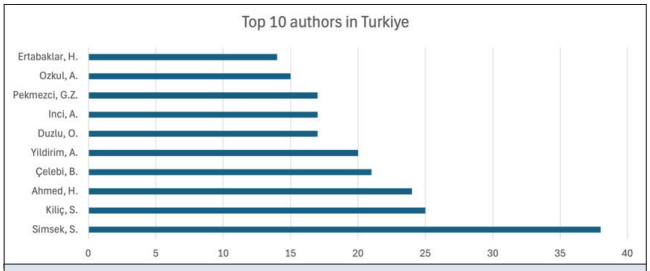

Fig 3. Top 10 authors in Türkiye

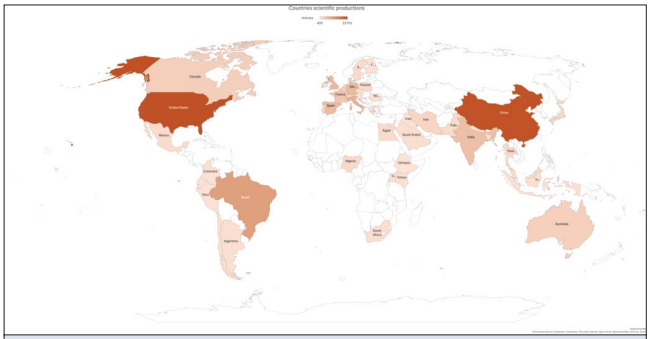

Fig 4. Scientific production per country. Scientific production was classified according to country, which shows the place that Türkiye holds

food safety, health policy, and climate change. This suggests a broader, interdisciplinary orientation on an international scale. The pathogenic associations in the global context span a wider spectrum, incorporating entities such as *Cryptosporidium, Blastocystis, Salmonella,* arboviruses, and bacteria. The Turkish approach prioritizes mammalian species, underscoring a focus on them within the framework of the "One Health" paradigm. *Fig. 6* provides an in-depth exploration of the interrelations inherent in conceptualizing "zoonosis." Within the Turkish context, this conceptual framework is notably entwined with various pathogens, including *Toxoplasma, Anthrax, Fasciola hepatica, Echinococcus, Brucella, Leishmania,* and Hantavirus. Additionally, there is an associative linkage to other pathogens, such as *Francisella, Bartonella, Coxiella*, Crimean Congo virus, and helminths. A global perspective unveils parallel associations between the "zoonosis" concept and analogous pathogens in Türkiye, albeit with nuanced distinctions. These distinctions include "hepatitis virus" and pathogens such as *Taenia solium, Rickettsia, Salmonella,* and *Giardia*, which are conspicuously absent in the author-associated keywords from Türkiye. Noteworthy is the linkage of the "zoonosis" concept to specific animal categories, including rodents, cats, dogs, humans, and cattle. Ticks emerge as principal vectors associated with zoonotic occurrences. The significance of wildlife and pets is underscored, emphasizing their substantial roles within the global zoonotic landscape. Moreover, the conceptual tapestry of zoonosis extends to tightly interconnected themes, including animal welfare, climate change, vector-borne and food-borne illnesses, and food safety.

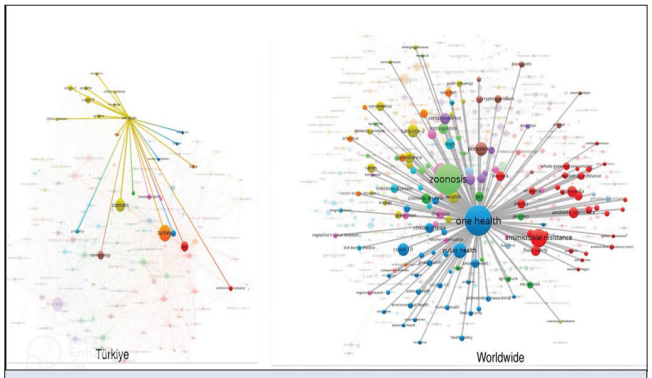

Fig 5. Keywords linked to the "One Health" concept in Türkiye vs worldwide. This figure is a word cloud that provides an overview of how the concepts are linked to "One Health", based on authors' keywords in articles. It was realised by VOSviewer software

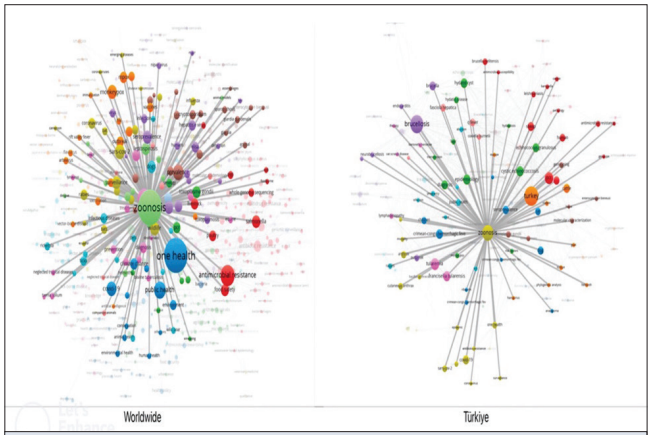

Fig 6. Authors' keywords linked to "zoonosis" concept worldwide vs in Türkiye. This figure is a word cloud that shows how the concepts are linked to "zoonosis", based on authors, keywords used in articles. It was realised by VOSviewer software

Discussion

We analyzed the concepts of "One Health," "zoonosis," and "zoonoses" to show the status of research conducted in the field by Turkish scientists and a comparative analysis with worldwide data by using bibliometric data. The quantity of publications serves as a crucial bibliometric indicator, providing insight into the level of attention a research field commands and its growth trajectory over time. The inaugural mentions of One Health in academic literature emerged predominantly from institutions and organizations in the United States, with subsequent contributions originating in Europe. Globally, nearly half of all scholarly publications on One Health are attributed to research conducted at North American institutions, with European institutions contributing approximately one-third of the total publications [8]. In Türkiye, academicians have been interested in this topic in the 2000s, and that interest continues to grow [9,10]. Globally, the One Health field has witnessed a consistent increase in publications in the last two decades, with a surge commencing around the 2000s. In terms of scientific production, Türkiye surpasses many countries by a significant margin. Most African nations, such as Egypt, Nigeria, and Kenya, and some European countries such as Romania and Ireland have demonstrated less production.

Academicians from various universities have conducted most of the research in Türkiye. The burgeoning global acceptance of the One Health framework underscores the indispensable role of academia in shaping and structing future professionals. The cultivation of a proficient global workforce proficient in bridging interdisciplinary gaps and fostering collaboration across diverse sectors is paramount for the improvement of health outcomes [13]. The conceptual understanding and implementation processes of the One Health approach in Türkiye are progressing more slowly compared to the United States and European countries. To ensure parallel progress in these processes, it is essential to embrace the One Health approach, make prompt political decisions, and allocate sufficient financial resources. In Türkiye, a "One Health" institute does not yet exist, nor is a curriculum dedicated

exclusively to it. Its creation could have a significant impact on the academic environment. Türkiye's strategic geographic position, the wide diversity of ecosystems, and the movement of human and animal populations lead to a high prevalence of human-animal interactions, thus increasing the risks associated with zoonotic diseases. The establishment of a One Health institute would serve as a multidisciplinary research hub, support the development of public policies in human, animal, and environmental health, and foster intersectoral coordination. Disease surveillance is a fundamental pillar of this approach; such an institute could significantly strengthen national capacities to detect and respond to priority diseases. An equally essential role would be to ensure the continuing training of health professionals and students to ensure a competent workforce aware of global health issues. The number of national and international multidisciplinary research networks focused on zoonotic diseases and the One Health paradigm is on the rise [8]. A global map delineates academic institutions, organizations, groups, and laboratories actively dedicated to advancing the concept of One Health. However, the low representation of Türkiye on this map raises attention [14]. It would be important for Türkiye to join the Global Early Warning System (GLEWS), which has national partners to assist in primary warning [15].

The bibliometric analysis highlighted a growing interest in One Health, showcasing its potential. However, it also brought to light a lack of involvement with the environmental sector in Türkiye. Findings suggest a necessity for more practical strategies to enhance collaboration across sectors and promote knowledge sharing. Effective strategies must be supported with funding [16]. To achieve a more holistic perspective, it is crucial to involve researchers with diverse expertise and disciplinary backgrounds. This interdisciplinary approach will enable a comprehensive examination of One Health, considering the human-animal-environment interface as an interconnected and unified entity rather than distinct and separate components [17].

Authors' keyword maps revealed various terms associated with zoonosis and One Health. Zoonosis consistently refers to specific diseases in Türkiye. Despite the World Health Organization cataloguing over one hundred zoonotic diseases, the significance attributed to these diseases varies among countries. In the Mediterranean region, zoonoses such as brucellosis, anthrax, and rabies are endemic. In Türkiye, the general directorate of public health focused on the following diseases: Ebola virus disease, tularaemia, brucellosis, anthrax, echinococcosis, West Nile disease, hantavirus disease, Crimean Congo haemorrhagic fever, rabies, and leishmaniasis [18]. However, research has revealed the presence of over thirty zoonotic diseases in the country, encompassing viral, bacterial, helminthic, protozoan, and fungal categories [15,19]. Some are vector-borne zoonotic diseases [17-21], and others are food-borne zoonotic diseases [15,22-23]. The prioritization of research and surveillance for zoonotic diseases differs among countries, depending on their epidemiological, economic, and health contexts. Certain nations prioritize the surveillance and control of specific zoonotic diseases, while others emphasize different ones, reflecting diverse public health priorities and contexts. In 2022, the European Union (EU) reported *Salmonella, Campylobacter*, and *Yersinia* as the top three zoonotic pathogens along with Shiga toxin-producing *Escherichia coli* and *Listeria monocytogenes* infections. Among vector-borne zoonoses, West Nile disease ranked at the top in Europe [24]. Research in the Horn of Africa has centered on diseases including hepatitis E, leptospirosis, brucellosis, Q fever, Rift Valley fever, trypanosomiasis, tuberculosis, toxoplasmosis, anthrax, echinococcosis, rabies, and leishmaniasis [25]. In South Africa, the top four zoonotic diseases prioritized are tuberculosis, brucellosis, Rift Valley fever, and cysticercosis [26]. Research in other parts of the continent has concentrated on a wider range of diseases, including anthrax, astrovirus, bartonellosis, borreliosis, brucellosis, cysticercosis, *Escherichia coli* infections, Ebola virus, echinococcosis, enterocytozoonis, Human T-lymphotropic virus (HTLV), influenza A, Lassa virus, leishmaniasis, leptospirosis, Marburg virus, Middle East Respiratory Syndrome Coronavirus (MERS-CoV), monkeypox, pentastomiasis, Q fever, rabies, rickettsiosis, Rift Valley fever, Simian Immunodeficiency Virus (SIV), toxoplasmosis, and tuberculosis [27].

Among the concepts associated with "zoonosis" is "animal welfare". For millennia, human-animal relations have played a crucial role in veterinary medicine and overall health and welfare. Better animal welfare is linked to less diseases [28]. In Türkiye, no studies associating "animal welfare" with zoonosis or the concept of One Health have been identified thus far, likely due to the recent introduction of this concept in veterinary curricula [29]. Climate change is also intertwined with the concept of zoonosis, as evidenced by the significant role played by ecological niche modelling (ENM) in determining the distribution of potential vectors responsible for diseases. ENM enables scientists to estimate the geographical areas where specific vectors are likely to be found. In Türkiye, a limited number of studies have explored the correlation between "climate change" and zoonotic diseases [28-33]. One of the most important concepts linked to "zoonosis" and "One Health", whether for Turkish or worldwide, is "Antimicrobial Resistance (AMR)". Indeed, the effectiveness of any therapeutic agent can be hindered by the possibility of tolerance or resistance developing

over time. Its negative implications extend across social, economic, and health domains, impacting individuals, animals, and the environment. Thus, AMR became a worldwide health concern integrated into the One Health approach [34]. Globally, the misuse of antimicrobials in veterinary and human medicine has led to a high level of AMR. In Türkiye, AMR is a huge concern due to its high level [35]. For this reason, the Ministry of Health (MoH) has instituted two key antimicrobial stewardship programs. The first program is tailored for hospitals, while the second program is designed to address antimicrobial stewardship in the community [36]. Researchers have been involved in this field and have focused more on public health pathogens such as *Campylobacter* [35-37], *Salmonella* [38-41], *Staphylococcus* [42-46], *Pseudomonas* [47], and *E. coli* [48]. AMR is one of the most obvious challenges that demands a One Health approach. In Türkiye, although research on antibiotic resistance has so far focused primarily on pathogens of public health interest, it is relevant to broaden investigations to domesticated animals, wildlife reservoirs, and environmental sources to obtain a more integrated and comprehensive view.

Bridging this gap requires strong advocacy at the administrative level. Additionally, there is a need to foster a multidisciplinary and interdisciplinary working culture across institutions at the central, regional, and local levels that aligns with the One Health approach. In this context, establishing a One Health institute at universities would be a crucial step in implementing the One Health approach and cultivating a One Health culture.

Conclusion

In conclusion, a bibliometric analysis of "One Health" and "zoonosis" research in Türkiye and globally revealed progress in these areas. While the interest and involvement of Turkish scientists in One Health research, particularly in addressing zoonotic diseases, is increasing, there are notable gaps, particularly in environmental aspects and interdisciplinary cooperation. The identification of various zoonotic diseases underlines the complexity of the issue and the need for comprehensive approaches. The interweaving of concepts such as animal welfare, climate change, and antimicrobial resistance with One Health highlights the interdependence of human, animal, and environmental health. This aspect deserves attention by researchers, decision-makers, politicians, and others. In parallel with worldwide developments, Türkiye needs to reduce the gaps in the fields of "One Health" and "zoonoses" and to carry out management coordination in accordance with the One Health approach. It is necessary to encourage interdisciplinary collaboration in the context of GLEWS, establish One Health institutes, expand research focus areas, implement antimicrobial resistance management, strengthen environmental and climate change efforts, investigate the epidemiology of other zoonotic diseases, demonstrate required political decisions, and create funding. The paradigmatic relationship between the One Health concept and the Sustainable Development Goals (SDGs) should be acknowledged and integrated into the academic framework of universities in Türkiye.

Declarations

Availability of Data and Materials: The datasets generated and analyzed during the study are available from the corresponding author (S. D. Diop) upon request.

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Authors Contributions: SDD developed the core ideas and designed the study framework. SDD, AI, ADK and ÖD devised and coordinated the methodology. SDD drafted the initial manuscript. AI, ADK, and ÖD contributed significantly to the writing and revision of the manuscript. All authors read and approved the final manuscript.

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Review Article

Epidemiology and Public Health Importance of Bovine Salmonellosis

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Abstract

Bovine salmonellosis caused by *Salmonella enterica* subsp. enterica serovar Dublin (S. Dublin) is a significant public health and economic concern globally. It leads to severe health issues in cattle, including enteritis, septicaemia, and abortion, with high mortality rates, especially in newborn calves. The disease not only impacts the well-being of the animals but also results in substantial economic losses through treatment costs, reduced milk production, and potential outbreaks. The transmission of S. Dublin is primarily through contaminated food, water, and environmental exposure, with the faecal route being the most significant mode of spread. The pathogenesis of *S.* Dublin involves complex interactions between the bacteria and the host immune system, with the bacteria capable of persisting in the herd as carriers, further complicating control measures. Effective control strategies are critical to minimizing its spread, and understanding the epidemiology, clinical signs, and diagnostic methods is key. This review demonstrates the public health importance, clinical manifestations, economic importance and diagnostic techniques of bovine salmonellosis.

Keywords: Bovine, Epidemiology, Food, Public health, Salmonellosis, S. Dublin

Introduction

Salmonellosis is a serious public health concern which affects multiple animal species and humans [1,2]. It is a foodborne bacterial infection that is caused by *Salmonella enterica* subsp. *enterica* serovar Dublin *(S.* Dublin) in bovines [3,4]. *S.* Dublin is a Gram-negative, non-spore-forming, oxidase-negative, motile and rod-shaped bacteria that belongs to family *Enterobacteriaceae* [5,6]. The survivability of *S.* Dublin depends on the environmental conditions, temperature, pH and other microflora [7,8]. It can survive for years in dried faecal matter and for months in different organic matters such as soil, cattle manure, and slurry [9,10]. However, *S.* Dublin does not resist antibiotics, sunlight, and disinfectants [11,12]. Multidrug-resistant strains can be isolated from dairy and beef sources [13,14]. The bacteria have the ability to reproduce in moist and warm conditions outside the host cell [15,16]. Salmonellosis cause severe health problems in bovines, which include 2 major syndromes: enteritis (inflammation of small intestine) and septicaemia (blood poisoning) [17,18]. However, other clinical signs include pyrexia, dysentery, and abortions in pregnant animals [19,20]. The severity of clinical signs of Salmonellosis depends on the age of the animal, infection dose, immune response, and physiological state of the host [1,17,21]. In newborn calves, septicaemia with enteritis is most commonly seen [18,22]. However, pneumonia and neurological signs may also occur [23,24]. In case of young ones or animals with age more than one-week, acute enteritis mostly occurs without systemic involvement [25,26]. The infection starts with pyrexia (40.5-41.5°C [105-107°F]) followed by dysentery and sometimes with tenesmus [27,28]. Mortality rates in both newborn calves and young ones may increase to 100%, depending on the virulence and infection load [29,30]. Milk production may drop in lactating animals [13,31]. Salmonellosis is a serious concern for economic losses and public health that needs critical attention [32-34]. However, different alternative therapeutics, mainly plant-based compounds, are under study to control multiple diseases [35,36]. To minimize the salmonellosis around the globe, we must understand the pathogenesis and epidemiology as drug resistance is increasing dramatically [37-39].

S. Dublin most commonly causes infection after direct transmission through contaminated food, water, and environment [40,41]. However, the severity of the disease depends upon the infectious dose of the pathogen [15,42]. *Salmonella* bacteria colonize the gut of the host, followed

by invasion in columnar enterocytes through the lymphatic system [43-45]. *Salmonella* can also enter the macrophages, which is a critical barrier [46,47]. The bacteria replicate in the macrophages, and during the replication they can easily enter the blood, lymph, lungs, liver, spleen, tonsils and lymph nodes [48,49]. *S.* Dublin can become the latent carrier, which leads to the persistence of the infection in the herd [50-52]. However, *S.* Dublin has been found in the internal organs of the animals while they show no signs of the infection [53-55]. Different stages of the *S.* Dublin infection have been reported, including per-acute, acute, chronic, passive carrier, active carrier and latent carrier stage [56-58]. The infected animal may or may not shed the bacteria in these different stages [59]. However, the *S.* Dublin may shed through urine, saliva, milk, faeces and vaginal discharge [60,61]. The duration and amount of bacterial excretion vary greatly among infected animals [62,63]. This is because the faecal material of the animal contains the highest number of bacteria, and they are produced in the large quantities [64,65]. So, the faecal route of the transmission is the most important route to cause the infection [66,67]. Humoral and cellular components of the immune system work together to fight pathogenic bacteria [68,69]. The first line of defence against the *S.* Dublin consists of neutrophils, polymorphonuclear leukocytes, macrophages, natural killer cells and their secreted cytokines [70,71]. This non-specific immune system activates the adaptive immune response [72,73]. IgG and IgM titres begin to increase, and IgG attains maximum titre between 6-11 weeks after the inoculation [74,75]. However, *S.* Dublin is host adopted to cattle, but there is a lack of agreement on the mechanism of the host adoption [76].

To prevent the *S.* Dublin infection, the mechanism of host adoption is not much important [77,78]. However, this feasibility of the host interaction with *S.* Dublin initiates the effective control programs without involving the other livestock sectors [4,79]. These effective control programs aid in preventing bovine salmonellosis and other important foodborne and public health important diseases from spreading [80,81]. This review emphasises the pathogenesis, public health importance and epidemiology of *S.* Dublin to adopt better preventive measures and control strategies. We will briefly discuss the economic importance, clinical signs, diagnosis, pathogenesis and public health aspect of bovine salmonellosis of *S.* Dublin.

Economic Importance of Bovine Salmonellosis

The high cost of treating clinical salmonellosis in farm animals leads to significant economic losses [82,83]. This includes the cost of diagnosis, treatments, cleaning and disinfectants, laboratory tests, cost of prevention and control, and death of the infected animal [84,85]. However, other related economic losses include a drop in the milk production in the lactating animals, poor growth and pregnancy loss in some severe cases of salmonellosis [86]. If one animal is diagnosed positive in a large herd of the animals, it would be difficult to diagnose all animals if they are infected with *S.* Dublin or not [87 88]. This will increase the cost of diagnostic tools and prevention strategies. The annual estimated loss due to bovine salmonellosis in the United States is billions of dollars, millions of pounds in United Kingdom and $160 million in Canada [89]. However, in North America, economic loss due to 5 outbreaks was $36.400-$62 million [90]. It is strictly suggested that every £1 spent on the investigation and control strategies can save £5 [91,92].

Clinical Manifestation of Bovine Salmonellosis

S. Dublin is usually endemic in bovine herds and bovines are the most important carrier of *Salmonella* infection [90,93]. They can carry *S.* Dublin for a longer period or sometimes it may be for a lifetime [94,95]. In calves, signs and symptoms of clinical ailment are present at the age of 2-6 weeks [96]. Signs and symptoms may vary with the infectious dose of the pathogen [97]. However, enteric form of salmonellosis is present in young calves which is characterized by dullness, pyrexia, and anorexia that is followed by severe diarrhoea [98]. Blood can also be present in the faecal material and faeces may become stringy because of the presence of necrotic mucosa of intestine [99]. While in adult animals, subacute or acute salmonellosis mostly occurs with abortion in the pregnant animals during the early stage of acute enteric disease [100]. Animals with severe infection show signs of pyrexia, depression and anorexia [101]. However, other important clinical manifestations include drop in the milk production, fowl smelling diarrhoea, bloody and mucoid faeces, with shreds of necrotic mucosa of intestine, dehydration, and congestion of mucous membrane [102]. Another most common clinical manifestation of bovine salmonellosis is retained placenta, which occurs in approximately 70% of the cases [103]. The acute phase of the bovine salmonellosis may only last for 1 week. However, the postmortem findings may vary [104,105].

The animal died in per-acute stage of the infection may have no gross lesions in the postmortem findings [106]. But extensive petechial haemorrhages are usually present in subserosal and submucosal layer [107]. In young calves, the mesenteric lymph nodes are enlarged in size, congested and oedematous [108]. The small intestine shows muco-haemorrhagic or diffused mucoid enteritis [109]. Necrotic enteritis, particularly affecting the ileum and large

intestine, occurs in adults [110]. Spleen and mesenteric lymph nodes are enlarged and swollen [111]. However, the wall of the intestine becomes thickened and covered grey-yellow necrotic material overlying granular, red surface [108].

Infectious Stages and Pathogenesis of S. Dublin

There are multiple infection stages of *S.* Dublin that include per-acute, acute, chronic, passive carrier, active carrier and latent carrier [112,113]. In per-acute stage of the infection, animals die within a very short period of time usually in 1-2 days [114]. Death of animals occurs even before they start to shed the bacteria [115]. While the acute phase may remain from 1 to 2 weeks to 5-9 weeks [116]. In this stage, animals shed bacteria in a large amount continuously or intermittently through faeces, urine, milk and vaginal discharge [101]. The amount of the bacteria in the acute stage of the infection varies from 1 to 10^8 CFU/g [51]. The chronic infection may remain for months, and the infected animal may or may not shed bacteria [117]. However, other carrier stages may remain from weeks to years and infected ones may or may not shed bacteria [118]. When the animals shed bacteria in the carrier stage, it may be in a continuous pattern or intermittent [119,120]. The amount of the bacteria may be low (10^1-10^4 CFU/g faeces), moderate (10^4-10^5 CFU/g faeces) and high (>10^5 CFU/g faeces). These amounts depend on the infection dose and pathogenesis of *S.* Dublin [121].

S. Dublin most commonly enters by ingestion of contaminated feed or water in cattle [122]. After entering the body of host, it directly goes to the rumen or stomach where it faces harsh conditions [123]. *S.* Dublin adopt to survive against the gastric acid and normal flora of the stomach/rumen [124]. After successfully adopting the conditions, the bacteria enter the epithelium of the intestine for colonization purpose [125]. Intestinal motility and mucus work as defence of the host in the intestine and *S.* Dublin have fimbriae lipopolysaccharide to overcome the host defence [126]. Host secretes neutrophils and macrophages when the bacteria evade the host defences [127]. *S.* Dublin have the ability to secrete the effector proteins by TTSS-1 & 2 (Type III secretion system) that combat the macrophages and neutrophils [25]. When the bacteria overcome the host defence system, the bacteria spread systemically, and enteritis has been induced in the host [128,129]. In response to systemic salmonellosis, pyrexia has been developed [130]. Detailed pathogenesis is explained in *Fig. 1*.

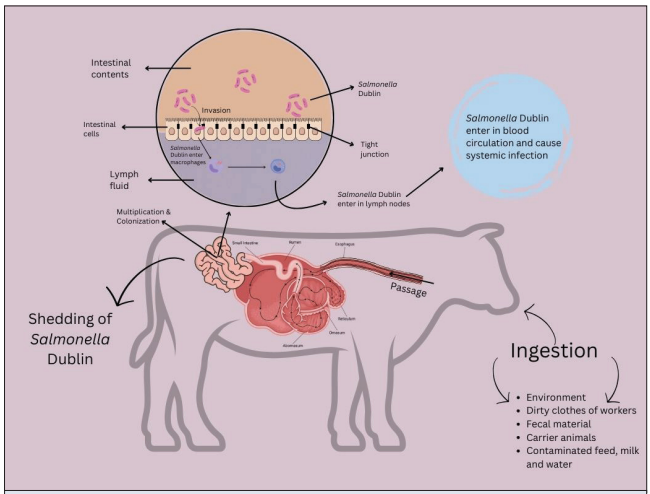

Fig 1. Pathogenesis of *Salmonella* Dublin in bovines

Public Health Importance of Bovine Salmonellosis

Salmonellosis is an important public health concern around the globe that causes a large amount of mortality and morbidity [131]. Although most of the cases are self-limiting and moderate, the serious cases may lead to death [132]. Over 3 million deaths of the humans are because of the salmonellosis annually [133,134]. In US, 500-1000 deaths occur out of 2-4 million cases annually [135]. Regardless of the hygiene, education and food processing, salmonellosis still remains an important public health concern that needs attention [136]. *S.* Dublin cause infection in average 10 humans per year in Ireland [137]. In Ireland, bovine salmonellosis is considered as the most important public health disease as it is a major threat to the livestock of the Ireland and because of the genetic evolutions of *S.* Dublin [138]. It is believed to have evolved recently due to the consistency in its multi-locus enzyme genotype and fliC flagellin DNA sequence analysis [139]. *S.* Dublin is considered being one step away from *S. enteritidis* (a common *Salmonella* serotype in humans and poultry) [140,141]. However, the genetic distinction between these two serovars is insignificant [18]. If *S.* Dublin have been diagnosed in the herd of cattle, there is a significant risk of persistent infection in the carrier cows for as long as the cow remains in the herd [52,142]. To diagnose the bovine salmonellosis in the herd, there are multiple options which are given in detail as follows.

Diagnosis

Diagnosis of the disease in the herd of the animal is the most important key to prevent the economic loss and maintaining health of the animals [143,144]. Pathological, postmortem findings and clinical signs are not adequate for diagnosing salmonellosis [145]. For definitive diagnosis, there are multiple techniques including cultivation of bacteria, clinical examinations and counting the antibodies against S. Dublin [146]. Detection of bacteria in fluids, body organs, faeces, and environmental samples can be done by

conventional culture methods of bacteria [147]. The main advantage of detecting bacteria is tracing infection in large groups of animals during the investigation of an outbreak. This method has a disadvantage of low sensitivity [148]. The new techniques for the detection of the bacteria are based on the genetic material, i.e., Polymerase chain reaction (PCR) techniques [149,150]. The PCR techniques are considered being the more sensitive techniques, but subsequent typing is not always possible [151,152]. The detailed diagnostic techniques are given as follows:

Culture Techniques

Culturing techniques are stepwise procedure in order to isolate the live bacteria in the sample [153]. The steps to isolate the bacteria in the sample include pre-enrichment, selective enrichment, plating and confirmation [154]. For positive results, bacteria must have the ability to grow in the enrichment steps [155]. This method should be able to detect the minimum CFU in the sample; up to 1 CFU [156]. However, this may not be always true in every case. For faecal samples of *S.* Dublin, the specificity is assumed to be 100% while the sensitivity in faecal samples is less than ideal [157]. For more accurate sensitivity test, faecal cultures should be used repeatedly to detect the active carriers [158].

PCR Techniques

PCR-based techniques are considered being more precise and accurate to detect the bacteria in the sample material [159,160]. These techniques are based on the detection of the genetic material of the bacteria (*S.* Dublin) [141]. However, there are 2 basic principles of PCR technique: real-time PCR and the traditional PCR [161,162]. The traditional PCR technique only gives qualitative results (whether the bacteria is present or not) while in real-time PCR, the exact amount of the DNA copied after every cycle is counted by the computer [149,163]. The performance of the test depends on the proper functioning of probes, internal control systems, and primers [164]. However, the PCR tests do not provide the information of the serotype of the *Salmonella* [165]. To detect the serotype *S.* Dublin in the tested sample, a follow-up culture test must be conducted on the positive samples [166]. Multiple studies show that the specificity and sensitivity of the PCR tests is not accurate [167,168]. In a case of naturally infected animals with *S.* Dublin, the sensitivity results of rt-PCR found poorer than compared to the conventional culture method [169].

Antibodies Detection

Antibodies detection has been proven very effective in checking the previous or current infection [170,171]. Enzyme-linked immunosorbent assays (ELISA) can be used to detect the O-antigens from *S.* Dublin in milk and blood samples [172]. This measures the humoral immune response that specifies the infection (previous or early) [173]. IgG antibody titre increases up to measurable amount in the animals after one too few weeks of the infection [174]. This method of diagnosis has multiple benefits of low cost and sampling feasibility. ELISA is used widely for the surveillance purpose, evaluation of control strategies and supporting decision [175]. In Denmark, bulk milk is collected regularly or alternative days for milk testing [176]. The samples have been tested for the surveillance of *S.* Dublin, Infectious Bovine Rhinotracheitis (IBR) and Bovine Viral Diarrhoea virus (BVDv) [177]. This routine testing helps in effective control strategies. Serum ELISA can also be performed to check the antibodies titre as its sensitivity is close to the individual milk ELISA testing [178]. However, other genomic and molecular methods are available for the differentiation of *S.* Dublin strain [179]. The details of these methods are beyond the scope of this review.

Conclusion

Bovine salmonellosis caused by *Salmonella enterica* subsp. enterica serovar Dublin (*S.* Dublin) is a significant public health and economic concern globally. It leads to severe health issues in cattle, including enteritis, septicaemia, and abortion, with high mortality rates, especially in newborn calves. The disease not only impacts the well-being of the animals but also results in substantial economic losses through treatment costs, reduced milk production, and potential outbreaks. The transmission of *S.* Dublin is primarily through contaminated food, water, and environmental exposure, with the faecal route being the most significant mode of spread. The pathogenesis of *S.* Dublin involves complex interactions between the bacteria and the host immune system, with the bacteria capable of persisting in the herd as carriers, further complicating control measures. Effective control strategies are critical to minimizing its spread, and understanding the epidemiology, clinical signs, and diagnostic methods is key. Current diagnostic tools, such as culture techniques, PCR, and antibody detection methods, while valuable, have limitations and require further refinement to enhance sensitivity and specificity. From a public health perspective, S. Dublin represents a major risk, especially due to its potential to evolve and cross-infect humans. As such, continued vigilance and research into prevention, control strategies, and early detection are essential for mitigating the public health risk posed by bovine salmonellosis. The development of more efficient surveillance systems and control measures will be vital in preventing the spread of this zoonotic pathogen, ensuring the health of both livestock and humans.

Declarations

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Review Article

Saponins and Their Role as Vaccine Adjuvant Against Coccidiosis in Poultry

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Abstract

Coccidiosis, induced by various *Eimeria* species, has been one of the most important health threats and performance of poultry around the world. Other than the current treatment, successful vaccination approaches have been realized. Advances in saponin biochemistry, from *Quillaja saponaria* and *Yucca schidigera* plants, have supported the development of vaccine adjuvants based on these natural glycosides. Saponins also activate innate immune pathways and can assist with antigen presentation, enhancing humoral and cell-mediated responses. Saponin-based adjuvants, including QS-21 and Quil A, can enhance adjuvant efficacy by inducing higher antibody responses and promoting long-lasting protective immunity privation. Nonetheless, challenges including toxicity issues about the saponin fractions and the variable adjuvant activity among different saponins, have also been reported. Future studies aim to improve saponin adjuvant formulations, determine their harmlessness, and investigate new transport systems, such as immunostimulating complexes. By enhancing poultry health, such advancements contribute to safer meat and egg products, directly supporting food safety. Moreover, reducing disease-related losses in poultry farms promotes food security by ensuring stable and efficient protein production. The focus of this review article is to highlight the role of saponins as vaccine adjuvants to enhance immunity against *Eimeria* species in poultry.

Keywords: Coccidiosis, *Eimeria*, Food safety, Phytochemicals, Quil A, Saponins, Vaccine adjuvants

Introduction

Coccidiosis is one of the most important health problems [1] affecting animals worldwide, causing disease with major economic losses [2]. It is caused by obligate intracellular parasitic protozoa known as *Eimeria*, belonging to the order Apicomplexa [3]. *Eimeria* remains one of the most economically important species that cause disease in livestock globally [4]. *Eimeria* is remarkably receptive in the poultry industry [5], this is because the transmission of parasites is highly favored by the bulk number of susceptible birds [6,7]. *Eimeria* spp. results in coccidiosis disease that hinders the expansion of the poultry industry [8]. The infection may get worse because of poor management techniques such as excessive stocking densities, contaminated feeders, drinkers, and damp litter that encourage oocyst sporulation, and inadequate ventilation facilities [9]. Among the most virulent *Eimeria* spp., *E. tenella* is the most common, followed by *E. acervulina* and *E. maxima* [10]. Due to increased mortality, stunted growth, and a low feed conversion ratio, the disease directly affects the production potential of affected livestock, resulting in significant financial losses [11,12]. In the poultry industry, globally there was a huge economic loss of more than 3 billion US$ annually [13]. Such economic losses not only reduce profitability but also threaten food security by limiting the availability of affordable protein sources. Additionally, disease-related contamination in meat and eggs poses a serious risk to food safety across the supply chain.

The coccidial parasites can enter various animal hosts and effectually exploit the immune system [4]. This creates a serious dispute against the action of control. Many drugs have been developed against the coccidia parasite [8]. In avian coccidiosis, *Eimeria* species develop frequent resistance when new and effective drugs are detected in the body [14]. Alongside commercial losses, using chemotherapeutics in poultry can also generate harmful residues in eggs and meat [15]. According to studies, the use of vaccines is the most efficient way to prevent and reduce the prevalence of infectious diseases [16,17]. Though

there is an emerging line of action as prophylactic vaccines, these are said to be a successful approach against coccidia, but these vaccines are minimal and in short supply [4]. Anticoccidial drugs have successfully prevented coccidiosis over the decades, with certain restrictions related to production costs [18]. There are various kinds of vaccines: live attenuated, inactivated vaccines (killed), and recombinant vaccines (subunits) [19]. Mostly, the commercial vaccines against coccidiosis are based on killed or live virulent organisms [20]. Nonetheless, the assurance of a live attenuated vaccine is uncertain because of the possibility of severe regression [21]. When comparing, it shows that a durable solution and safer options are provided by subunit vaccines [22,23]. However, isolated antigens from different host systems can be less antigenic and immune stimulating than killed or live attenuated vaccine variants [24]. To stimulate an effective immune response by delivering these antigens to the immune system is a challenging task [25]. In this matter, it is generally acknowledged that to increase efficacy and immunity, we need further components to be added to vaccines. These components are macromolecules and their complexes, either compounds or molecules, commonly known as adjuvants [26,27].

Adjuvants were first discovered in 1920 by Gaston Ramon [28], a French scientist who observed that if aluminum salts are included in a vaccine, they increase its potential [29]. It is derived from the Latin word "Adjuvare" which means to aid [30,31]. Vaccine adjuvants are used to increase the potential of the immune system response to combined antigens [32]. Most adjuvants are chemicals, macromolecules, or compounds that improve innate immunity by combining with the antigens, and enhance the immune response [16,33]. Many efforts have been made to reduce the intricacy of antigens, such as pure antigens, recombinants [34], artificially manufactured peptides, and proteins, as an alternative use of whole inactive organisms without adjuvants to induce immunity [35,36]. The non-toxic adjuvants can boost and direct immune response. Certain adjuvants, including mineral gels or water in oil emulsions, regulate the antigen at the injection site [37]. Adjuvants increase the secondary type of immune response by slowly releasing the restrained antigen into the immune system [38, 39]. The enhancement in the action of vaccines using adjuvants is shown in *Fig. 1*.

Although the mechanism of action of adjuvants is still under investigation, significant progress has been made in recent years to identify them [40]. The potential and virulent nature of adjuvants should be maintained in order to provide protected stimulants with very fewer reactions [41], it depends on how adjuvants are being used [36,42]. In several years, adjuvants have been used in many experimental subunit vaccines that are often too weak to stimulate

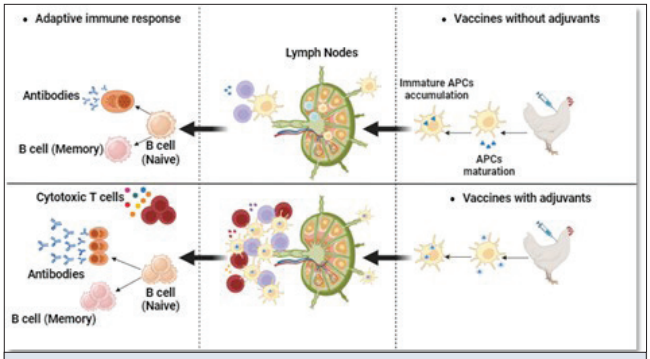

Fig 1. Vaccine enhancement using adjuvants

immune response alone [31], however, not all vaccines need adjuvants [36].

Many studies have shown that the plant-derived compounds of herb spices known as phytochemicals, play a vital role in antimicrobial, coccidiostat activities in animals [43-46], as they improve gut health, immunity, growth enhancement, and adsorption of nutrients [47,48]. Adjuvants derived from phytochemistry, such as saponins, carbohydrates,protein lectins, and heat-shock proteins, are efficient immune stimulants with little toxicity [49]. Different studies marked the effectiveness of phytochemicals as adjuvants against various diseases. These have proved to be useful as potential adjuvants against coccidiosis. Saponins are the most significant phyto-biotics for use as adjuvants in vaccines among all other phytochemicals [50]. They are natural compounds produced by plants that play an important role in defense mechanisms due to their antimicrobial, fungicidal, and insecticidal properties. Furthermore, many plant saponins can activate the immune system, which leads to considerable interest in their potential as vaccine adjuvants [3]. This review deliberates the potential application of plant-derived saponin compounds in the development of vaccines against poultry coccidiosis. It also comprehensively explains the immunological effects, functions, constraints, and postulated mechanisms of action of saponins.

Saponins

Saponins are amphiphilic, heat-stable, and glycosidic secondary metabolites derived from plants [51]. These steroidal aglycones and triterpenoids are significantly used in the pharmaceutical industry [52]. They have immunomodulatory and antioxidant qualities that mainly make them useful as immunizing adjuvants against coccidiosis [53,54]. The bark, roots, leaves, and seeds of the *Quillaia Saponaria* tree yield an extract known to have immune-modulatory qualities. This tree is widely distributed throughout South America [3,52,55]. Non-polar

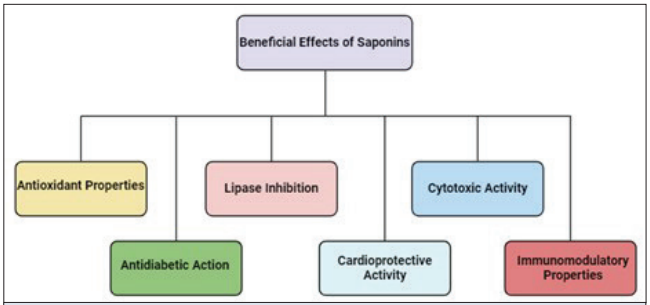

Fig 2. Properties of Saponins

Table 1. *Adjuvant active saponins*

Source	Adjuvant	Poultry	*Eimeria* spp.	Antigen	Route	Results	Ref.
Saponins	QCDC/RT	Broiler	*E. acervulina*	rProfilin	Subcutaneous	Weight gain, antibody production, and intestinal lesions were reduced	[63]
Saponins	QCDC-R	Chicken	*E. acervulina*	rProfilin	Subcutaneous	Mitogen-induced lymphocyte production and increased body weight Reduced intestinal lesions. No impact on oocyst shedding.	[64]
QS-21	QS-21	Chicken	*E.* spp.	IgG2a	Intramuscular and Intranasal	Purified saponin from *Quillaja saponica*, used to stabilize lipid emulsions	[65]
Saponins	QCDC	Chicken Embryo	*E. maxima*	rProfilin	Non-encapsulated	Increased growth. Reduced oocyst shedding	[66]
Anemone radiant Saponins	3- monodesmoside, 3,28-bisdesmoside	Chicken	*E.* spp.	IgGs	Subcutaneous	The serum antibody titer in chicken activates macrophages	[55, 67]
Saponins	QCDC	Broiler	*E. acervulina*	rProfilin	Subcutaneous	No effect on oocyst, IgG increased	[68]
Saponins	ISCOMs from endemic plants	Broiler	*E. tenella*	*E. tenella* all antigens	Intranasal	Increased IgGs protected against infection	[69]
Saponins	ISCOMs	Broiler	*E. tenella*	Antigens from sporozoite	Intranasal	Decreased lesion score and oocyst shedding	[70]
Saponins	ISCOMs	Broiler	*E. tenella*	AgP27	Diet Supplement	Increased total body weight Conferred partial protection against infection	[71]

aglycones make up the chemical structure of saponins, which is connected with chains of carbohydrates (polar) [56,57]. The presence of both polar and non-polar groups provides surface-active properties [58]. Saponins have a lot of beneficial effects as shown in *Fig. 2*.

They can stimulate a broad range of cytokine secretions [59], thereby enhancing both humoral and cellular immune responses [60]. Their ability to modulate innate immunity suggests that they could be used to design new vaccines that induce specific immune responses tailored to various pathogens and can reduce the drug resistance problem [61,62]. Adjuvant active saponins are shown in *Table 1*.

Saponin-Derived Compounds

Summarized in *Table 2*.

Immunostimulating Complexes (ISCOMs)

One innovative approach in utilizing saponins while mitigating their toxicity involves formulating them into Immunostimulating complexes "ISCOMs" [72]. ISCOMs are particulate adjuvant systems that combine saponins with cholesterol and phospholipids [73], significantly reducing toxicity while retaining potent adjuvant properties [74]. They provide a sophisticated approach to coccidiosis vaccination in poultry, taking advantage of their unique structure and composition to efficiently boost immune responses [3]. It mimics the immune system's innate detection of bacterial and viral structures to trigger potent humoral and cellular immune responses in poultry [75]. It is essential to activate both immune systems while treating intracellular parasites such as *Eimeria* species [76].

Table 2. *Saponin-Derived Compounds*

Source	Common Name	Adjuvant	Mode of Action	Benefits	Ref.
Peptasan	Sikakai	*Acacia concinna*	Immune modulation enhances adaptive and innate immune reactions Antioxidant and anti-inflammatory characteristics	Enhanced immune response Reduced parasite load Natural and safe for poultry	[91]
Yucca schidigera	Yucca	*Y. schidigera*	Saponins boost the immune response by boosting cytokine synthesis and T-cell proliferation Antioxidant characteristics	Improved gut health Reduced ammonia levels Enhanced vaccine efficacy	[92]
Quillaja Saponaria	Soapbark	*Q. Saponaria*	Saponins boost the immune response by boosting dendritic cell development and antigen presentation Adjuvant for vaccinations	Increased vaccine efficacy Enhanced antibody production Natural adjuvant	[50]
Norponin XO2	Yucca and Fenugreek	*Yucca schidigera* and *Trigonella foenum-graecum*	Yucca and Fenugreek saponins work together to enhance immunological response Improved intestinal health and decreased parasite load	The synergistic effect enhances immune response Improved gut health	[93]

Research has demonstrated encouraging outcomes in terms of improving vaccination efficacy and offering a more comprehensive defense against many *Eimeria* strains that are common in the production of chickens [45,77,78].

Quil A

Dalsgaard purified an adjuvant derived from *Q. saponaria* called Quil A containing a mixture of saponins in 1978 [79]. The mixture is enriched to use as an adjuvant for immunity induction via other isotypes of antibodies [80]. It is also used commercially in many vaccines [81,82]. The toxicity differs significantly between the components of Quil A [83,84]. The efficacy of Quil A as an adjuvant has been hindered by its apparent toxicity [85], which has been found in small animals following parenteral immunization with this adjuvant, and such toxicity may limit its utility [86]. However, nontoxic immunostimulatory fractions of Quil A have been identified, which may reduce or eliminate this issue [87]. While Quil A does not appear to be an effective mucosal adjuvant, its use as part of ISCOMs appears to be critical to the system's efficacy [88]. Structural and functional relationships of Saponin-adjuvant compounds are of interest because various fractions are used in immunological research.

QS-21

It can play an important role in poultry coccidiosis by improving antigen presentation and helping in T cells and antibody development [75]. Regardless of its efficacy, it is linked to formulation stability issues and some toxicity problems [89], which has prompted ongoing research to address these issues. The goal of continuing research is to create QS-21 variations that are less toxic while maintaining immunostimulatory activity to maximize its potential as an adjuvant in vaccines against coccidiosis [90]. QS-21 shows promising results as an adjuvant in boosting immune responses against coccidiosis. However, their efficacy and safety profiles in this setting necessitate additional research and optimization for their efficient application in poultry vaccination programs [44].

Mechanism of Action

Immunomodulatory Effects and Immune Boosting

In poultry, saponins enhance the immune response by affecting immune organ maturation, increasing antibody levels, and providing better defense against coccidiosis [94]. They are well-recognized for their potent immune modulatory effects [95]. The primary mechanism involves their interaction with immune cells, leading to the enhancement of the immune response [96]. They attract a variety of cells including macrophages, DCs (dendritic cells), and lymphocytes [55]. Saponins activate DCs by enhancing their capacity to process T lymphocytes. Maturation of DCs and upregulation of co-stimulatory chemicals allow for the efficient activation of T-cells [73,95]. B cells can create large amounts of antibodies. Saponins do this by boosting antibody isotype switching, resulting in a more effective immune response to pathogens.

Antioxidant Properties

Saponins have a great antioxidant capacity, which helps to manage reactive oxygen species and minimize diseases linked with oxidative stress, a major role in coccidiosis [97,98]. Reactive oxygen species (ROS) are produced during coccidia infection, resulting in oxidative stress and tissue damage. Saponins' antioxidant activities serve to neutralize ROS, lowering oxidative stress and its related

diseases [99]. This antioxidant activity not only protects the host from ROS-induced damage but also improves the immune response by lowering pro-inflammatory cytokine production and increasing overall antioxidant levels [100-102].

Mechanisms at the Cellular Level

Saponins regulate several essential systems that underpin the immune response. These include the effects on antigen presentation and cytokine generation [103]. Saponins have a crucial role in antigen presentation, which initiates the adaptive immune response [104]. When antigen-presenting cells (APCs) are enhanced, dendritic cells (DCs) and macrophages can efficiently process and present antigens, thereby initiating immune responses [78,105]. It involves the upregulation of major histocompatibility complex (MHC) molecules on the surface of APCs, that are important for presenting antigens to T-cells. Boosting antigen presentation by APCs leads to activation of helper T cells (Th cells) and cytotoxic T cells [106].

Comparative Studies with Other Adjuvants

Comparative studies help to position saponins within the broader context of coccidiosis control strategies and highlight their advantages and limitations. A comparative study by [107] assessed the efficacy of saponins compared to synthetic anticoccidial drugs in broiler chickens. The results demonstrated that saponins were as effective as synthetic drugs in controlling coccidiosis, with the added benefit of being natural and reducing the risk of resistance development. This study also emphasized the potential of saponins to be used in combination with other control methods to enhance their efficacy [107]. In another study, saponins were compared with ionophore anticoccidials, which are widely used in the poultry industry. The findings indicated that saponins were comparable to ionophores in reducing lesion scores and oocyst shedding. Moreover, saponins exhibited a more favorable safety profile, with fewer side effects reported in treated birds. This highlights the potential of saponins as safer alternatives to traditional anticoccidials [78,105].

Limitations of Phytochemical Adjuvant Vaccination of Coccidiosis

Phytochemicals may have anti-nutritional qualities, resulting in decreased feed intake, growth suppression, and negative effects on body growth [108]. Some may be harmful at high quantities but harmless at lower levels [109]. The effectiveness of phytochemicals in treating coccidiosis varies depending on the specific plant compounds employed, their quantities, and their ratios [110-112]. The lack of standardization has the potential to undermine the dependability and efficacy of phytochemical adjuvants [113]. The extensive use of phytochemicals, like anticoccidial chemical compounds, has the potential to cause resistance in *Eimeria* spp. [108]. As a result, it is critical to apply techniques to prevent resistance development, such as alternating phytochemical adjuvants with other management methods and employing them in conjunction with other interventions [114,115]. More research is needed to understand the mechanisms of action of phytochemical adjuvants and to identify the most effective chemicals for coccidiosis reduction.

Prospects

Advances in biotechnology and synthetic biology offer exciting opportunities for producing saponins with enhanced properties [112], genetic engineering techniques can be used to modify microorganisms to produce saponins with specific structural features that enhance their stability and immunostimulatory effects. Moreover, the integration of saponins with advanced delivery systems, such as nanocarriers and immunostimulating complexes, holds great potential. These delivery systems can protect saponins from degradation, enhance their targeting of immune cells, and provide a controlled release of antigens [115]. The task at hand involves developing standardized animal models that replicate the illnesses of the intended species for use in vaccination regimens and determining the parameters that must be assessed accurately to determine the effectiveness of vaccines [3].

Conclusion

In summary, the reviewed data suggest that saponins as adjuvants allow the opportunity to achieve the main objective of adjuvant research in vaccines, as a safe option that is mostly non-toxic and able to boost immune response as it may be included in many vaccine formulations against coccidiosis. However, the difficulty lies in choosing the best adjuvant that is most suitable for immunization protocols. Despite that, their decreased immunogenicity eventually became a crucial component as a refined antigen of several vaccinations. In addition to their amazing qualities, the non-toxic nature of most saponins eases the main worry about manufactured compounds having severe effects. Saponins can increase the efficacy of coccidiosis vaccines, which are made using live attenuated, inactivated, and recombinant techniques by increasing immune response. Formulations such as Quil A, QS-21, and ISCOMs have shown considerable promise in increasing vaccine efficacy while minimizing toxicity when appropriately modified. Unlike synthetic adjuvants, saponins are derived from plants and often exhibit fewer side effects, with added antioxidant and immunomodulatory benefits. However, comparative

studies suggest that saponins can be as effective as conventional anticoccidials, while also reducing the risk of drug resistance. It is demonstrated that they can dramatically boost the immune response, increasing protection against illness and saponins are safer substitutes for traditional adjuvants. All things considered, the use of saponins in vaccines is an essential tactic in the prevention and control of coccidiosis, and future research should concentrate on the creation of safer and more effective adjuvants and vaccines to tackle this serious health issue.

Declaration

Availability of Data and Materials: Data and materials for this research are available upon request.

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Research Article

Inhibitory Effect of Doxycycline on MMP2/9 and its Improvement in Cardiac Function and Left Ventricular Remodeling After Myocardial Infarction in Rats

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Abstract

The objective of this research was to investigate the inhibitory effect of doxycycline (DOX) on MMP2/9 and the improvement in cardiac function and left ventricular remodeling (LVR) after myocardial infarction (MI) in rats. For this purpose, thirty-six rats were randomly divided into control (group A), model (group B) and experimental (group C) groups. A MI model was established in groups B and C by ligation of the left anterior descending (LAD) of the coronary artery, while the rats in group A underwent thoracotomy without ligation only. The rats in groups A and B were injected with normal saline, and the rats in group C were injected with DOX. Two weeks after the operation, the cardiac function of the rats was evaluated by color Doppler ultrasound, myocardial infarct size by Masson staining, collagen content and the ratio of type I/III collagen by immunohistochemistry and immunoblotting respectively and activities of MMP2/9 by gelatin enzyme method. The results revealed DOX to cause reduction in the degree of ventricular enlargement and cardiac wall thinning, reduce the collagen content, increase the ratio of type I/III collagen, decrease the activities of MMP2/9, and reduce myocardial destruction and remodeling in rats after MI. However, further clinical research is recommended for the evaluation of the effectiveness of DOX.

Keywords: Doxycycline, Left ventricular remodeling, Myocardial infarction, Matrix metalloproteinases

Introduction

Ventricular remodeling after myocardial infarction (MI) is an important pathogenesis of heart failure that results from neurohumoral and other factors [1]. The mortality of patients with MI has gradually decreased, but the incidence of cardiac dysfunction caused by left ventricular remodeling (LVR) after MI has significantly increased. Heart failure after MI is gradually becoming the main cause of death in patients with MI [2]. This is due to the reason that the treatments fail to specifically address the underlying pathophysiological mechanisms [3]. The activities of many kinds of matrix metalloproteinases (MMPs), especially MMP-9, in the myocardium increase after MI [4]. Increased MMPs promote the development of LVR after MI by regulating extracellular matrix metabolism, which in turn aggravates cardiac function damage in patients and model animals after MI [5]. The inhibition of MMP activity in the myocardium after MI may reduce LVR and improve cardiac function in patients with MI [6,7].

LVR after acute MI (AMI) is a common pathophysiological process of progressive development in the clinic and begins within a few hours after AMI. Uncoordinated elongation and thinning of the necrotic myocardium and eccentric hypertrophy of the non-infarcted area lead to ventricular wall dilation, ventricular enlargement and changes in ventricular chamber geometry in the infarcted area [8]. This process increases the ventricular volume and leads to the impairment of LV function, which is considered to be the most important factor in determining the survival and prognosis of patients [9]. Doxycycline (DOX) is a tetracycline antibiotic and a broad-spectrum inhibitor of matrix metalloproteinases that can reduce the expression of MMPs by binding to the active center of MMPs and inhibiting their transcription [10]. However, it is not clear whether DOX can inhibit myocardial matrix metalloproteinases-2 and-9 and improve cardiac function and LVR in rats after MI. Therefore, the purpose of this study was to explore the effects of DOX on cardiac function and LVR after MI in rats and the possible underlying mechanism.

Materials and Methods

Ethical Statement

This study was approved by the Xi 'an Daxing Hospital committee (Approval No: 20230911).

Experimental Animals

Thirty-six male SD rats weighing 260±10 g were purchased from the Experimental Animal Center of Anhui Medical University and raised in the laboratory of the Animal Center. The temperature ranged from 20-22°C, and the humidity ranged from 45-55%.

Experimental Instruments and Reagents

Small ventilators and high-speed centrifugal freezers were purchased from the Eppendorf Company of Germany, IE33 echocardiography machines were purchased from the Shanghai Ohaus Company, and a ZD-9556 horizontal decolorizing shaker, horizontal electrophoresis instrument and inverted microscope were purchased from the Jiangsu Guosheng Experimental Instrument Factory. DOX was purchased from Haikou Kangliyuan Pharmaceutical Co., Ltd. (national drug standard: H20060405), a Masson staining kit was purchased from Beijing Biotechnology Co., Ltd. (production batch number: ZC00126), and a GENMED matrix metalloproteinases and gelatin zymography electrophoresis analysis kit was purchased from Shanghai Jiemei Genome Pharmaceutical Technology Co., Ltd.

Establishment of a Rat Model of MI

Thirty-six rats were anesthetized by intraperitoneal injection of pentobarbital sodium (PB). An electrophysiological instrument was used to monitor the electrocardiogram, endotracheal intubation and ventilation. The respiratory rate was set at 45 beats per minute, and the tidal volume was 30 mL. After the thoracotomy test, the anterior descending branch was marked, and the LAD of the coronary artery was ligated 1 mm below the root of the left atrial appendage. The rats whose electrocardiogram showed characteristic STT changes of MI after the operation that lasted for more than half an hour were judged to be successful in establishing the MI model. The successful rats were randomly divided into model (group B) (n = 12) and experimental (group C) groups (n = 12). Three days after the establishment of the MI model, group C was given 15 mg kg-1 body weight (BW) DOX with a 1 mL saline dilution and then injected intraperitoneally twice. In group B, only 0.5 mL of saline was injected intraperitoneally twice a day. In the other 12 rats, the chest was only opened to expose the heart, but the coronary artery was not ligated. In group a, 0.5 mg of normal saline was injected intraperitoneally twice a day for 5 days.

Ultrasonic Electrocardiogram Examination

Two weeks later, three groups of rats with MI were anesthetized and weighed by intraperitoneal injection of PB, and the heart was examined by HP-5500 color Doppler echocardiography. The LVAW, LVPW and LVDd were recorded by M-mode echocardiography at the LV short axis papillary muscle plane. The LVEF and LVFS were calculated by the Teichholtz method. All the data were analyzed offline after recording, and each parameter was measured for 3 cardiac cycles and averaged.

Specimen Handling and Masson Staining

After blood was collected from the right ventricle of the open chest, the heart was washed, the fat was removed with PBS solution, and the whole heart weight (HW) was measured. Compared with BW, the proportion of HW/BW was calculated. A 2 mm thick tissue layer was cut from the middle part of the rat heart along the cross section and fixed with 10% formalin, while the apical part was quickly frozen and preserved in liquid nitrogen.

After the myocardial tissue was fixed, it was embedded in paraffin and cut into 5 μm thick sections. A Masson staining kit was used to dye the slices. Images were taken under a 10x microscope and saved in TIF format. The images were analyzed with imaging software to calculate the ratio of the infarct area to the LV area.

Determination of the Total Amount of Collagen and the Ratio of Type I/III Collagen

According to the hydroxyproline digestion kit and type I/III collagen immunohistochemical detection kit, the images were analyzed and processed by a CMIAS true color medical image analysis system. By observing the ratio of collagen to the area of the heart in the visual field, the content of collagen can be estimated indirectly, and then the ratio of type I/type III collagen can be calculated.

Zymography and Immunoblotting

The myocardial tissue samples of 6 rats in the three groups were treated, ground in lysis buffer, and centrifuged at 12.000 rpm for 15 min, after which the supernatant was collected for subsequent analysis. The protein content in the supernatant was determined by a bicinchoninic acid (BCA) protein concentration assay. Samples with a protein content of 60 μg were selected, and the activities of the two target enzymes were determined according to the instructions of the MMP-2 and MMP-9 activity detection kits. The image with the target band was captured by a Bio-Rad imaging analysis system, and the grayscale value of the target band was analyzed by Quantity-One software. The gray values obtained were compared with those in group A to evaluate the degree of MI. After protein extraction, the frozen rat myocardium was extracted by immunoblotting and subjected to transmembrane staining, incubated with primary and secondary antibodies and treated with a DAB chromogenic agent.

Table 1. LVR and cardiac function in rats in each group

Group	N	LVAW (mm)	LVPW (mm)	LVDd (mm)	LVSd (mm)	LVFS (%)	LVEF (%)
A	12	0.26±0.08	2.39±0.34	4.56±0.90	3.46±0.64	40.32±9.56	74.14±9.63
B	12	1.93±0.14[a]	2.25±0.42[a]	6.90±1.08[a]	5.05±0.92[a]	27.21±4.10[a]	52.40±6.51[a]
C	12	1.43±0.17[ab]	2.42±0.43[ab]	5.82±0.92[ab]	4.26±0.83[ab]	33.53±4.72[ab]	66.73±5.22[ab]
F		481.730	0.620	17.490	11.700	11.86	27.080
P		<0.001	0.543	<0.001	<0.001	<0.001	<0.001

** Parameters' values across different groups in the same column having similar superscripts are statistically non-significant*
LVAW = Left Ventricular Anterior Wall, LVPW = Left Ventricular Posterior Wall, LVDd = Left Ventricular End-Diastolic Diameter, LVSd = Left Ventricular End-Systolic Diameter, LVFS = Left Ventricular Fractional Shortening, LVEF = Left Ventricular Ejection Fraction

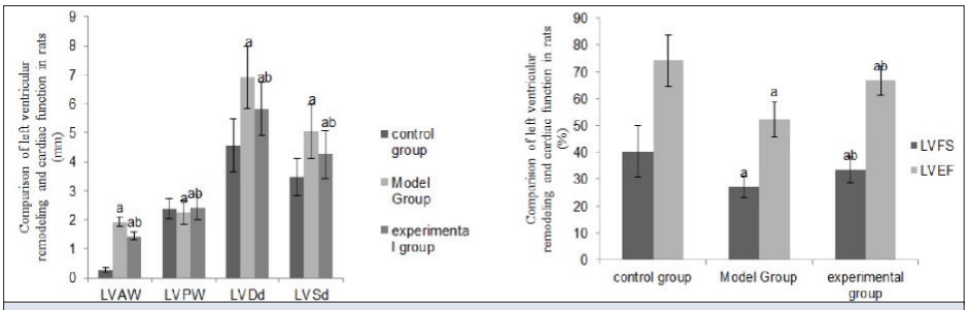

Fig 1. Comparison of LVR and cardiac function in rats

Table 2. MI size in rats

Group	N	HW/BW
A	12	3.92±0.11
B	12	5.03±0.16[a]
C	12	4.27±0.14[ab]
F		202.320
P		<0.001

** Parameters' values across different groups in the same column having similar superscripts are statistically non-significant*
HW/BW = Heart Weight Body Weight ratio

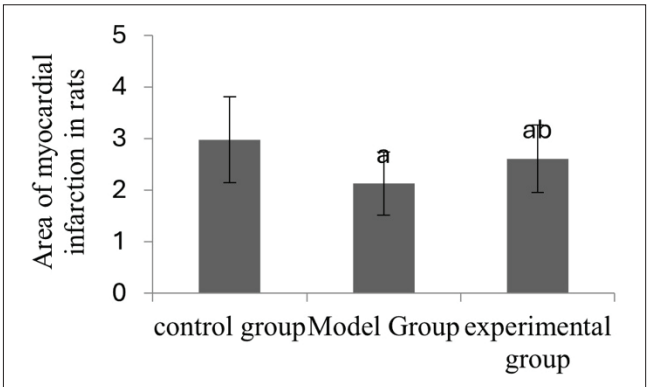

Fig 2. Comparison of MI size among the rats in each group

Statistical Analysis

All the data are represented by (`x±s), and the experimental data were analyzed by SPSS 20.0 statistical software. Compared with that of group A, the [a]P of group B was less than 0.05. Compared with that of group B, the [b]P of group C was less than 0.05.

Results

Effects of DOX on LVR and Cardiac Function After MI in Rats

The results of echocardiography showed that the LV cavity of rats after MI was enlarged, the LV anterior wall membrane became thinner, and many dilated into ventricular aneurysms. The LVAW, LVDd and LVSd in group B were greater than those in group A, while these values decreased, and the LVFS and LVEF increased in group C compared with those in group B *(Table 1, Fig. 1)*.

Analysis of Myocardial Infarct Size by Masson Staining

The results of Masson staining showed that the infarcted myocardial tissue was replaced by collagen, which was light blue, while the normal myocardial tissue was red. In group B, the ventricular wall in the infarcted area became thinner, and the LV cavity became enlarged, while the LV wall thinning and bulging in group C improved compared with those in group B. The HW/BW ratio of group B was increased group A, and the HW/BW ratio of group C decreased than group B *(Table 2, Fig. 2)*.

Comparison of the Ratio of Myocardial Collagen and I/III Collagen

The collagen content of group B was greater than that of group A, and the ratio of I/III collagen decreased, while that of group C decreased, and the ratio of I/III collagen increased compared with that of group B *(Table 3, Fig. 3)*.

Table 3. *The ratios of myocardial collagen and I/III collagen in rats*

Group	N	Collagen Content (μg/mg)	I/III Collagen
A	12	34.62±1.53	2.98±0.83
B	12	38.65±2.12[a]	2.13±0.61[a]
C	12	36.17±2.05[ab]	2.61±0.65[ab]
F		13.480	4.410
P		<0.001	0.020

** Parameters' values across different groups in the same column having similar superscripts are statistically non-significant*
HW/BW = Collagen type I and III ratio

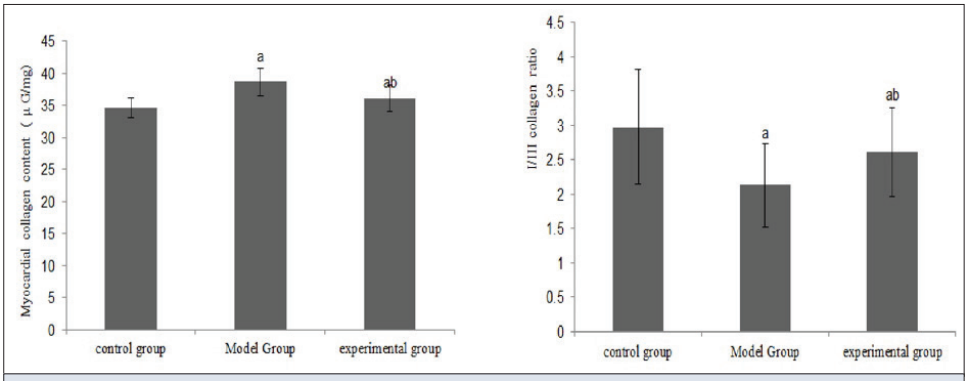

Fig 3. Comparison of the ratios of myocardial collagen and I/III collagen

Table 4. *Comparison of myocardial MMP-2 and MMP-9 activities in rats*

Group	N	MMP-2	MMP-9
A	12	1.03±0.02	1.02±0.03
B	12	1.87±0.14[a]	2.18±0.16[a]
C	12	1.52±0.12[ab]	1.34±0.14[ab]
F		186.310	28.300
P		<0.001	<0.001

** Parameters' values across different groups in the same column having similar superscripts are statistically non-significant*
MMP-2 = Matrix Metalloproteinase-2, MMP-9= Matrix Metalloproteinase-9

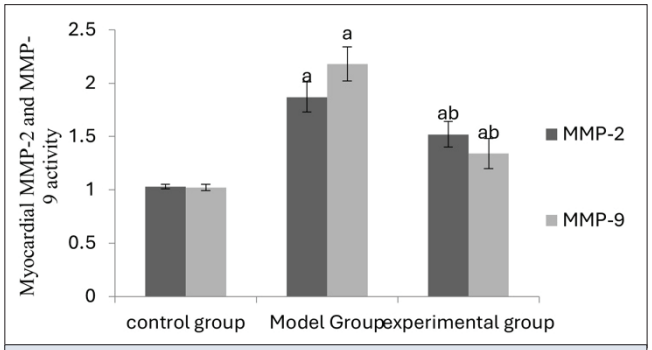

Fig 4. Comparison of myocardial MMP-2 and MMP-9 activities in rats

Comparison of Myocardial MMP-2 and MMP-9 Activities

The MMP-2 and MMP-9 in group B were greater than those in group A, while those in group C were less than group B *(Table 4, Fig. 4)*.

Discussion

MI can lead to severe myocardial injury. Gene expression changes after injury, resulting in increased expression of inflammatory factors, MMPs and growth factors; cardiomyocyte hypertrophy; necrosis; apoptosis; fibroblast proliferation; extracellular matrix degradation; and collagen accumulation, that is, fibrosis [11,12]. Exploring the mechanism of ventricular remodeling caused by MI and to identify effective therapeutic drugs is important.

The results showed that the ratio of LV weight to BW in group C was lower than group B, indicating that DOX could inhibit the development of cardiac remodeling to some extent. The results of Masson staining showed that the infarcted myocardial tissue was replaced by collagen, which was light blue, while the normal myocardial tissue was red. In group B, the ventricular wall in the infarcted

area became thinner, and the LV cavity became enlarged, while the LV wall thinning and bulging in the DOX group improved than group B. These findings suggest that DOX therapy can inhibit the development of ventricular remodeling to some extent. The ultrasound results also showed that DOX could improve LVR, reduce the thinning of the ventricular wall in the infarcted area and reduce the enlargement of the LV cavity. After MI, the process of LVR develops with a decrease in cardiac function, which may eventually lead to heart failure. The cardiac function of the rats was evaluated by color Doppler echocardiography. The LVAW, LVDd and LVSd in group C were reduced than group B. These findings suggested that DOX can inhibit the process of LVR, reduce the degree of LV dilatation and improve the cardiac function of MI rats to some extent.

LVR is the result of many factors, especially an increase in MMP activity, which promotes intercellular matrix degradation and fibrosis [13]. DOX is a tetracycline antibiotic that not only can inhibit bacterial protein synthesis but is also a broad-spectrum and efficient MMP inhibitor [14,15]. The activities of MMP-2 and MMP-9 in group C were lower than those in group B. Analysis revealed that DOX can reduce the expression of MMP-2 by reducing the half-life of mRNAs related to MMP-2 expression [10]. DOX can also inhibit the transcription of MMPs and reduce the activity and expression of MMPs in tissue by binding to the active center of MMPs [16]. Moreover, it has been shown that DOX can also reduce both the local and systemic inflammation as observed in various animal models [17,18]. This is achieved through factors such as C-reactive protein and myeloperoxide that further inhibit the progression of LVR. DOX can also inhibit cardiomyocyte apoptosis, thus inhibiting the development of ventricular fibrosis and improving LVR [19,20].

Collagen is the extracellular matrix and plays an important role in the functional unit of cardiomyocytes, especially type I and type III collagen [21]. After MI, ventricular remodeling occurs, and the early repair process is mainly characterized by an increase in the expression of type III collagen [22]. However, water tissue composed of type III collagen is not as strong as that composed mainly of type I collagen. Therefore, changes in collagen composition after MI can lead to cardiac enlargement [23]. Hydroxyproline accounts for 13.4% of collagen, very little in elastin, and does not exist in other proteins [24,25]. After treatment with DOX, the collagen content of group C was reduced than group B, and the ratio of group I/III collagen was optimized. Thus, DOX can reduce the degradation of collagen and inhibit LVR.

In summary, the activation of MMPs in myocardial tissue after MI promotes the development of LVR and heart failure. DOX can improve LVR and cardiac function after MI to some extent by inhibiting the activity of MMPs.

Declarations

Availability of Data and Materials: All data generated or analyzed during this study are available from the corresponding author (J. Li) upon reasonable request.

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Ethical Statement: This study was approved by the Xi 'an Daxing Hospital committee (Approval No: 20230911).

Competing Interests: The authors declare that they have no competing interests.

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Authors' Contribution: YC and JL designed the experiment, carried out research collected data and performed data analysis whereas KL was involved in manuscript drafting, improving the language of the manuscript and reviewing of the final draft. All authors read and approved the final manuscript and agree to be accountable for all aspects of the work. This statement complies with the"ETHICAL PRINCIPLES AND PUBLICATION POLICY/Authorship and Authors Rights"guidelines of the journal.

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RESEARCH ARTICLE

Dendrobium officinale Polysaccharide Regulates the Immune Function of RAW264.7 Cells Via the NF-κB and MAPK Signaling Pathways

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Abstract

To investigate the immunomodulatory effects of *Dendrobium officinale* polysaccharide (DOP) on RAW264.7 macrophages. DOP was purified from *Dendrobium officinale* using water extraction-alcohol precipitation and gel column chromatography. The molecular weight distribution of the polysaccharide was analyzed by high-performance gel permeation chromatography (HPGPC). The effects of DOP on RAW264.7 cells were evaluated as follows: cell viability was assessed via MTT assay to screen appropriate treatment concentrations; ELISA was used to detect the secretion of inflammatory cytokines (IL-10, IL-12 p40, and IL-6); RT-qPCR was employed to measure the expression of inflammatory genes *(IL-1β, IL-10, IL-12 p40*, and *IL-6);* and Western blotting was performed to analyze the expression of P65, phosphorylated P65 (p-P65), JNK, phosphorylated JNK (p-JNK), ERK, and phosphorylated ERK (p-ERK). The purified DOP exhibited a yield of 8.24% and a total sugar content of 95.33±3.11%. HPGPC analysis revealed three peaks corresponding to molecular weights of 1.89 x 10^5 Da, 549 Da, and 211 Da. DOP showed no cytotoxicity toward RAW264.7 cells at concentrations ranging from 3.125 to 400 μg/mL. All tested concentrations of DOP significantly enhanced the mRNA expression of *IL-1β, IL-10, IL-12 p40*, and *IL-6* ($P<0.05$, $P<0.01$, $P<0.001$) and promoted the secretion of IL-10, IL-12 p40, and IL-6 in a dose-dependent manner. Regarding the NF-κB and MAPK signaling pathways, DOP markedly increased the expression of p-JNK, p-ERK, and p-P65 proteins ($P<0.001$), with sustained effects observed for up to 120 minutes ($P<0.001$). DOP activates RAW264.7 cells by promoting inflammatory gene expression, stimulating cytokine secretion, and triggering the NF-κB and MAPK pathways, thereby exerting immunomodulatory effects.

Keywords: *Dendrobium officinale* polysaccharide, RAW264.7 Macrophages, Cytokines, Immunomodulation

INTRODUCTION

Dendrobium officinale, a perennial herb of the Orchidaceae family, is revered as the foremost among the "Nine Sacred Herbs" in traditional Chinese medicine and is listed in the Pharmacopoeia of the People's Republic of China (2020 edition). It is renowned for its pharmacological effects, including assisting in clearing internal heat, regulating gastric function, and enhancing digestive absorption capacity [1]. Modern pharmacological studies have identified diverse bioactive components in *Dendrobium officinale*, such as polysaccharides, alkaloids, flavonoids, phenylpropanoids, and lignans, with polysaccharides being the most abundant [2]. Research on polysaccharides in China began relatively late, starting in the 1970s, but has rapidly advanced, emerging as a focal point in modern pharmacological investigations. Recent studies have confirmed that polysaccharides exhibit multifaceted biological functions, including antibacterial, antioxidant, gut microbiota-modulating, and immunomodulatory activities [3,4], positioning them as a valuable resource for novel drug development.

Macrophages, extensively distributed across various tissues in organisms, play a dual role in both phagocytosing foreign pathogens/tumor cells and mediating the immune response via cytokine release (e.g., tumor necrosis factor and interleukins), thereby serving as primary agents of immune functionality [5]. Research demonstrated that polysaccharide components derived from traditional Chinese medicine can stimulate macrophages, enhancing secretion of cytokines such as nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) [6].

This study involved the extraction of crude polysaccharides from *Dendrobium officinale* via ethanol precipitation, followed by purification and separation through gel column chromatography, yielding *Dendrobium officinale* polysaccharide (DOP) with a total polysaccharide content exceeding 90%. The aim was to analyze the immunomodulatory effects of these DOP on RAW264.7 cells, providing a scientific foundation for further exploration and development of the active components within DOP.

Material and Methods

Ethical Statement

This study does not require ethical permission.

Materials and Chemicals

Dendrobium officinale was collected from the Dendrobium base of West Anhui University and identified by Professor LU Baowei of West Anhui University. RAW264.7 mouse macrophage cells were procured from the Shanghai Institute of Cell Research. The required fetal bovine serum, high-glucose DMEM culture medium, 0.25% trypsin with EDTA, and PBS phosphate-buffered saline were purchased from Shanghai Pufei Biotech Co., Ltd. Lipopolysaccharide (LPS) and MTT assay reagents were acquired from Sigma, USA. Sinopharm Chemical Reagent Co., Ltd supplied anhydrous ethanol, chloroform, and isopropanol. Mouse IL-10, IL-12p40, IL-6 ELISA kits, along with antibodies for GAPDH, NF-κB p65, Phospho-NF-κB p65, c-Jun N-terminal kinase (JNK), p-JNK, ERK, and p-ERK, were obtained from Wuhan Boster Biological Technology, Ltd. TRIzol reagent for RNA isolation was sourced from Shandong Seko Biotech Co., Ltd. For cDNA synthesis and qPCR analysis, NovoScript® Plus All-in-one 1st Strand cDNA Synthesis SuperMix (gDNA Purge), and NovoScript® SYBR qPCR SuperMix Plus were acquired from Coastal Biotech Co., Ltd. The antibodies targeting GAPDH, NF-κB p65, Phospho-NF-κB p65, JNK, p-JNK, ERK, and p-ERK were also sourced from Wuhan Boster Biological Technology, Ltd.

Preparation of DOP

In this study, 300 g of fresh *Dendrobium officinale* stems were initially blended with 200 mL of water, then mixed with an additional 400 mL of water for heat reflux extraction (2 x 2 h). The extract was filtered through filter paper, and the filtrate was subsequently concentrated under reduced pressure to a volume of 600 mL, achieving a relative density of 1.07. During stirring, 95% ethanol was added to achieve an alcohol concentration of 80%, and the mixture was allowed to stand at 4°C for 24 h. The resultant precipitate was collected through vacuum filtration, redissolved in water, and subjected to alcohol precipitation three times by the same method to yield crude DOP. The polysaccharides were then defatted with ethanol, acetone, and petroleum ether, followed by deproteinization using the Sevage method [7]. For purification, the DOP underwent DEAE Sephadex A50 gel column chromatography, eluting in a sequence with distilled water and escalating concentrations of NaCl solutions (0.1, 0.3, 0.5, 0.7, 1.0, and 2.0 M) at a flow rate of 0.5 mL/min. An automatic fraction collector gathered 7.5 mL samples every 15 min. The phenol-sulfuric acid assay was utilized to determine the absorbance at 490 nm of each eluate, with tube numbers plotted against absorbance values to establish an elution curve. Sampling followed an alternating tube sequence (1, 3, 5, 7, 9, 11, 13, etc.), with positive peaks merged based on the elution curve. These combined eluates were then concentrated to a smaller volume under reduced pressure at 45°C using a rotary evaporator and dialyzed in a dialysis bag with a molecular weight cutoff of 7.000 Da against tap water for 48 h, refreshing the water every 6 h, before further dialysis against distilled water for 48 h The dialysate was freeze-dried to obtain purified DOP.

Determination of Total Polysaccharide Content of *Dendrobium polysaccharides*

Using phenol-sulfuric acid method [8] to detect the total sugar content of DOP. Weigh 10.1 mg of anhydrous glucose and dissolve it in double-distilled water, adjusting the volume to 100 mL to prepare a glucose standard solution with a concentration of 0.101 mg/mL. Sequentially transfer 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mL of this solution into 10 mL test tubes with stoppers, and dilute each to 2.0 mL with double-distilled water. To each tube, add 1.0 mL of 6% phenol solution, followed by 5 mL of concentrated sulfuric acid. Thoroughly mix by vortexing and allow the mixture to stand for 20 minutes for color development before measuring the absorbance at 490 nm. Construct a standard curve plotting glucose concentration on the x-axis against absorbance on the y-axis. For the polysaccharide sample analysis, weigh 5 mg of the sample and dissolve it in water, adjusting the total volume to 25 mL. Transfer 1 mL of this solution into a 10 mL stoppered test tube. Measure the absorbance of this solution using the established method.

Analysis of Molecular Weight Distribution of DOP

An Agilent 1260 HPLC system equipped with a Shodex KS805 column (8.0 x 300 mm) was utilized for the analysis. The mobile phase was ultrapure water, with detection conducted via a differential refractive index detector. The settings included an injection volume of 50 µL, a column temperature of 35°C, and a flow rate of 1.0 mL/min. Glucan standards, with molecular weights of 10, 40, 70, and 500 kDa, were individually prepared in ultrapure water to a concentration of 1 mg/mL. A linear

regression analysis was conducted with the retention time on the x-axis and the logarithm of molecular weight (log MW) on the y-axis, using the glucan standards to establish a linear regression equation. The samples were prepared at a concentration of 1 mg/mL in ultrapure water and filtered through a 0.22 μm microfiltration membrane prior to injection for chromatographic analysis.

MTT Analysis of the Effect of DOP on RAW264.7 Cells Proliferation

MTT assay was used to detect the cell proliferation of RAW264.7 cells [9]. RAW264.7 cells were plated at a density of 1.5×10^5 cells per well and underwent a 24-h culture period to ensure proper adhesion. Subsequently, 100 μL of varying concentrations of DOP solution were introduced to the cells, which were then incubated at 37°C in a CO_2 incubator for 20 h. Following this, MTT solution was administered, and the cells were incubated for an additional 4 h. Post-incubation, the 96-well plate was centrifuged at 2.000 rpm for 5 min, the supernatant was removed, and 150 μL of acidic DMSO was added to each well. The plate was then subjected to shaking on a micro-vibrator for 15 min, after which the optical density (OD) at a wavelength of 490 nm was recorded.

ELISA Detection of Inflammatory Cytokine Secretion in RAW264.7 Cells

In a separate set of experiments, RAW264.7 cells were seeded at a concentration of 2×10^5 cells per well in a 24-well plate and cultured for 24 h to promote cell adhesion. Following this, the cells were treated with equal volumes of DOP solution at various concentrations and incubated at 37°C in an atmosphere of 5% CO_2 for 24 h. After incubation, the supernatants from the cell cultures were harvested, and the concentrations of IL-10, IL-12p40, and IL-6 were quantified using an ELISA, in accordance with the instructions provided in the kit's manual.

RT-qPCR Detection of Inflammatory Gene Expression

RAW264.7 cells were cultured in 24-well plates at a density of 1.5 × 10^5 cells per well and treated with varying concentrations of DOP solution. Incubation was carried out at 37°C in a 5% CO_2 incubator for durations of 1, 2, and 4 h. Subsequent to the removal of the cell culture supernatant, each well was treated with 1 mL of TRIzol for cell sample collection. The total RNA was extracted from these samples using the chloroform-isopropanol extraction technique [7]. Following RNA extraction, cDNA synthesis was performed using a reverse transcription kit. The RT-qPCR was then conducted by adding 1 μL of cDNA template, specific primers (detailed in *Table 1*), and the reaction mixture under the following amplification conditions: an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min, with a final extension step at 95°C for 5 sec and 65°C for 5 sec. Primer specificity for each gene was validated through dissociation curve analysis and agarose gel electrophoresis. GAPDH served as the internal control. The relative gene expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method, with primer sequences listed in *Table 1*.

Western Blot Analysis of the Expression of Relevant Proteins

RAW264.7 cells were cultured at a concentration of 2×10^5 cells per well in 24-well plates and incubated for 24 h prior to treatment with DOP (400 μg/mL) at five designated time points: 0, 15, 30, 60, and 120 min. Following treatment, 30 μL of lysis buffer was added to each well. The mixture was thoroughly mixed by pipetting and then transferred to 1.5 mL centrifuge tubes. The bromocresol green with albumin (BCA) assay was used to measure the total protein concentration in each sample. For protein separation, samples were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (with an initial voltage of 70 V for the stacking gel for 30 min and 110 V for the resolving gel for 60 min). Proteins were then transferred to Polyvinylidene fluoride (PVDF) membranes at 300 mA for 90 min. Post-transfer, membranes were blocked in blocking solution at room temperature for 2 h on a shaker, then washed with TBST. The primary antibody, diluted 1:1000, was applied and

Table 1. *Primer sequence*

Genes	Primer Sequence		Product Size (bp)
GAPDH	F	5'-ATCCTGTAGGCCAGGTGATG-3'	104
	R	5'-TATGCCCGAGGACAATAAGG-3'	
IL-1β	F	5'-TTGACAGTGATGAGAATGACCTG-3'	137
	R	5'-GCTCTTGTTGATGTGCTGCT-3'	
IL-10	F	5'-GCTCTTACTGACTGGCATGAG-3'	105
	R	5'-CGCAGCTCTAGGAGCATGTG-3'	
IL-12p40	F	5'-GGAAGCACGGCAGCAGAATAAAT-3'	180
	R	5'-AACTTGAGGGAGAAGTAGGAATGG-3'	

left to incubate overnight at 4°C. After primary antibody incubation and subsequent washes, the secondary antibody was applied and incubated with shaking at room temperature for 2 h. After this incubation period, membranes were washed and subjected to development. ImageJ software was utilized to quantify the grayscale values of the protein bands.

Statistical Analysis

All data were analyzed using a one-way analysis of variance with LSD test and expressed as the mean ± standard error of the mean (SD). SPSS software (SPSS 22.0, IBM, USA) was used and statistical significance was set at $P<0.05$.

Results

Preparation of DOP

Crude polysaccharides from *Dendrobium officinale* (CDOP) were isolated via water extraction, followed by concentration under reduced pressure, ethanol precipitation, and deproteinization, resulting in an extraction efficiency of 19.22%. The total sugar content was determined to be 67.53±4.32%, using the phenol-sulfuric acid method. Subsequent separation of CDOP employed DEAE Sephadex A50 column chromatography, utilizing varying concentrations of NaCl solution for elution. An elution curve was generated, mapping eluent tube numbers against optical density (OD) values *(Fig. 1)*. Peaks identified as positive from this curve were pooled, concentrated via reduced pressure to a minimal volume, and subjected to desalination by dialysis in a 7000 Da molecular weight cutoff bag. This process was followed by freeze-drying the dialysate to yield DOP, achieving a yield of 8.24% and a total sugar content of 95.33±3.11%.

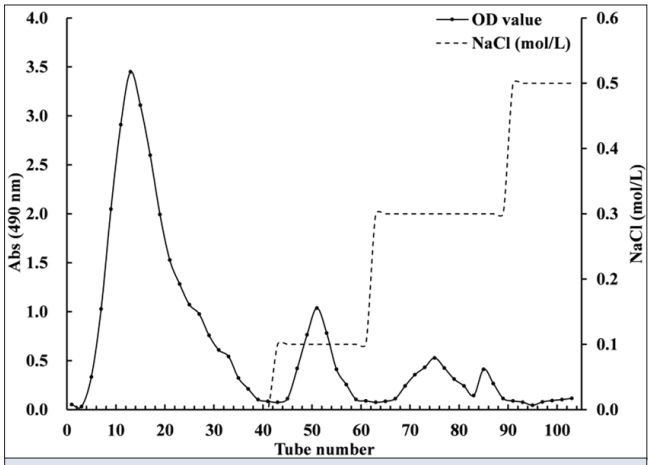

Fig 1. Elution curve of DOP DEAE-A50

The Molecular Weight Distribution of DOP was Analyzed Using HPGPC

A standard curve was constructed by plotting the logarithm of molecular weights of standard polysaccharides against their retention times. Analysis of the sample revealed three distinct peaks: the primary peak (Peak 1) with a molecular weight of 1.89×10^5 Da, and the subsequent peaks, Peak 2 and Peak 3, with molecular weights of 549 Da and 211 Da, respectively *(Fig. 2)*.

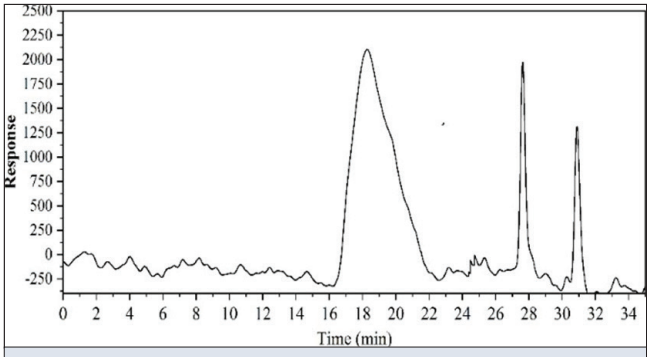

Fig 2. The HPGPC chromatogram of DOP

Impact of DOP on RAW264.7 Cells Proliferation

The *Fig. 3* demonstrates that DOP do not exhibit cytotoxicity towards RAW264.7 cells across a concentration range of 3.125 to 400 μg/mL, showing no significant deviation from the normal control group's results ($P>0.05$). Nonetheless, at a concentration of 600 μg/mL, DOP significantly reduces the proliferation of RAW264.7 cells ($P<0.05$).

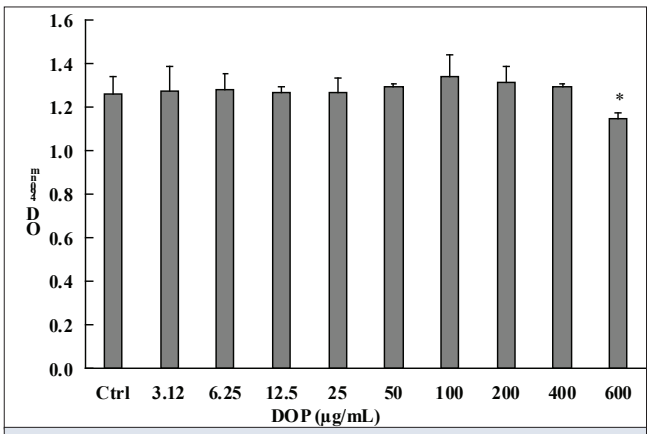

Fig 3. Effect of DOP on the proliferation of RAW264.7 cells. Compared to the control group, *P<0.05, **P<0.01

Effects of DOP on the Expression of Inflammatory Cytokine Genes in RAW264.7 Cells

As shown in *Fig. 4*, the mRNA expression levels of *IL-1β*, *IL-6*, *IL-10*, and *IL-12 p40* in RAW264.7 cells were analyzed by quantitative real-time PCR after 1, 2, and 4 hours of DOP treatment. All tested concentrations of DOP significantly upregulated the mRNA expression of IL-1β, IL-10, IL-12 p40, and IL-6 in RAW264.7 cells ($P<0.01$). The stimulatory effects of DOP on these cytokines exhibited a clear dose-dependent manner, with progressively

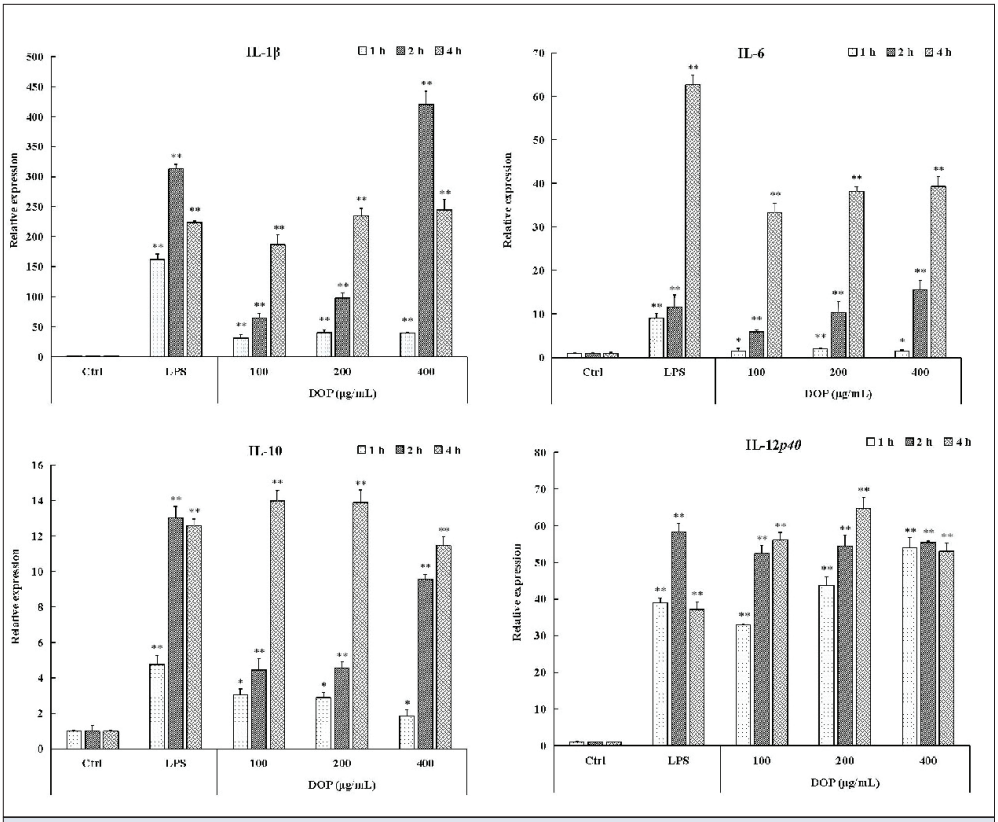

Fig 4. Effect of DOP on the expression of inflammatory cytokine mRNA in RAW264.7 cells. Compared to the control group, *P<0.05, **P<0.01

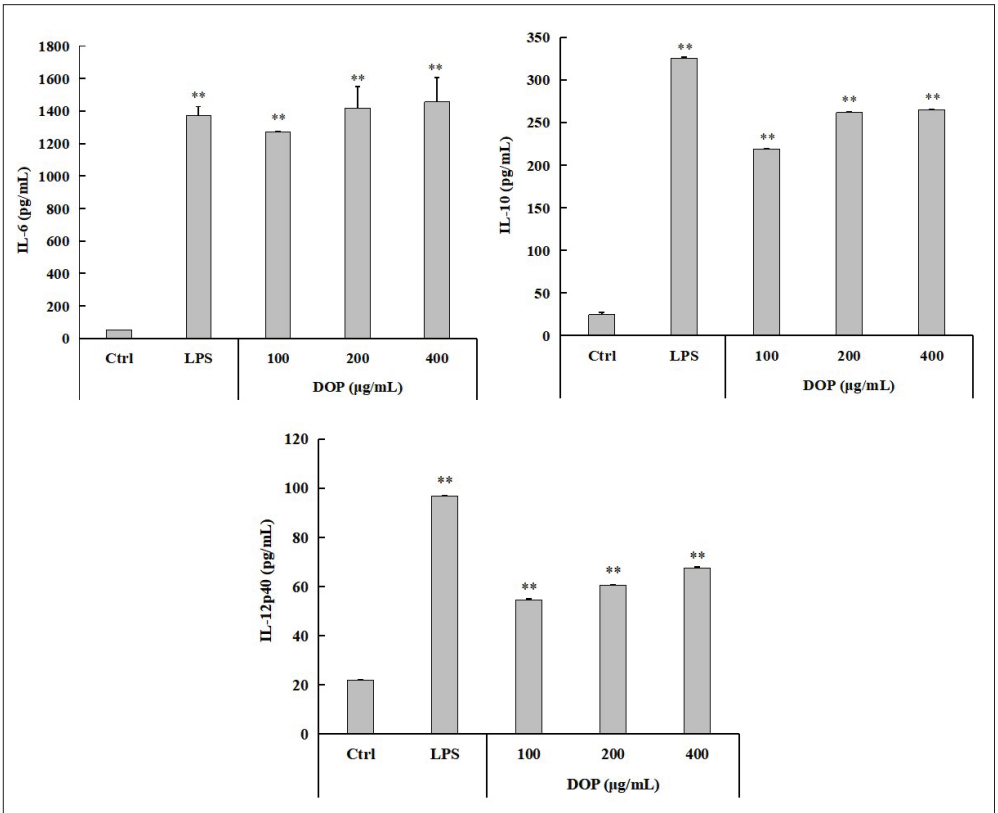

Fig 5. Effect of DOP on the expression of inflammatory cytokine mRNA in RAW264.7 cells. Compared to the control group, *P<0.05, **P<0.01

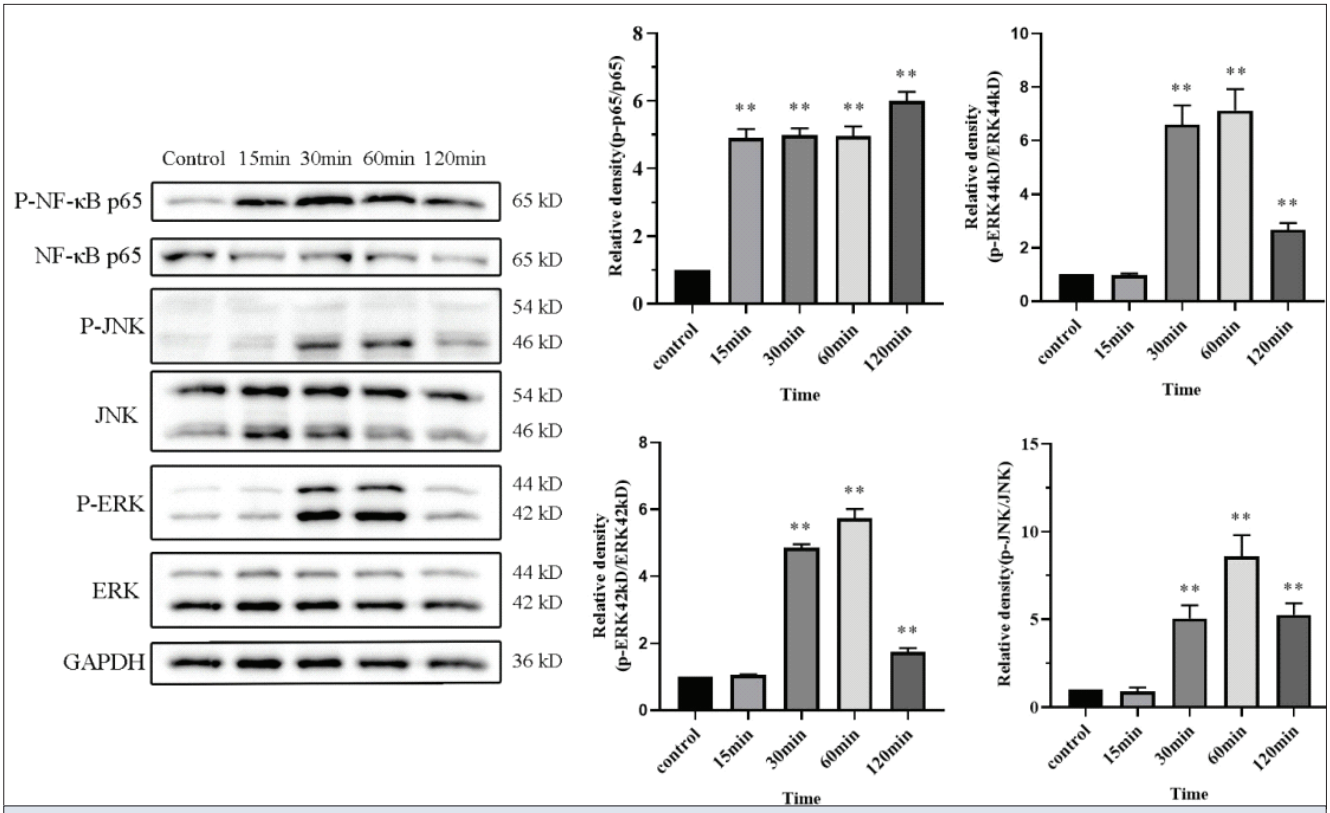

Fig 6. Effects of DOP on the expression of NF-κB/MAPK-related proteins in RAW264.7 cells. Compared to the control group, *P<0.05, **P<0.01

enhanced mRNA upregulation as the DOP concentration increased. Furthermore, prolonged treatment duration (up to 4 h) resulted in a time-dependent elevation of mRNA expression for IL-1β, IL-12 p40, IL-10, and IL-6, with the highest fold increase observed at the 4-h time point (P<0.01).

Influence of DOP on Inflammatory Cytokine Release in RAW264.7 Cells

As shown in *Fig. 5*, the cytokine levels in the culture supernatant of RAW264.7 cells treated with DOP for 24 h were measured using ELISA. The results demonstrated that DOP at various concentrations could significantly promote the secretion of IL-10, IL-12 p40, and IL-6 in RAW264.7 cells (P<0.01). With increasing administration concentrations, the enhancing effect of DOP on the secretion of these cytokines gradually strengthened, showing a favorable dose-dependent relationship.

Impact of DOP on the Expression of Relevant Proteins in RAW264.7 Cells

As shown in *Fig. 6*, compared with the control group, the protein expression levels of p-JNK, p-ERK, and p-p65 were significantly increased in all drug-administered groups (P<0.01). At 15 min after administration, only p65 protein expression showed a significant increase (P<0.01). After 30 and 60 min of drug treatment, the protein expression levels of p-JNK, p-ERK, and p-p65 were all significantly elevated (P<0.01). By 120 min post-administration, while the expression levels of p-JNK and p-ERK decreased compared to those at 1 h, they still remained significantly higher than the control group (P<0.01).

Discussion

Dendrobium officinale, a valued traditional Chinese medicinal herb known as "life-saving fairy grass," has shown significant biological activities in recent pharmacological research [10]. Studies suggest its extracts enhance hemolysin antibody levels and carbon clearance index [11]. However, the exact immune-regulatory mechanisms and target cells of DOP remain unclear. This study investigates DOP's effects on inflammatory factor release and gene expression in RAW264.7 macrophages to clarify its immune-regulatory actions and lay the groundwork for future research.

In this research, DOP were purified using a method that combined water extraction, ethanol precipitation, and gel column chromatography [12], yielding a polysaccharide product with a total sugar content of over 95%. Molecular weight analysis of the purified polysaccharides revealed three distinct peaks, suggesting that, although the total sugar content was high, the molecular weight distribution of the DOP was heterogeneous.

Macrophages, originating from bone marrow hematopoietic

stem cells, differentiate from monocytes in the bloodstream into macrophages in tissues. RAW264.7 cell proliferation aids in understanding immune cell differentiation and roles in immune responses [13]. Macrophages are divided into M1 (classically activated) and M2 (alternatively activated) types, with their balance being critical for immune response outcomes [14]. Investigating the activation process of macrophages and identifying compounds that effectively stimulate this process are key to enhancing immune response. Previous studies, including those by Li et al.[15] and Wei et al.[16] have demonstrated that *Saccharum* polysaccharides and *Astragalus* polysaccharides, respectively, up-regulate the mRNA expression of cytokines such as IL-1β, TNF-α, and IL-6 in RAW264.7 cells. This study's findings reveal that DOP at various concentrations notably enhance the expression of *IL-1β, IL-10, IL-12p40*, and *IL-6* mRNA in RAW264.7 cells, with a dose-dependent increase in the up-regulation of these cytokines' mRNA expression. Furthermore, ELISA results indicate that DOP significantly boosts the secretion of IL-10, IL-12 p40, and IL-6, echoing findings by Meng et al. [17] This demonstrates DOP's potential in modulating macrophage activation and contributing to the immune response.

IL-10, a biomarker for M2 macrophages, suppresses cytokine secretion, including from M1 macrophages, thus reducing inflammation [18]. It downregulates MHC-II and co-stimulatory molecule expression in monocytes/macrophages, indirectly limiting T-cell proliferation and cytokine production [19]. Additionally, IL-10 weakens macrophage antigen presentation and cytokine production, helping to control inflammatory diseases. Wongchana et al. showed that IL-10 inhibits immune complexes and suppresses disorders caused by autoimmune hyperactivity [20]. This study found that DOP markedly increased IL-10 secretion in RAW264.7 cells. IL-12, a heterodimer consisting of p35 and p40 subunits, is predominantly synthesized by macrophages, B lymphocytes, and dendritic cells. It plays a pivotal role in cellular immune responses by activating T cells and NK cells [21]. The release of IL-12 also boosts the cytotoxic actions of NK cells and cytotoxic T lymphocytes (CTLs) against target cells and fosters lymphocyte proliferation, underscoring its significance in orchestrating cellular immunity [22]. Ablimit et al. [23] demonstrated that *Momordica charantia* polysaccharide significantly enhanced the secretion and expression of IL-12 in rats. In parallel, Huang et al. discovered that *Radix Rehmanniae* polysaccharide prompted dendritic cells to secrete a spectrum of cytokines, including IL-12. Hence, it is evident that IL-12 secretion boosts the immune system's function to a certain degree [24].

NF-κB is a pleiotropic transcription factor present in almost all cell types and participates in numerous biological processes, including inflammation, immunity, differentiation, cell growth, tumorigenesis, and apoptosis [25]. NF-κB p65, a member of the NF-κB family, is frequently involved in inflammatory responses [26]. Our experimental results demonstrated that DOP stimulation induced phosphorylation of the p65 protein in RAW264.7 cells, thereby activating the NF-κB signaling pathway. The MAPK signaling pathway serves as a critical hub for cellular responses to external stimuli and regulates cell growth and death. It not only influences proliferation, differentiation, and survival but also plays a pivotal role in pathological processes such as cancer, neurodegenerative diseases, and inflammation [27]. In mammals, MAPK can be categorized into three major subfamilies: ERK (extracellular signal-regulated kinase), JNK (Jun N-terminal kinase), and p38/SAPKs (stress-activated protein kinases) [28]. Among these, ERK is one of the most representative pathways in the MAPK signaling cascade, primarily responding to growth factors and mitogens to induce cell growth and differentiation [29]. The JNK module is crucial for apoptosis, inflammation, cytokine production, and metabolism. In this study, both ERK and JNK protein expressions were upregulated, leading to MAPK pathway activation. Multiple studies have indicated that the MAPK signaling pathway contributes to NF-κB activation [30]. Our findings revealed that DOP significantly promoted the expression of p-JNK, p-ERK, and p-p65 proteins in RAW264.7 cells, effectively activating both the MAPK and NF-κB signaling pathways.

Dendrobium officinale polysaccharides (DOP) exhibit high affinity and binding stability with Toll-like receptor 4 (TLR4), suggesting that they may play a significant role in intestinal immune regulation through the TLR4 signaling pathway. As a key receptor in the innate immune system, TLR4 can recognize pathogen-associated molecular patterns (PAMPs) and activate downstream signaling pathways, thereby inducing the release of inflammatory cytokines and immune responses [31]. Our *in vivo* experiments further confirmed that DOP significantly modulates the expression levels of TLR4 and its downstream key proteins in the colonic mucosa of mice, which may help alleviate excessive inflammatory responses and maintain intestinal homeostasis. Additionally, based on literature reports, there is a close interaction between the intestinal microbial community and the TLR4 signaling pathway [32]. We speculate that DOP may indirectly influence TLR4 activity by regulating the microbiota-host immune axis, thereby playing a dual role in intestinal immune defense and balance.

While this study demonstrates the immunomodulatory effects of DOP through NF-κB/MAPK pathways, several methodological limitations warrant discussion. Firstly, although DEAE Sephadex A50 chromatography achieved

95.33% total sugar content (Section 2.2), the HPGPC analysis revealed three distinct molecular weight peaks (1.89×10^5 Da, 549 Da, and 211 Da) in the DOP preparation *(Fig. 2)*. This heterogeneity suggests potential interactions between different polysaccharide fractions that might confound the observed bioactivities. Secondly, batch-to-batch variations inherent to plant-derived polysaccharides were not systematically evaluated. Minor fluctuations in ethanol precipitation conditions (80% ethanol at 4°C for 24 h) and NaCl gradient elution parameters (0.1-2.0 M) during purification could potentially alter polysaccharide composition across production batches. To address these limitations, future studies should: Employ orthogonal purification strategies (e.g., sequential ultrafiltration and HILIC chromatography) to isolate homogeneous polysaccharide fractions; Establish quality control metrics beyond total sugar content, including monosaccharide composition analysis and molecular weight distribution profiling; Conduct inter-batch comparisons using at least three independently prepared DOP batches to verify reproducibility.

In summary, *Dendrobium officinale* polysaccharide (DOP) enhances the immune function of RAW264.7 cells by promoting the secretion of inflammatory cytokines and the expression of inflammatory genes, mediated through modulation of the MAPK and NF-κB signaling pathways.

Declarations

Availability of Data and Materials: The original data of the paper are available upon request from the corresponding author (C. Sun).

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Ethical Statement: This study does not require ethical permission.

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

Declaration of Generative Artificial Intelligence (AI): The authors declare that the article and/or tables and figures were not written/created by AI and AI-assisted technologies.

Author Contributions: GX and YH contributed to the design of this study. GX, LY and JC participated in the sample collection, data analysis. CS provided funding and analytical tools. PJ wrote the original draft. All authors contributed to data collection and discussion.

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Research Article

The Effect of Quercetin Application on Desmin and Vimentin Levels in Ovariectomized Rats with Cyclophosphamide-Induced Cardiotoxicity

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Abstract

The aim of our study was to investigate the effect of quercetin on cyclophosphamide-induced cardiotoxicity. A total of 35 female rats were used in the study, divided into five groups of seven rats each. All of the rats, except those in the control group, underwent ovariectomy and had their ovaries removed. This eliminated the potential impact of hormones such as oestrogen and progesterone on the study. All rats were fed ad libitum. While the control and ovariectomy groups received no treatment, 50 mg/kg quercetin (oral) was administered to the quercetin group, 100 mg/kg cyclophosphamide (intraperitoneal) to the cyclophosphamide group, and 50 mg/kg quercetin (oral) and 100 mg/kg cyclophosphamide to the quercetin + cyclophosphamide group was applied. electrocardiography (ECG) measurements were taken before toxicity induction (day 0) and on day 5 after toxicity induction. At the end of the study, the rats were euthanized under anesthesia in accordance with ethical rules, and tissue and blood samples necessary for analysis were taken. Troponin, creatine kinase (CK), and creatinine kinase MB (CK-MB) parameters were measured in the blood samples taken. Histopathological and immunohistochemical analyses (desmin and vimentin) were performed on heart tissue. According to the analysis, an increase in troponin, CK, and CK-MB parameters was observed in the cyclophosphamide group, while a decrease was observed in the quercetin group. In desmin and vimentin immunoreactivity, a decrease was observed in the cyclophosphamide group, while an increase was observed in the quercetin group. In conclusion, in our study, we demonstrated that quercetin has a positive effect against cyclophosphamide-induced cardiotoxicity.

Keywords: Cyclophosphamide, Troponin, Creatine kinase, CK-MB, Desmin, Vimentin

Introduction

The phenomenon of drug-induced cardiotoxicity represents a significant threat to human health. Furthermore, the growing prevalence of cardio-oncology in the context of cardiotoxicity represents a significant contemporary concern [1,2]. Cyclophosphamide is an alkylating anticancer substance that was first discovered in experimental studies on rat tumours. The substance exhibits significant cytotoxic and immunosuppressive properties, classifying it as an oxazaphosphorine-substituted nitrogen mustard [3]. It serves as the foundation for the majority of organ transplant preparatory procedures. In addition to its utilisation in combination chemotherapy for Hodgkin's disease, non-Hodgkin's lymphoma, leukemia, rheumatoid arthritis, Burkitt's lymphoma, lupus erythematosus, multiple sclerosis, neuroblastoma, multiple myeloma, endometrial cancer, breast cancer, and lung cancer, it is also a widely active anticancer and immunosuppressive agent. Cyclophosphamide is administered in high dosages for the treatment of lymphomas and solid tumours. The combination of cyclophosphamide and bone marrow transplantation is a viable therapeutic option [1,4,5]. Cyclophosphamide has been demonstrated to induce cardiac damage when administered in high

doses. Patients who receive doses in excess of 150 mg/kg per day are particularly vulnerable to cardiotoxicity [6]. Cardiotoxicity of an irreversible nature has been observed in conjunction with cyclophosphamide treatment [1]. Fatal cardiomyopathy has been reported in 2-17% of patients treated with cyclophosphamide [7]. Cardiotoxicity, a potential complication of cyclophosphamide treatment, has been observed in 7 to 28% of patients receiving the drug [4].

Quercetin is a polyphenol that is found in abundance in nature. It is a prevalent ingredient in numerous plant-based products. Onions are reported to contain the highest concentration of quercetin. It is hypothesised that this plant component exerts an anti-aging effect in addition to its antioxidant capabilities. It is recognised to exist in both conjugated and free forms. Quercetin has been shown to possess a range of biological activities, including antiviral, antioxidant, anti-inflammatory, anti-proliferative, anti-carcinogenic, and anti-diabetic properties. Quercetin, a bioavailable chemical compound, has been demonstrated to be efficacious in addressing a number of health issues [8]. Quercetin is a potent antioxidant that is currently employed in a variety of pharmaceutical products. Quercetin has been demonstrated to have therapeutic benefits in the treatment of various pathologies, including cancer, allergic reactions, inflammation, arthritis, and cardiovascular disorders [9]. A diet abundant in quercetin has been demonstrated to engender a number of health-promoting advantages. Its function as a coagulation, hyperglycaemia, inflammatory, and hypertension-lowering agent has been demonstrated. The supplementation of quercetin has been demonstrated to be efficacious in the treatment and prevention of a number of chronic conditions, including cardiovascular issues [10].

It is generally accepted that myocardial contusion is suspected due to high creatinine kinase MB (CK-MB) activity, whether it is expressed as a fraction of electrocardiography (ECG) findings and creatine kinase (CK)-total activity [11,12]. The presence of artefacts in the blood, such as creatine phosphokinase and CK macroenzymes, can complicate the measurement of CK-MB activity. It has been demonstrated that measurements of CK-MB mass remain unaltered by these effects. However, it is important to note that elevations in CK-MB mass and CK-MB activity have also been observed in cases of severe skeletal muscle damage. Another typical diagnostic method is a 12-lead ECG. However, in the initial hours following a significant injury, an aberrant ECG reading may simply be indicative of metabolic alterations [13].

Vimentin and desmin, the primary components of fibroblastic intermediate filaments, are present in the majority of mesenchymal cells. The distribution and localization of vimentin and desmin have been investigated in several neuromuscular diseases using monoclonal antibodies. Vimentin has been reported to be expressed in the fibres that regenerate in several neuromuscular diseases, despite being rarely found in normal human muscle fibres. Furthermore, it is mentioned that these fibers exhibit a high level of desmin antibody positivity. In standard muscle fibres, desmin is localised exclusively at the level of the Z line. It has been hypothesised that desmin and vimentin may be overexpressed during muscle regeneration processes due to their importance in the structural organisation of the sarcomere [14]. Concurrently, a mutation in the desmin gene has been demonstrated to be a causative agent for an inherited heart and skeletal muscle disease [15].

The objective of this study was to demonstrate quercetin's protective effect on desmin and vimentin levels against cyclophosphamide-induced cardiotoxicity.

Materials and Methods

Ethical Approval

The study was initiated subsequent to the acquisition of the permission number KAÜ-HADYEK, 2022-018 from the Ethics Committee of Kafkas University for Animal Experiments.

Animals

In the present study, a total of 35 female Wistar albino rats, with a weight range of 250-350 g, were utilised, with seven rats assigned to each group. Prior to the commencement of the experiment, the rats underwent ovariectomy. The rats selected for inclusion in the study were accommodated in standard cages, with an ambient temperature maintained at 22±2°C, a photoperiod of 12 h of light and 12 h of darkness, and access to ad-libitum tap water.

Ovariectomy

The study involved the performance of ovariectomy operations on a total of 28 rats, which were divided into four groups. The rats were anaesthetized using a combination of ketamine HCl (75 mg/kg) (Ketalar®, Pfizer) and xylazine HCl (15 mg/kg) (Rompun®, Bayer) for the ovariectomy procedure. Subsequent to the administration of anaesthesia, the rats were positioned in a supine position, and the operative area was prepared by means of shaving. The operation was performed with a median incision; the skin, muscle layers, and peritoneum were cut, the abdominal cavity was reached, and the suspensory ligaments and veins of the right and left ovaries were ligatized and removed using 3.0 polyglactin 910 (Vicryl® Ethicon). Subsequent to this procedure, the peritoneum and muscles were sutured using simple continuous stitches, while the skin was sutured using horizontal U stitches. In order to prevent postoperative complications, the administration of antibiotics (Iespor®, MENARINI)

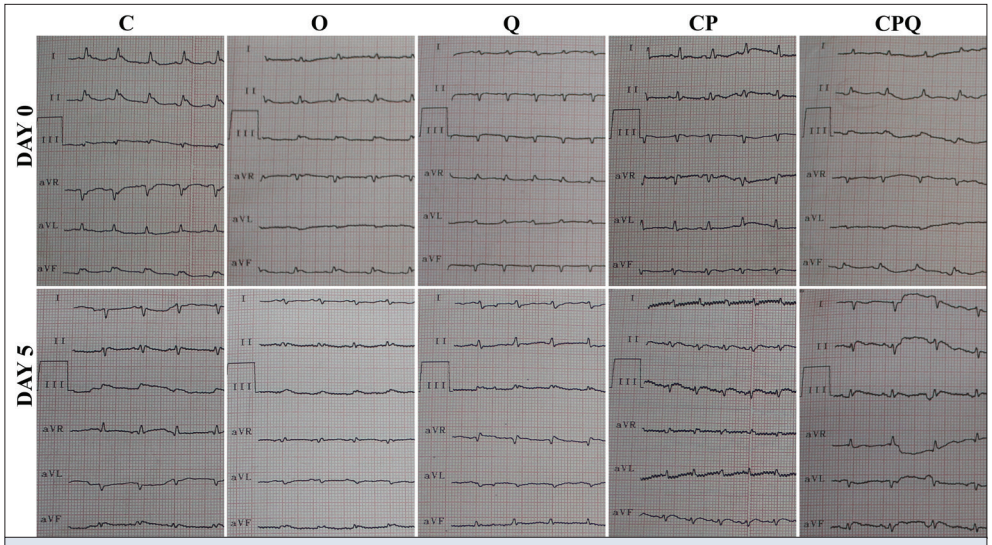

Fig 1. All groups electrocardiogram by days. C: Control group, O: Ovariectomy group, Q: Quercetin group, CP: Cyclophosphamide group, CPQ: Quercetin + cyclophosphamide group

was initiated for a period of four days. Thereafter, the incision site of the rats was examined on a daily basis for the presence of peritonitis and inflammation.

The commencement of the experimental study was scheduled 10 days subsequent to the ovariectomy procedure.

Creation of the Experimental Model

Control group (C, n = 7): Rats in this group will be fed standard feed and water.

Ovariectomy group (O, n = 7): Rats in this group were fed standard feed and water and then underwent ovariectomy.

Quercetin group (Q, n = 7): Rats in this group underwent ovariectomy, and 50 mg/kg quercetin was administered by oral gavage once a day for a period of 5 days [16].

Cyclophosphamide group (CP, n = 7): Rats in this group underwent ovariectomy, and 100 mg/kg cyclophosphamide was administered by intraperitoneally once daily for a period of 5 days [17].

Quercetin + cyclophosphamide group (CPQ, n = 7): Rats in this group underwent ovariectomy and started to administer quercetin 50 mg/kg by oral gavage 72 hours before cyclophosphamide administration, followed by 100 mg/kg cyclophosphamide [17] by intraperitoneally thread and 50 mg/kg quercetin oral gavage once a day for a period of 5 days [16].

At the conclusion of the experiment, the rats were euthanized under anesthesia by cervical dislocation. Blood (intracardiac) and tissue sampling were then performed on the rats. Following the centrifugation of the blood samples at 3000 RPM, the extracted sera were stored at -20°C until analysis. The presence of heart tissues was detected in a 10% formol solution for the purposes of histological and immunohistochemical analysis.

Electrocardiogram Measurement

Electrocardiography *(Fig. 1)* measurements were obtained from each animal in the experiment while it was under anesthesia. ECG measurements were taken prior to the induction of toxicity (day 0) and on day 5 following the induction of toxicity. The Nihon Kohden Cardiofax S ECG-1250 device was utilised for this purpose. Digital ECG records were obtained using the following leads: I, II, III, aVR, aVL, and aVF. The velocity of the ECG was set at 50 mm/s, and the calibration was set at 1 mV=10 mm. A 50-Hz filter was also applied *(Fig. 1)*. It is evident that no supplementary computations were conducted, as the device automatically calculates the QTc data.

Biochemical Analysis

In serum samples, troponin and CK-MB parameters were measured spectrophotometrically with a Beckman DXI 800 autoanalyzer, while the CK parameter was measured spectrophotometrically with a Beckman-Coulter AU5800 autoanalyzer.

Histological Examinations

Following the completion of the tissue follow-up procedure, the tissues are to be processed for paraffin embedding. Subsequently, hematoxylin-eosin staining will be performed on 5-µm sections taken from the paraffin blocks. A histological evaluation of the heart tissues will then be conducted.

Immunohistochemical Examinations

The streptavidin-biotin peroxidase method was applied to the sections taken on slides coated with chromium alumine gelatin. PBS (0.1 M, pH 7.2) buffer was utilised for the purpose of washing throughout the procedure. Sections: In order to inhibit endogenous peroxidase activity, the sample

was incubated in 3% H_2O_2 prepared in 0.1 M PBS for a period of 15 min. Subsequently, the citrate buffer solution was subjected to microwave heating for a period of 10 minutes at its maximum temperature setting, with the objective of releasing antigens. Subsequently, the specimen was subjected to an incubation with Large Volume Ultra V Block solution for a period of 10 min. Subsequently, the anti-desmin (SC-271677, 1/500 dilution) and anti-vimentin (SC-6260, 1/100 dilution) primary antibodies were applied to the sections at room temperature and in a humid environment for a period of 1 h. Subsequently, the samples were subjected to an incubation process. This involved the application of a biotin-conjugated solution comprising goat anti-B polyvalent antibodies, along with a streptavidin-streptavidin peroxidase complex, to the samples at ambient temperature. The duration of this incubation was set at 15 min for each solution. The dihydrochloric acid-hydrogen peroxide (DAB-H_2O_2) chromogen substrate solution was administered for the purpose of staining. A modified Gill III hematoxylin solution was utilised for the purpose of contrasting staining. In order to ascertain whether the immunoreactivity of desmin and vimentin was specific, all procedures were applied to the sections stored in PBS without the addition of primary antibodies. For the purpose of immunohistochemical evaluation, the target cells were evaluated by two independent observers according to the characteristics of non-staining (0), weak staining (1), moderate staining (2), or severe staining (3). This evaluation was conducted with consideration for the staining characteristics and density, and values ranging from 0 to 3 were assigned. Sections that had been prepared for histological and immunohistochemical studies were evaluated and photographed under a light microscope [18].

A total of three preparations were selected from each group for the purpose of counting immunopositive cells. The experimental units were then subdivided into four equal segments, and cell counts were made from a total of 12 areas in each group. The ImageJ software was utilised for the purpose of cell counts.

Statistical Analyses

Prior to the commencement of the study, power analysis was conducted using G-Power 3.1.9.7. The analysis indicates that the sample size was determined in accordance with the test power of 0.95 and the significance level of 0.05. A two-way analysis of variance (ANOVA) was conducted on ECG parameters in order to ascertain whether there was a difference between the groups under consideration. A one-way analysis of variance (ANOVA) was conducted on the biochemical and immunohistochemical parameters in order to ascertain whether there was a difference between the groups. A P-value of less than 0.05 was considered to be statistically significant. The statistical analyses were conducted using GraphPad 8.1 (San Diego, CA, USA).

Results

ECG Results

A comparison of the heart rate *(Fig. 2)* on day 5 reveals a significant increase in the CP group compared to the other groups. Concurrently, a statistical increase is observed in the CP group on day 5 in comparison with day 0.

With regard to the results of the PR analysis *(Fig. 2)*, a significant decrease was observed in the CP group on day 5 in comparison to the O and CPQ groups. Furthermore, a statistically significant decrease was observed in the CP group on day 5 in comparison with day 0.

An analysis of the QRS data *(Fig. 2)* revealed a statistically significant increase in the O group on day 5 compared to day 0.

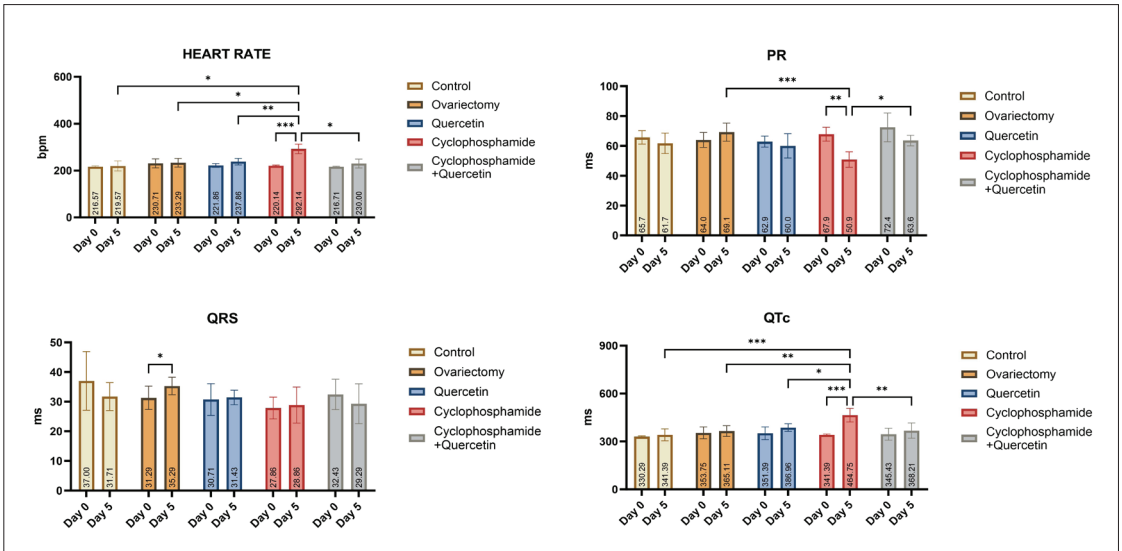

Fig 2. Means and standard deviation of the four groups for electrocardiography. *: P<0.05, **: P<0.01, ***: P<0.001, ****: P<0.0001

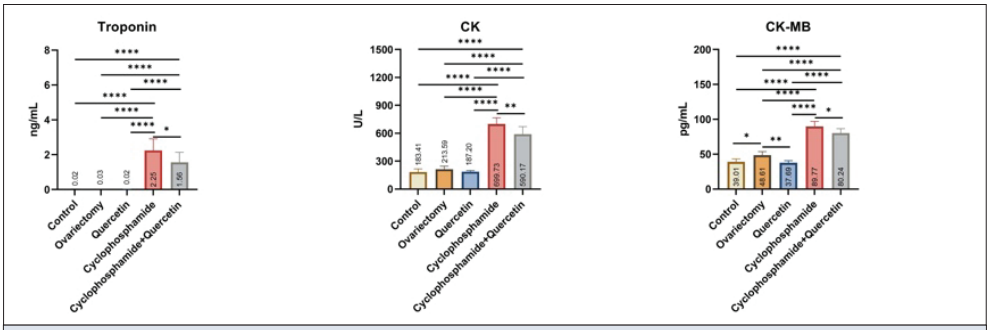

Fig 3. Means and standard deviation of the four groups for biochemical parameters. *: P<0.05, **: P<0.01, ***: P<0.001, ****: P<0.0001. CK: Creatinine kinase (CK), CK-MB: Creatinine kinase MB

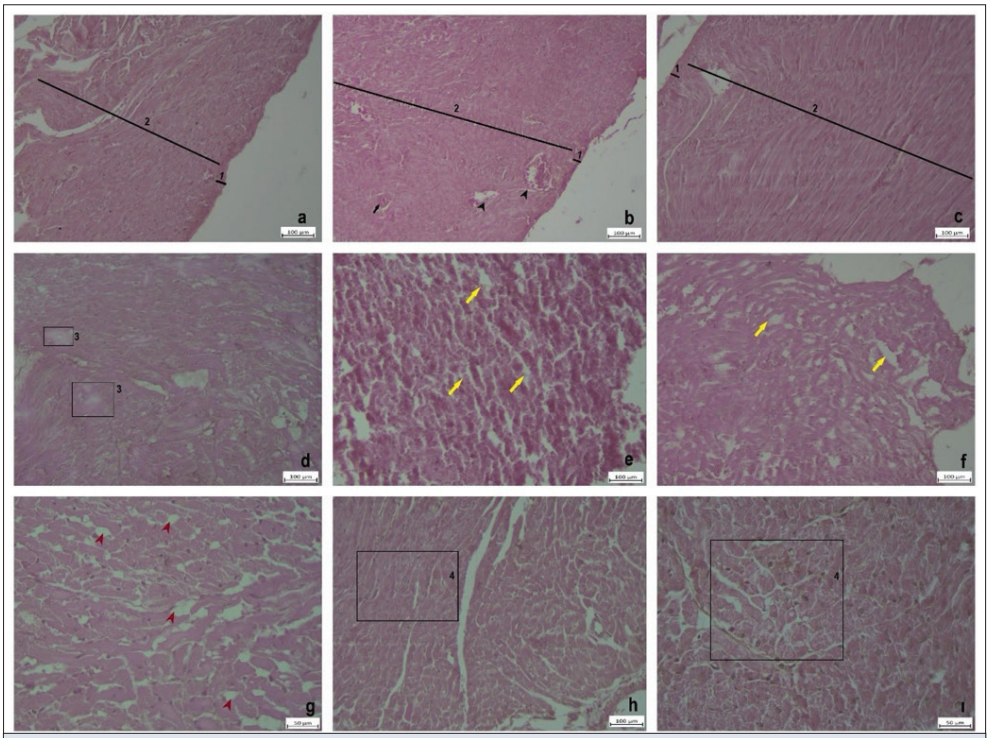

Fig 4. H&E staining of heart tissue. Control group (a), ovariectomy group (b), quercetin group (c), cyclophosphamide group (d, e, f, g), quercetin + cyclophosphamide group (h, i). Epicardium (1), myocardium (2), artery *(arrow),* vein *(arrowhead),* necrotic areas in heart tissue (3), fragmentation of myofibrils *(yellow arrow),* increased intercellular spaces (red arrow), histological appearance close to the control group (4)

In the QTc data *(Fig. 2),* a significant increase was determined in the CP group compared to the other groups when the day 5 data was analysed. However, a statistical increase was observed in the CP group on day 5 in comparison with day 0.

Biochemical Results

Biochemical analysis included the measurement of troponin, CK and CK-MB parameters *(Fig. 3).* In this context, a statistical increase in troponin, CK and CK-MB is observed in the CP group when the CP group is compared with other groups. When the CPQ group was compared with the control and O, as well as Q, groups, a significant increase in troponin, CK and CK-MB was determined in the CPQ group. Furthermore, a substantial increase in CK-MB levels was observed in group O in comparison to groups C and Q.

Histopathological Results

In groups C, O and Q, the heart tissue was observed to have a normal histology *(Fig. 4-a, b, c).* In the CP group, the presence of fragmentation of myofibrils, an increase in the intracellular space, and an increase in the spaces between cardiomyocytes and necrotic areas was detected *(Fig. 4-d, e, f, g).* In the CPQ group, the heart tissue exhibited a histological appearance analogous to that of group C. Myofibrillar fragmentation, intercardiomyocyte gaps, and necrotic areas were found to be present, though at low levels *(Fig. 4-h, i).*

Immunohistochemical Results

Strong desmin immunoreactivity was detected in the cytoplasm of cardiomyocytes in groups C, O and Q *(Fig. 5-a, b, c).* Weak immunoreactivity was detected in the CP

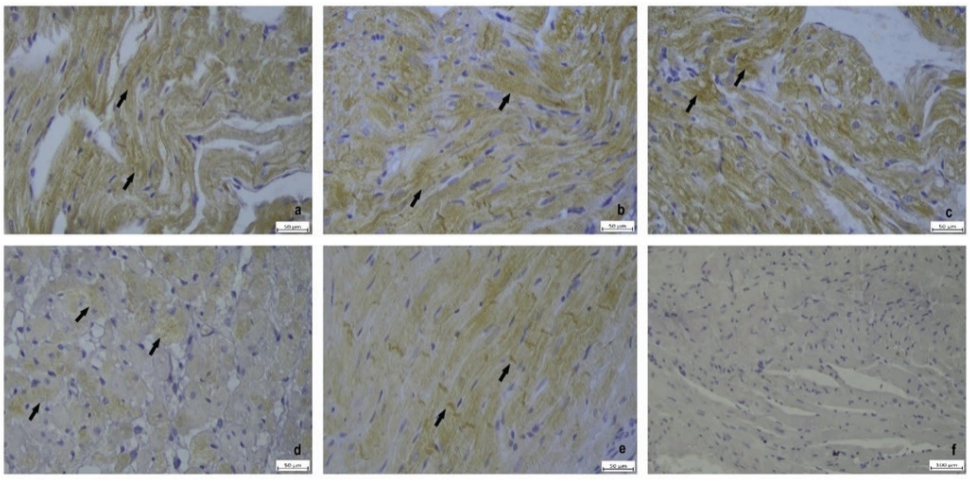

Fig 5. Desmin immunoreactivity in heart tissue. Control group (a), ovariectomy group (b), quercetin group (c), cyclophosphamide group (d), quercetin + cyclophosphamide group (e), Negative control (f). Cardiomyocytes *(arrows)*

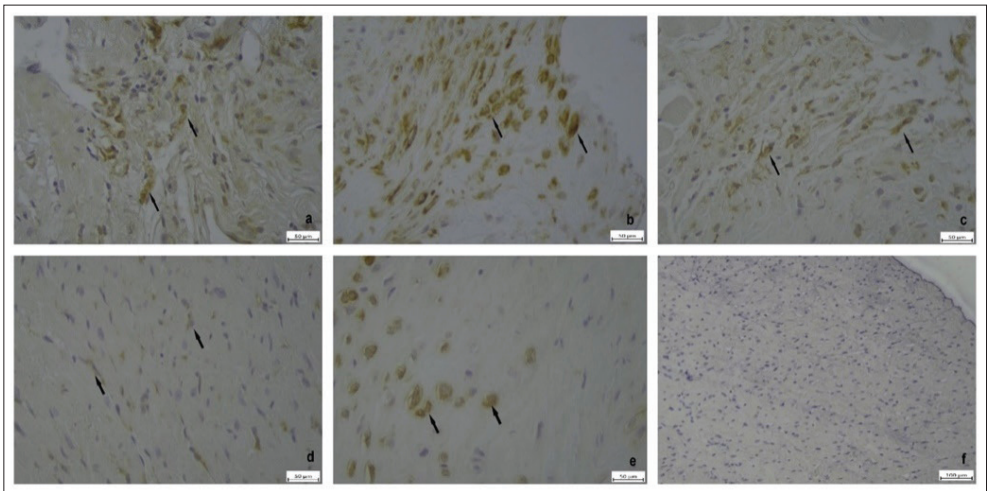

Fig 6. Vimentin immunoreactivity in heart tissue. Control group (a), ovariectomy group (b), quercetin group (c), cyclophosphamide group (d), quercetin + cyclophosphamide group (e), Negative control (f). Cardiomyocytes *(arrows)*

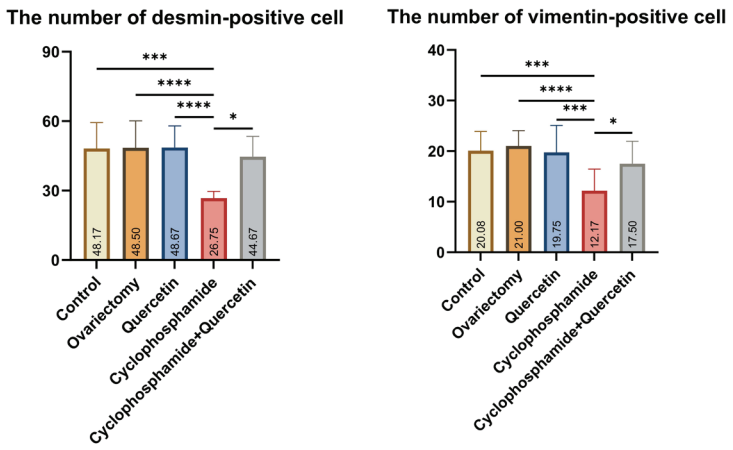

Fig 7. Means and standard deviation of the four groups for desmin and vimentin immunoreactivity parameters. *: P<0.05, **: P<0.01, ***: P<0.001, ****: P<0.0001.

group and moderate immunoreactivity was detected in the CPQ group *(Fig. 5-d, e)*.

Diffuse cytoplasmic and strong vimentin immunoreactivity was detected in cardiomyocytes in groups C, O and Q *(Fig. 6 a, b, c)*. Weak immunoreactivity was detected in the CP group and moderate immunoreactivity was detected in the CPQ group *(Fig. 6 d, e)*. In addition, moderate immunoreactivity was observed in the artery and vein walls of the heart tissue in all groups. In addition, when the number of vimentin positive cells was examined, a significant decrease was observed in the CP group compared to the other groups *(Fig. 7)*.

It was noted that desmin immunoreactivity was diffusely cytoplasmic and banded. Additionally, when the number of desmin positive cells was examined, a significant decrease was observed in the CP group compared to the other groups *(Fig. 7)*.

Discussion

Cyclophosphamide is a widely employed anticancer pharmaceutical agent. The medication has been employed in a wide range of clinical settings; however, it has been observed that it can result in cardiotoxicity, which is a significant cause for concern. The observed cardiotoxic properties were reported at the therapeutic dose. Consequently, this condition frequently results in a high mortality rate and suboptimal clinical outcomes [19] Patients receiving cancer chemotherapy can generally be considered to be at high risk of heart failure or in the stage A heart failure group [1].

The primary objective of this study was to investigate the potential protective role of quercetin in modulating the expression of desmin and vimentin in cardiac tissue subjected to cyclophosphamide-induced cardiotoxicity.

The pathophysiology of cardiac damage caused by cyclophosphamide is not fully known [20]. It is hypothesised that proteins, blood cells and hazardous metabolites may extravasate as a result of oxidative stress and direct endothelial capillary damage caused by its byproducts. The presence of toxic metabolites has been demonstrated to induce the degradation of endothelial cells, resulting in the impairment of the myocardium and the formation of capillaries. This process is associated with the occurrence of oedema, interstitial bleeding and microthrombosis [1,21]. Endothelial cells exhibit a heightened sensitivity to damage induced by cyclophosphamide in comparison to other cell types [22]. It has been hypothesised that this phenomenon may be associated with the elevated proliferation rate [23]. It has been reported that the formation of reactive oxygen species (ROS) caused by cyclophosphamide may lead to a decrease in nitric oxide bioavailability, leading to deterioration of endothelial function [24]. As demonstrated in the extant literature, there is evidence to suggest that cyclophosphamide can induce endothelial damage through the process of direct or oxidant damage.

Quercetin is a flavonoid found in various plants such as *Polygonum cuspidatum* Sieb. et Zucc. In addition to having strong antioxidant properties, it also has an anti-inflammatory effect. It is reported to have a therapeutic effect on cardiovascular diseases, metabolic disorders, neurodegenerative diseases, diabetes, cancer and obesity [25,26].

Endothelial dysfunction is a broad term and refers to impairment of nitric oxide (NO) production and/or imbalance of relaxation and contraction factors such as endothelium-derived endothelin-1 (ET-1), angiotensin and oxidants [27]. Endothelial dysfunction is one of the basic mechanisms in the atherosclerotic process. Classical and newly identified risk factors create chronic damage to the endothelium, leading to a decrease in the vasodilator response. Thus, events such as vasoconstriction in the endothelium, accumulation of inflammatory cells, migration of smooth muscle cells, and an increase in cytokine production cause atherosclerotic plaque formation [28,29]. As is known, cardiac muscle consists of smooth muscle cells. In this context, in our study, troponin, CK and CK-MB parameters were evaluated to determine cardiac muscle damage to support endothelial muscle damage caused by cyclophosphamide. The increase in these parameters in the CP group indirectly supports endothelial damage, according to the above literature information. In addition, the decrease in these parameters in the Q group compared to the CP group indicates ($P<0.05$) that quercetin reduces endothelial damage. It is thought that the reason for this is that quercetin reduces endothelial damage by preventing oxidant damage with its antioxidant properties.

A significant constituent of the cytoskeleton is constituted by desmin and vimentin filaments, which are classified as either homopolymers or heteropolymers, and are formed by smooth muscle filaments. Vimentin and desmin proteins are classified as type III filaments due to their structural and sequence similarities. The protein desmin, unique to muscles, is expressed by skeletal muscle, cardiac muscle and smooth muscle [30].

The oxidant effect of cyclophosphamide, particularly on the heart muscle, should be given due consideration. The absence of desmin in cells has been demonstrated to result in mitochondrial dysfunction. This is due to the fact that mitochondria play a pivotal role in the execution of necrotic and apoptotic mechanisms, as well as the synthesis of macroergic chemicals in oxidative muscle cells. There is also mounting evidence that certain types of myopathies and heart failure may be attributable to mitochondrial

dysfunction associated with dysregulation of desmin or vimentin cytoskeletal structures [31]. It is also reported that desmin and vimentin proteins directly participate in signal transduction [32]. A perusal of the study reveals that, on the day 5, QTc is prolonged in the CP group in comparison to the other groups. The rationale behind this phenomenon can be elucidated by referencing the deceleration in signal transmission, particularly during the transition from the atrioventricular nodule to the bundles of His. This decline is attributable to the diminution in desmin and vimentin cells, whose concentrations were reduced in the CP group within the ambit of our study. As demonstrated in our study, a number of cardiotoxicity studies demonstrate that QTc is prolonged during toxicity [33,34]. Conversely, the decline in the PR interval can be attributed to the robust signal emanating from the sinoatrial node, which rapidly reaches the atrioventricular nodule, yet subsequently decelerates due to a deficiency of desmin and vimentin. However, the results of this study suggest that the antioxidant effect of quercetin, which was utilised in the present study, mitigates the mitochondrial dysfunction induced by reactive oxygen species generated by cyclophosphamide. This observation resulted in an enhancement in the parameters observed in the QCP group compared to the CP group ($P<0.05$).

Quercetin has antioxidant, anti-inflammatory, and anticancer properties. It especially scavenges superoxide anion, singlet oxygen, and lipid peroxy radicals [35]. Within ROS-mediated cardiomyopathy, QCT has been reported to scavenge ROS and prevent the activation of mitogen-activated protein kinase and extracellular signal-regulated kinase [36,37]. In the present study, the positive effect on troponin, CK and CK-MB parameters is particularly noteworthy, and the findings are highly effective in light of the existing literature on cardiotoxicity. Consequently, it was determined that quercetin exhibited a therapeutic effect on cyclophosphamide-induced cardiotoxicity.

Declarations

Availability of Data and Materials: The data that support the findings of this study are available on request from the corresponding author (MM). The data are not publicly available due to privacy or ethical restrictions.

Ethical Statement: The study has been approved by the Institutional Animal Care and Use Committee of Kafkas University (KAÜ-HADYEK, 2022-018).

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

Declaration of Generative Artificial Intelligence (AI): Authors declare that the article and/or tables and figures were not written/created by AI and AI-assisted technologies.

Author Contributions: SYA, MM, MK, EKS, AG and SEY analysed and interpreted the data. SYA, MM, MK, and EKS was a major contributor in writing the manuscript. MM analysed and interpreted the ECG data. MO analysed and interpreted the biochemistry data. SYA, EKS, AG, SEY analysed and interpreted the histopathology data. All authors read and approved the final manuscript.

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Research Article

Seroprevalence and Risk Factors Associated with Caprine Arthritis Encephalitis Virus Infection in Goats: Insights from Three Egyptian Governorates

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Abstract

This study aimed to determine the seroprevalence of caprine arthritis encephalitis virus (CAEV) in goats in three northern Egyptian governorates (Kafr El Sheikh, Beheira, and Alexandria) and assess the associated risk factors. A total of 415 goats were sampled, with serological analysis conducted using an cELISA test. The overall seroprevalence of CAEV was found to be 9.6%, with significant variation observed based on sex, age, breed, production system, and farm management practices. The results indicated higher seroprevalence in females, older goats, and certain breeds such as the Egyptian Baladi, as well as in dairy goats. Additionally, poor biosecurity management and contact with other herds were identified as significant risk factors. This study provides valuable insights into the epidemiology of CAEV in Egypt and underscores the need for improved control measures to mitigate the spread of the virus. Further research is recommended to explore the full extent of CAEV infection in goat populations and to enhance the understanding of its impact on goat farming in the region.

Keywords: Caprine Arthritis Encephalitis Virus, cELISA; Risk factor, Goat, Egypt

Introduction

Caprine arthritis and encephalitis (CAE) is a fetal viral disease of goats, caused by the caprine arthritis-encephalitis virus (CAEV), a lentivirus belonging to the *Retroviridae* family [1]. It induces multisystemic diseases in sheep and goats [2]. Lentivirus infections produce persistent, progressive, and fatal infections in many target organs, including carpal joints, the mammary gland, lungs and central nervous system [3,4]. This can affect milk production and increased the risk of developing mastitis [5,6].

The disease causes mastitis, severe arthritis, and possibly interstitial pneumonia in adults, while causes encephalitis in young goats [7]. Also, CAEV infections can cause synovitis, lameness, joint enlargement and reduction in growth rate [3,7,8]. The disease causes economic losses resulting from reduced yield from subclinical infections, decreased value of culled animals, mortality from clinical sickness, and ultimately a shorter economic lifespan of affected goats [9,10].

Transmission of CAEV can occur both horizontally and vertically [11]. Horizontal transmission results from direct contact with intimate contact of diseased animals, body fluids, and excretions, while vertical transmission happens through consumption of contaminated milk or colostrum [12-14].

Once the virus enters the body, the host becomes permanently infected. Additionally, cases of interspecies transmission, especially, goat-to-sheep and sheep-to-goat can occur spontaneously within mixed populations [15].

CAEV is distributed worldwide [16]. It has been reported in numerous places of the world since its first identification in goats in 1974 [17]. The prevalence of CAEV varies by country, ranging from 1.9% in Turkey and 3.6% in Mexico to 82% in Australia and 73% in the USA [18]. In Thailand, the prevalence of CAEV was 12.4% [19].

Diagnosis of the diseases depends mainly on clinical signs, postmortem lesions and histopathological findings [20]. Serological tests are the primary method for detecting CAEV due to the persistence of antibodies against virus in circulation. Serological techniques to detect CAEV include enzyme-linked immunosorbent assay (ELISA), agar gel immunodiffusion (AGID) and indirect immunofluorescence [7,9,21]. In addition, the most widely used serological test in for identifying CAEV infection is the ELISA, because it is more sensitive than AGID [3].

Currently, there is no effective therapy or vaccination for the condition and improving diagnostic test quality and efficacy could potentially eliminate it. However, early diagnosis of CAEV infection using serological approaches remains crucial for prevention, eradication and control [22-24].

The study aimed to determine the seroprevalence of CAEV in goats across three Egyptian governorates and assess the risk factors associated with the infection to provide valuable insights into the epidemiology of the disease in the region.

Materials and Methods

Ethical Statement

The research protocol was reviewed and approved by the Institutional Animal Care and Use Committee at Faculty of Veterinary Medicine, Benha University, ensuring adherence to guidelines. (Ethical No: BUFVTM 20-11-2024). Informed consent was obtained from all goat owners prior to sample collection. The procedures involved, including blood sample collection, were performed by trained veterinarians to minimize animal discomfort and ensure their welfare. No animals were subjected to unnecessary harm, and all efforts were made to reduce stress during handling. All methods are reported in accordance with ARRIVE guidelines.

Study Area

The study was conducted from January to December 2023 in three Egyptian governorates: Kafr El-Sheikh, Beheira, and Alexandria *(Fig. 1)*. These governorates are situated in the northern part of Egypt, each with distinct geographical and climatic characteristics. These governorates (Kafr El-Sheikh, Beheira, and Alexandria) were selected because they are agricultural areas with a high animal population.

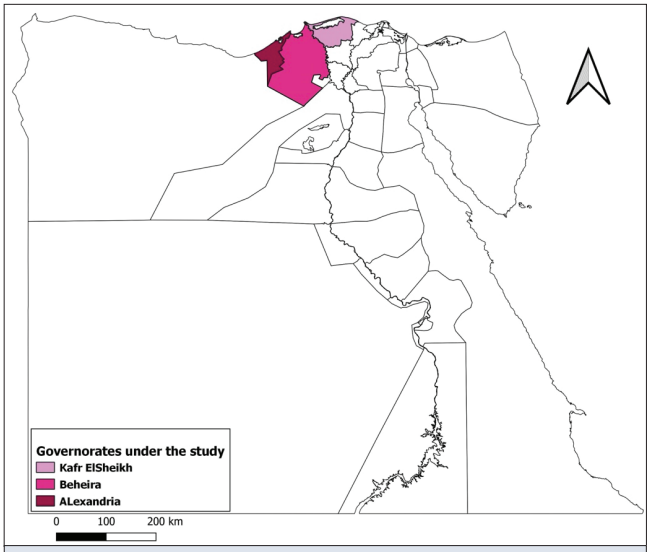

Fig 1. Map showed the governorates under the study (MAP generated by QGIS software)

Kafr ElSheikh is located in the Nile Delta, bordered by the Mediterranean Sea to the north. The governorate has a Mediterranean climate with mild, wet winters and hot, dry summers, which supports extensive agricultural activities and livestock farming. Beheira is situated in the western part of the Nile Delta and like Kafr El-Sheikh, it has a Mediterranean climate, with higher humidity levels due to its proximity to the sea and irrigated lands. In addition, Alexandris is located along the Mediterranean coast, it is Egypt's primary port city and has urban and peri-urban areas where livestock farming exists. It has a temperate Mediterranean climate with relatively higher humidity and moderate rainfall during winter, compared to the inland governorates.

Sample Size and Sampling

To determine the sample size for assessing the sero-prevalence of CAEV in goats, the formula by Thrusfield [25] was applied:

$$N=Z^2P_{exp}(1-P_{exp})/d^2$$

Where:

N = Required sample size

Pexp = Expected prevalence (assumed at 8.52% as previously reported by Baraka, Khadr [26])

Z = Standard normal deviate for a 95% confidence interval (1.96)

d = Desired margin of error (5% or 0.05)

The calculated sample size for determining the seroprevalence of CAEV was 119, based on the formula by Thrusfield. However, to enhance precision and reliability, the sample size was increased to 415 to increase precision. Blood samples were collected from the jugular vein of goats. Blood samples were centrifuged at 3000 rpm for 10 min to separate sera. Sera were then carefully aliquoted and preserved at −20°C until they were subjected to serological examination.

Serological Analysis

A commercial ELISA kit (ID Screen® MDV/CAEV Indirect, ID vet, France) was used to screen all collected sera for anti-CAEV antibodies in accordance with the manufacturer's instructions. A microplate reader (ALLSheng A101; China) was used to read the OD value at 450 nm.

Samples were considered positive for the CAEV if their percentage of inhibition (PI) was greater than 60%. The kit's sensitivity and specificity rates for identifying CAEV antibodies in small ruminants are 99.3% and 99.7%, respectively [27].

Statistical Analysis

Statistical analysis was performed using SPSS software (IBM SPSS 24, USA). To assess the statistical significance between various factors and the seroprevalence of CAEV infection, the Chi-square (χ^2) test was used. A logistic regression analysis was then carried out to explore the relationship between CAEV antibodies and risk factors such as locality, sex, age, breed, production system, flock size, rearing with sheep, contact with other goat herds and biosecurity status. Variables that had a P-value of less than 0.25 in the univariable analysis were included in a multivariable logistic regression model. This model provided the odds ratios (ORs) and confidence intervals (CIs) for each significant variable. The model's goodness-of-fit was assessed using the Hosmer-Lemeshow test.

Results

The overall seroprevalence of CAEV in goats was found to be 9.6% (40/415), with no statistically significant differences (P=0.301) observed among the examined governorates. However, Alexandria governorate exhibited the highest seroprevalence at 12.4%, (18/145) *(Table 1)*.

Table 1. *The seroprevalence of Caprine Arthritis Encephalitis Virus in goats in relation to different studied factors*

Variable		Total Examined Animals	No of Positive	No of Negative	% of Positive	95% CI	Statistic
Locality	Kafr ElSheikh	130	9	121	6.9	3.68-12.63	χ2=2.403 df=2 P=0.301
	Beheira	140	13	127	9.3	5.51-15.24	
	Alexandria	145	18	127	12.4	8-18.76	
Sex	Male	80	4	76	5.0	1.96-12.16	χ2=2.448 df=1 P=0.118
	Female	335	36	299	10.7	7.87-14.52	
Age	≤1	111	4	107	3.6	1.41-8.9	χ2=9.085 df=2 P=0.011*
	1-2	179	17	162	9.5	6.02-14.69	
	>2	125	19	106	15.2	9.95-22.52	
Breed	Egyptian Baladi	166	25	141	15.1	10.41-21.29	χ2=9.797 df=2 P=0.007*
	Zaraibi	140	10	130	7.1	3.92-12.65	
	Damascus (Shami)	109	5	104	4.6	1.98-10.29	
Production system	Meat	134	8	126	6.0	3.06-11.34	χ2=3.058 df=1 P=0.080
	Dairy	281	32	249	11.4	8.18-15.64	
Flock size	<30	320	36	284	11.3	8.24-15.18	χ2=4.168 df=1 P=0.041*
	>30	95	4	91	4.2	1.65-10.33	
Rearing with sheep	Yes	264	27	237	10.2	7.13-14.47	χ2=0.289 df=1 P=0.591
	No	151	13	138	8.6	5.1-14.17	
Contact with other goat herds	Yes	245	31	214	12.7	9.06-17.4	χ2=6.240 df=1 P=0.021*
	No	170	9	161	5.3	2.81-9.75	
Biosecurity status	Good	153	7	146	4.6	2.24-9.15	χ2=7.134 df=1 P=0.008*
	Poor	262	33	229	12.6	9.08-17.1	
Total		415	40	375	9.6	7.16-12.86	

**Results are significant if P<0.05*

Table 2. *Multivariable logistic regression analysis for risk factors associated with seropositivity of Caprine Arthritis Encephalitis Virus in goats*

Variable		B	S.E.	OR	95% C.I. for OR		P Value
					Lower	Upper	
Sex	Female	0.873	0.567	2.4	0.8	7.3	0.012
Age	1-2	0.757	0.590	2.1	0.7	6.8	0.020
	>2	1.329	0.589	3.8	1.2	12.0	0.024
Breed	Egyptian Baladi	1.295	0.527	3.6	1.3	10.3	0.014
	Zaraibi	0.423	0.585	1.5	0.5	4.8	0.036
Production system	Dairy	0.917	0.437	2.5	1.1	5.9	0.036
Flock size	<30	0.879	0.561	2.4	0.8	7.2	0.011
Contact with other goat herds	Yes	0.921	0.413	2.5	1.1	5.6	0.026
Biosecurity status	Poor	1.216	0.452	3.4	1.4	8.2	0.007

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

The seroprevalence of CAEV was higher in females (10.7%) compared to males (5%) and was significantly elevated in goats older than two years (15.2%). Furthermore, the seroprevalence varied among breeds, with the Egyptian Baladi breed showing the highest rate (15.1%), followed by Zaraibi (7.1%) and Shami (4.6%). Notably, goats bred for dairy production exhibited a higher seroprevalence (11.4%) compared to other purposes *(Table 1)*.

Interestingly, goats that had contact with other goat flocks or were raised in small flocks of fewer than 30 animals demonstrated significantly higher seropositivity for CAEV. Additionally, inadequate biosecurity measures were associated with a significantly increased likelihood of CAEV infection, underscoring the critical role of effective management practices in controlling the spread of the virus.

The multivariable logistic regression analysis revealed several significant factors associated with the likelihood of CAEV infection in goats. Female goats were at higher risk (OR = 2.4, 95% CI: 0.8–7.3), as were older animals over two years of age (OR = 3.8, 95% CI: 1.2-12). The Egyptian Baladi breed showed a markedly increased likelihood of infection (OR = 3.6, 95% CI: 1.3-10.3), and goats bred for dairy production were also more susceptible (OR = 2.5, 95% CI: 1.1-5.9) *(Table 2)*.

Furthermore, goats raised in smaller flocks with fewer than 30 animals exhibited a higher risk (OR = 2.4, 95% CI: 0.8-7.2). The risk was also elevated for goats in contact with other herds (OR = 2.5, 95% CI: 1.1-5.6) and those kept under poor biosecurity conditions (OR = 3.4, 95% CI: 1.4-8.2) *(Table 2)*.

Discussion

Goats are susceptible to caprine arthritis and encephalitis, which has a major negative economic impact and causes chronic weariness. Although a small percentage of goats may display specific symptoms, the disease usually manifests as subclinical. Goats infected with CAEV are more likely to suffer from neurological problems, arthritis, chronic mastitis, and prolonged pneumonia. These circumstances considerably lower productivity and reproductive potential, which raises the illness and mortality rates among afflicted goats [28-31].

This study aimed to determine the seroprevalence of CAEV in goats across three northern Egyptian governorates-Kafr El Sheikh, Beheira, and Alexandria-and to evaluate the associated risk factors influencing infection rates.

The overall seroprevalence of CAEV in the current study was 9.6%, aligning closely with the previously reported prevalence in Egypt (8.52%) [26] but showing variability when compared to other regions. It was lower than the prevalence reported in Jordan (8.9%) [18], Somalia (6.0%) [32], Syria (12.1%) [33], and Brazil (14.1% and 8.2%) [34,35]. Significantly higher rates have been reported in regions like Norway (42%) [36] and the United States (31%) [37].

Conversely, the prevalence in this study was higher than those documented in Saudi Arabia (0.8%) [38], Mexico (0.4%) [39], Turkey (1.9%) [40], and Italy (4.0%) [41]. This variation reflects the influence of diverse geographical, management, and climatic factors, as well as differences in diagnostic methodologies and population characteristics [42-46].

Regarding sex-based differences, the seroprevalence of CAEV was notably higher in females, consistent with previous findings reported by Ratanapob, Rukkwamsuk [19]. This could be attributed to physiological stressors like pregnancy, lactation, or hormonal changes, which may predispose females to higher susceptibility or immune responses to CAEV infection [7,32]. Interestingly, contrary

findings were noted in a study conducted in Thailand, where male goats, specifically exhibited higher seroprevalence [43]. This variability highlights the importance of considering local factors, management practices, and sample population characteristics when interpreting sex-related differences in CAEV seroprevalence [47-51].

This study observed that the seroprevalence of CAEV antibodies increased with age, rising from 3.6% in goats under one year to 15.2% in those older than two years. Similarly, Cutlip, Lehmkuhl [37] and Al-Qudah, Al-Majali [18] also reported that seroprevalence increased with age, with the latter noting a significantly higher prevalence in goats older than three years.

The age-related increase in seroprevalence can be attributed to prolonged exposure to risk factors over time and the lifelong persistence of CAEV in infected hosts [43]. Contrasting findings have also been reported. For instance, Dawson and Wilesmith [52] observed the highest prevalence in yearlings, suggesting that local management practices, herd dynamics, or diagnostic methodologies could influence age-related seroprevalence patterns.

Goat breeds demonstrated significant associations with seropositivity for CAEV infection in the univariate analysis. Notably, the seroprevalence was significantly higher in native breeds compared to others. Contrarily, Baraka, Khadr [26] observed seropositivity exclusively in Barki breed goats, highlighting potential breed-specific vulnerability or differences in exposure risks. This suggesting that genetic or environmental factors specific to these breeds might influence susceptibility [9,43].

Regarding the production system, the seroprevalence of CAEV is notably higher in dairy goats, which aligns with previous findings [53]. This study suggests that management practices and replacement policies on dairy farms may contribute to the higher seroprevalence of CAEV infection. Meat goats, which are often sold seasonally, have less prolonged exposure within the herd, reducing their risk of infection. In contrast, dairy goats are typically kept for longer periods, increasing their likelihood of contracting and transmitting CAEV within the herd [26,43].

Herd size, particularly herds with fewer than 30 animals, was identified as a significant risk factor for CAEV seropositivity in goats. This finding contrasts with the results reported by Nyi Lin, Ngarmkum [43], which suggested a different relationship between herd size and seroprevalence. In this study, an increase in herd size was inversely proportional to the odds ratio of seropositivity, indicating that smaller herds posed a greater risk for CAEV infection. The higher risk in smaller herds might be attributed to close contact among animals, which facilitates the transmission of CAEV through direct interactions or contaminated environments [54,55].

Another important risk factor in the current study was contact with goats from other herds, which has been reported to be a risk factor in earlier research [18,39]. This can be attributed to the farm management system, where farms in close proximity often share common grazing grounds. Additionally, some adjacent farms practiced the sharing of a common buck, which further facilitates the transmission of CAEV between herds. These practices increase the likelihood of cross-herd contact and, consequently, the spread of the virus [35,40,43].

In the present study, farms with poor biosecurity management showed higher seropositivity for CAEV compared to those with good biosecurity practices. This aligns with findings by Rahman, Akther [2], who reported that inadequate biosecurity and sanitation practices significantly contribute to the spread of CAEV. Farms with effective biosecurity measures typically limit external exposure, such as restricting the introduction of infected animals and minimizing contact with contaminated equipment or personnel, leading to lower seroprevalence rates [56-58].

This study provides an overview of the seroprevalence and associated risk factors of CAEV infection in goat herds raised in northern Egypt. The findings highlight the significant factors influencing the prevalence of CAEV, such as age, sex, breed, production system, flock size and farm management practices. To gain a more complete understanding of the epidemiology of CAEV, further research is needed to examine additional farms and expand on the factors influencing CAEV transmission. Such studies would be crucial for improving the management and development of goat farming in the region, ensuring better control measures and strategies for mitigating the impact of CAEV.

Declarations

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

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Ethical Approval: The research protocol was reviewed and approved by the Institutional Animal Care and Use Committee at Faculty of Veterinary Medicine, Benha University, ensuring adherence to guidelines (Ethical No: BUFVTM 20-11-2024).

Competing Interests: The authors declare that they have no conflicts of interest.

Declaration of Generative Artificial Intelligence (AI): The article

and/or tables and figures were not written/created by AI and AI-assisted technologies

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RESEARCH ARTICLE

Effects of *In-Ovo* Chrysin Injection to Quail Eggs on Hatchability, Production Parameters, and Immunity

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Abstract

This study investigated the effects of in ovo Chrysin injection on hatchability, embryonic mortality, body weight, feed conversion ratio (FCR), and immune responses in quails. A total of 720 fertilized eggs were randomly assigned to four groups: Control (0.1 mL distilled water) and Chrysin-treated groups (0.1 mL containing 0.25 mg, 0.50 mg, or 0.75 mg Chrysin per egg). Hatchability and hatching efficiency were unaffected by Chrysin treatment, although late embryonic mortality was significantly higher in the 0.75 mg group. Post-hatch evaluations showed significantly greater body weights on days 14 and 42 in the 0.25 mg and 0.50 mg groups compared to the control. These groups also exhibited improved FCR values, while feed intake remained unchanged across groups. Immunological analysis revealed a significant increase in IgA levels in the 0.75 mg group, with no significant effects on IgM or IgG levels. Overall, *in ovo* Chrysin supplementation at 0.25 mg and 0.50 mg enhanced growth performance and feed efficiency without adversely affecting hatchability, while the 0.75 mg increased embryonic mortality, indicating a need for dose optimization. These findings suggest that Chrysin holds potential as a biotechnological tool in poultry production, though further studies are required to refine dosing strategies and investigate its long-term impacts on performance and immunity.

Keywords: *Chrysin, Feed conversion ratio, Hatchability, Immunity, In-ovo*

Introduction

Avian embryonic development differs fundamentally from that of mammals, as it relies entirely on the nutrients stored within the egg, with no additional natural sources available to meet the embryo's nutritional needs [1]. Although the egg is nutritionally complete and capable of supporting the embryo throughout the incubation period [1,2], recent intensive selection in commercial poultry has resulted in increased metabolic rates during embryogenesis, thereby elevating the nutritional demands of the developing embryo. Insufficient nutrition during this critical period can impair embryonic development, reduce hatchability, and adversely affect post-hatch growth [3]. Moreover, the limited nutrient supply during incubation can constrain the growth potential of newly hatched chicks, preventing them from reaching their optimal developmental capacity [1,4].

To address these challenges, a number of studies have explored strategies to supplement the limited nutritional reserves available to embryos [1,2,5]. One promising approach is *in ovo* feeding, which involves the injection of liquid nutrients into specific areas of the egg, such as the albumen, amniotic fluid, yolk sac, or air sac, at various stages of incubation [4,6-8]. *In ovo* feeding has emerged as a viable strategy to enhance embryonic nutrition, potentially mitigating perinatal nutritional deficiencies and improving hatchability [9,10].

Among the various substances explored for *in ovo* feeding, plant-derived flavonoid compounds have garnered particular attention due to their pharmacological effects, antioxidant activities, and enzyme inhibition properties [11]. Chrysin *(5,7-dihydroxyflavone)*, a flavonoid found in plants such as *Passiflora caerulea, Passiflora incarnata*, and *Oroxylum indicum*, is noted for its wide range of biological effects, including antioxidant, anti-inflammatory, anti-cancer, anti-aging, anti-allergic, anti-diabetic, and neuro-protective properties [11-15]. Chrysin has also been shown to exhibit potent antioxidant activity, and numerous studies have investigated its effects of adding Chrysin to the diets of animals on animal performance and its protective

effects against various toxic agents [16-19]. However, research on the effects of *in ovo* Chrysin administration remains limited. A study by Khaligh et al.[20] indicated that *in ovo* administration of Chrysin did not significantly impact hatchability or the post-hatch performance of broiler chicks from 0 to 11 days of age. Conversely, Kurt et al.[21] observed a significant reduction in cataract formation in Chrysin-treated embryos, suggesting that its antioxidant and anti-apoptotic properties may contribute to this beneficial effect.

This study aims to investigate the effects of *in ovo* Chrysin administration on immune function and performance in quails up to 42 days of age. Specifically, it evaluates the impact of Chrysin injection into fertilized quail eggs on hatchability, embryonic mortality, production parameters such as body weight and feed conversion ratio (FCR), as well as immune responses. This study seeks to provide further insight into the potential of Chrysin as an *in ovo* nutrient supplement that could enhance both embryonic development and post-hatch health.

Material and Methods

Ethical Statement

The research was conducted at the Atatürk University Food and Livestock Application and Research Center Poultry Unit and was ethically approved by the Atatürk University Local Ethics Council of Animal Experiments (29.03.2022, 2022/4, Decision number: 64).

Experimental Procedures

A total of 750 eggs were collected from Japanese quail breeders *(Coturnix coturnix japonica)* aged 16 to 20 weeks, comprising 60 males and 180 females, over a period of 7 days. Prior to incubation, 30 eggs exhibiting broken or cracked shells and those not meeting ideal shape and size criteria were discarded. The experiment was designed with four groups (one control and three differing doses of Chrysin). A total of 720 eggs were randomly allocated into four groups of 180 eggs each. These eggs were placed in a single-stage incubator during the improvement stage (0-15 days), where the temperature was maintained at 37.5±0.3°C and relative humidity at 65±5%. The eggs were turned 90 degrees forward and backward at a 45-degree angle from the vertical axis every hour.

Before transitioning to the hatching period (the last 3 days of incubation), the following *in-ovo* injection treatments were administered:

Group 1 (Control): 0.1 mL of distilled water

Group 2: 0.1 mL injection containing 0.25 mg Chrysin per egg

Group 3: 0.1 mL injection containing 0.50 mg Chrysin per egg

Group 4: 0.1 mL injection containing 0.75 mg Chrysin per egg

Chrysin doses were dissolved in a physiological saline solution to a final volume of 1 mL. Eggs were disinfected with 70% ethanol and pierced at the flatter end (air cell) before manual injection using a 26 G syringe to a depth of approximately 5 mm. After injection, the holes were sealed with nail polish and disinfected again with 70% ethanol [7,21]. The injected eggs were then placed back into the single-stage incubator.

During the final 3 days of incubation, the temperature was adjusted to 36.5±0.3°C, with relative humidity set at 75±5%. Upon hatching, chicks were individually weighed to calculate average body weight. Ten chicks per treatment group were euthanized using mild sevoflurane anesthesia, and liver samples were collected and stored at -20°C for subsequent biochemical analyses.

Hatchability Parameters

Hatchability was calculated as the proportion of successfully hatched chicks relative to the total number of eggs incubated. Fertilization rate was determined as the proportion of fertile eggs among the total eggs incubated. Hatching success, also referred to as hatching efficiency, was defined as the proportion of chicks hatched from the total number of fertile eggs.

Non-hatched eggs were broken to assess the presence of embryos, and embryonic mortality was classified according to the stage of incubation as early (1-6 days), intermediate (7-14 days), or late (15-18 days).

Raising Chicks

Healthy chicks were reared in separate brooder cages according to their respective treatment groups for the initial two weeks. To ensure post-hatch uniformity, a total of 360 chicks -90 from each experimental group- were placed in cages. During this period, a starter diet containing 23% crude protein (CP) and 3.000 kcal/kg metabolizable energy (ME) was provided. After the second week, the chicks were transferred to multi-tier rearing cages, with mixed gender assigned according to their treatment groups. The housing system consisted of four cage units, each comprising three tiers, with five cage cells per tier. Six chicks were housed in each cage cell. The grower diet was designed to meet the nutritional needs of quails, containing 20% CP and 3.250 kcal/kg ME. Feed and water were provided ad libitum throughout the study. Environmental conditions were controlled: the ambient temperature was maintained at 32-33°C for the first three days, then gradually decreased by 1-2°C per week until stabilized at 24°C. A lighting regimen of 23 h of light and 1 h of darkness per day was implemented for the duration of the experiment. Daily feed intake was monitored, and body weight along with weight gain were recorded

at 10:00 AM on the first day of each week. Feed intake and FCR were calculated. The study concluded when the quails reached six weeks of age, at which point the age at sexual maturity, defined as the age of first egg-laying, was documented.

Liver Biochemical Analyses

Standards and samples of immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobulin M (IgM) were pipetted into wells of a microplate pre-coated with specific antibodies. The IgA, IgG, and IgM present in the samples bound to the immobilized antibodies. Enzyme-linked secondary antibodies were then added to bind with the primary antibodies, followed by the addition of an enzyme substrate, resulting in a color change proportional to the concentrations of IgA, IgG, and IgM. The color intensity was measured at 450 nm [22].

The calculation was performed using a regression equation derived from a standard curve, with absorbance values plotted on the X-axis and corresponding standard concentrations on the Y-axis. The experiment, including reagent preparation and calculation procedures, was conducted using consistent protocols, the same brand of reagents, and identical measurement steps throughout.

Statistical Analysis

Data were analyzed using SPSS 12.0. The chi-square test was used for embryonic mortality and hatchability analysis; One-Way ANOVA for body weight changes, feed conversion ratios, and average feed intake; and General Linear Model for biochemical data. Results with $P<0.05$ were considered significant.

Results

The research findings indicated that the 0.75 mg Chrysin treatment group exhibited the lowest hatchability and hatching efficiency rates; however, these differences were not statistically significant ($P>0.05$). The hatchability rates for the control, 0.25 mg, 0.50 mg, and 0.75 mg Chrysin treatments were 72.78%, 72.22%, 74.44%, and 62.78%, respectively. Similarly, the hatching efficiency rates were 86.75%, 83.87%, 84.81%, and 75.84%, respectively *(Table 1)*.

As the experimental treatment was applied on the 15th day of incubation, it had no effect on fertility rates or early- to mid-term embryonic mortality. Regarding embryonic mortality, the highest incidence was observed during the late embryonic period. Late embryonic death rates for the experimental groups were 8.61%, 11.61%, 10.76%, and 18.79%, respectively. The rate of late embryonic death was significantly higher in the 0.75 mg treatment group compared to the other groups ($P<0.05$) *(Table 1)*.

A statistically significant difference in body weight was observed between treatment groups on days 14 and 42 ($P<0.05$). On day 14, quails in the 0.25 mg Chrysin group exhibited the lowest body weight but demonstrated the highest weight gain throughout the experiment. By day 42, quails in all Chrysin treatment groups (0.25 mg, 0.50

Table 1. *Hatchability performance and embryo mortality rates (%) as a function of treatment*

Parameter		Control	0.25 mg	0.50 mg	0.75 mg	P
Hatchability Performance	Hatchability (%)	72.78	72.22	74.44	62.78	0.065
	Fertilization rate (%)	83.89	86.11	87.78	82.78	0.544
	Hatching efficiency (%)	86.75	83.87	84.81	75.84	0.062
Embryo mortality rate (%)	Early (1 to 6 d)	1.32	2.58	1.90	2.68	0.755
	Intermediate (7 to 14 d)	3.31	1.94	2.53	2.68	0.908
	Late (15 to 18 d)	8.61^{b}	11.61^{b}	10.76^{b}	18.79^{a}	0.044

$^{a, b}$*Different letters within one row are significantly different (P<0.05)*

Table 2. *Average weekly body weights and standard errors of the trial groups ($\bar{x} \pm SE$)*

Day	Body Weight (g)				
	Control	0.25 mg	0.50 mg	0.75 mg	P
14	55.43±1.28^{ab}	52.51±1.85^{b}	56.97±1.46^{ab}	59.69±1.31^{a}	0.015
21	97.39±1.53	95.39±1.08	98.65±2.61	99.89±1.89	0.324
28	136.39±1.94	139.18±1.80	141.22±3.71	141.88±2.67	0.407
35	170.58±2.09	178.88±2.39	180.42±4.17	176.49±1.88	0.055
42	202.62±1.22^{b}	214.87±2.70^{a}	215.15±3.69^{a}	208.58±1.80^{ab}	0.001

$^{a, b}$ *Different letters within one row are significantly different (P<0.05)*

Table 3. *Average weekly daily feed intake and standard errors of the trial groups ($\bar{x}$± SE)*

Day	Daily Feed Intake (g/quail)				
	Control	0.25 mg	0.50 mg	0.75 mg	P
14-21	20.24±1.16	21.67±1.19	23.46±1.94	22.74±1.46	0.411
21-28	23.70±1.24	27.10±1.06	25.78±1.03	25.53±1.01	0.165
28-35	35.55±1.09	33.14±1.30	32.05±0.96	34.53±1.14	0.165
35-42	36.43±0.81	35.49±1.40	32.04±1.18	36.47±0.50	0.734
14-42	28.98±0.54	29.35±0.47	29.08±0.87	29.82±0.53	0.778

Table 4. *Average weekly feed conversion ratio and standard errors of the trial groups ($\bar{x}$± SE)*

Day	Feed Conversion Ratio				
	Control	0.25 mg	0.50 mg	0.75 mg	P
14-21	3.45±0.22	3.57±0.19	3.95±0.30	3.99±0.28	0.320
21-28	4.39±0.27	4.47±0.29	4.32±0.19	4.53±0.40	0.968
28-35	7.34±0.29[a]	5.92±0.29[b]	5.98±0.37[b]	7.22±0.46[a]	0.004
35-42	8.32±0.47	7.12±0.30	7.38±0.41	8.00±0.21	0.085
14-42	5.51±0.56[a]	5.08±0.97[b]	5.15±0.10[b]	5.61±0.85[a]	<0.001

[a, b] *Different letters within one row are significantly different (P<0.05)*

Table 5. *Average IgA, IgM and IgG levels and standart errors of the trial groups ($\bar{x}$± SE)*

Type	Immunoglobulin Levels (mg/ml)				
	Control	0.25 mg	0.50 mg	0.75 mg	P
IgA	62.30±1.20[b]	63.10±1.18[b]	61.64±1.18[b]	69.55±1.18[a]	<0.001
IgM	483.61±16.61	483.32±16.38	447.79±16.38	504.36±16.38	0.118
IgG	377.79±12.25	364.37±12.08	357.42±12.58	394.84±12.08	0.148

[a, b] *Different letters within one row are significantly different (P<0.05)*

mg, and 0.75 mg) had higher body weights compared to the control group. Specifically, the final body weights at the end of the experiment were 202.62 g for the control group, 214.87 g for the 0.25 mg group, 215.15 g for the 0.50 mg group, and 208.58 g for the 0.75 mg group. The highest body weights were recorded in the 0.50 mg and 0.25 mg Chrysin treatment groups, with a significant difference (P<0.01) observed between these groups and the control *(Table 2)*.

The daily feed intake of quails across treatment groups is presented in *Table 3*. Feed intake exhibited an increasing trend over time in all groups; however, the differences among the groups were not statistically significant (P>0.05). During the experimental period (14-42 days), the average daily feed intake was recorded as 28.98 g, 29.35 g, 29.08 g, and 29.82 g for the control group and the groups receiving 0.25 mg, 0.50 mg, and 0.75 mg of Chrysin, respectively.

FCR are presented in *Table 4*. No significant differences in FCR were observed among the groups during individual weeks, except for the 3rd week (P>0.05). However, statistically significant differences were found in FCR values over the entire experimental period (14-42 days) (P<0.001). The average FCR values for the control group and the groups receiving 0.25 mg, 0.50 mg, and 0.75 mg of Chrysin were 5.51, 5.08, 5.15, and 5.61, respectively. The 0.25 mg and 0.50 mg Chrysin groups demonstrated better FCR values compared to the control and 0.75 mg Chrysin groups. Additionally, FCR values increased over time in all groups.

The IgA levels in liver tissues of chicks were measured as 62.30±1.20 mg/ml, 63.10±1.18 mg/mL, 61.64±1.18 mg/mL, and 69.55±1.18 mg/mL for the control group and the groups receiving 0.25 mg, 0.50 mg, and 0.75 mg of Chrysin, respectively. Statistically significant differences in IgA levels were observed among the groups (P<0.001). However, no significant differences were found in IgM and IgG levels *(Table 5)*.

The age at sexual maturity, defined as the age at first egg, was recorded as 40, 41, 39, and 37 days for the control group and the 0.25 mg, 0.50 mg, and 0.75 mg Chrysin groups, respectively.

Discussion

This study examined the effects of *in ovo* Chrysin injection at three different doses. As the injections occurred on the 15th day of the embryonic period, they had no impact on fertilization rates, early- and mid-term embryonic mortality.

The results revealed no statistically significant relationship between *in-ovo* Chrysin injections and hatchability or hatching efficiency. Although a trend towards lower hatchability was observed, the lack of statistical significance suggests that the impact of Chrysin on hatching success is relatively mild within the tested dose ranges. Notably, the 0.75 mg dose was associated with lower hatchability rates and increased late-stage embryonic mortality (18.79%), indicating potential developmental challenges at higher doses. In contrast, previous studies have reported improved hatching outcomes with other substances. For instance, Genc et al.[7] found that a 0.25 mg Rutin injection yielded the highest hatching performance in quail eggs, while Ghane et al.[23] demonstrated that in-ovo feeding with vitamins C and E significantly improved hatchability in broiler eggs. Similarly, Taha et al.[24] reported that a 0.5 mL *in-ovo* royal jelly injection on the 7th day of incubation enhanced hatching performance.

Late embryonic deaths observed in this study may be attributed to the timing and method of *in-ovo* application rather than the antioxidant properties of Chrysin itself. Since the injections were performed after the first 15 days of incubation, they could have disrupted critical stages of embryonic development. This is consistent with findings from Subramaniyan et al.[25], who reported that *in-ovo* L-arginine injection on the 14th day of incubation in chicken eggs positively influenced survival and hatching rates, whereas applications closer to hatching showed diminished benefits.

The increased late embryonic mortality in the 0.75 mg Chrysin group (18.79%) compared to the control (8.61%), 0.25 mg (11.61%), and 0.50 mg (10.76%) groups highlights a dose-dependent effect. While Chrysin's antioxidant and anti-inflammatory properties may offer potential benefits, higher concentrations could negatively affect embryonic development during the later stages of incubation. These adverse effects may stem from overdose of Chrysin, which can disrupt the balance of oxidative stress, alter cellular metabolism, and affect gene expression. Excessive doses might impair tissue and organ formation, leading to higher mortality in the final stages of embryogenesis [26,27].

The weekly body weight measurements demonstrate the potential of Chrysin as an *in ovo* dietary supplement to enhance the growth performance of quails. The results indicate that *in ovo* administration of Chrysin significantly improves growth performance, particularly in the groups receiving 0.25 mg and 0.50 mg doses. At the end of the trial (42 days), the highest body weights were observed in these two groups, whereas the control group exhibited the lowest final body weight. These findings are consistent with previous studies highlighting the beneficial effects of flavonoids on growth performance through their antioxidant, anti-inflammatory, and metabolic regulatory roles [25,28]. Coskun et al.[28] reported that *in ovo* DL-methionine injection increased chick weight by 3.8%. Considering the positive correlation between chick weights and subsequent body weights [29], it is likely that *in ovo* injection of Chrysin can similarly enhance adult body weights. Furthermore, Hassan et al.[30] observed that *in ovo* injection of different Cu sources improved broiler chicken body weights, while Dang et al.[31] reported improved development in goose embryos with disaccharide and methionine injections. Similarly, Abdel-Halim et al.[32] demonstrated that *in ovo* injection of folic acid and glucose (0.2 mg folic acid + 125 mg glucose) enhanced carcass characteristics by increasing growth rates. The observed improvements in body weight in the Chrysin-treated groups can be attributed to the compound's ability to reduce oxidative stress during critical periods of embryonic development and early growth [27]. By scavenging free radicals and supporting cellular functions, Chrysin likely enhances energy efficiency and nutrient utilization, resulting in improved growth rates [27]. Reduced oxidative stress may promote better cell proliferation and organ development, potentially explaining the superior weight gain observed in the Chrysin-treated groups compared to the control. A noteworthy aspect of this study is the dose-dependent response. While the 0.25 mg and 0.50 mg doses of Chrysin optimized growth, the 0.75 mg dose resulted in slightly lower final body weights compared to the other Chrysin-treated groups. This suggests that the 0.75 mg dose may exceed the optimal range, potentially causing suboptimal effects due to metabolic imbalances or mild toxicity. This phenomenon is consistent with the hormesis theory, which proposes that moderate doses of bioactive compounds elicit beneficial effects, whereas higher doses may inhibit growth or induce stress [33].

Regarding feed intake no significant differences found between the groups, suggesting that Chrysin does not influence appetite or feed intake behavior in quails. However, a significant difference was observed in FCR maintenance during the experimental period (14-42 days). The lack of variation in feed intake, coupled with differences in FCR, may underscore Chrysin's potential role in enhancing nutrient utilization efficiency. The improved FCR values in the Chrysin-treated groups can likely be attributed to the compound's antioxidant properties, which may reduce oxidative stress during critical

developmental stages [34]. By improving cellular energy efficiency and nutrient absorption, Chrysin supports better growth performance without altering feed intake. Consistent with previous research, the positive effects observed in this study support the role of flavonoids and *in ovo* administration of bioactive compounds in enhancing metabolic health, feed intake, and growth performance in poultry [35-38]. The dose-dependent effects observed in this study are particularly noteworthy. The optimal FCR values in the 0.25 mg and 0.50 mg Chrysin groups suggest that these doses enhance nutrient metabolism and energy utilization. In contrast, the slightly higher FCR observed in the 0.75 mg group may indicate potential metabolic disruptions or mild toxicity at this dose [26,27]. This finding highlights the importance of determining an optimal dosage range for Chrysin supplementation to maximize its efficacy while avoiding adverse effects.

Immunity, or the immune response, serves as the defense mechanism against microorganisms such as viruses, bacteria, fungi, and parasites in all vertebrates, including poultry. It is crucial for preventing disease development and begins to develop during incubation. Chicks receive maternal antibodies from the yolk sac, which provide protection against microorganisms during early life [39]. The development of immunity during the chick period is vital, as it significantly impacts their survival and later performance [40,41]. Williams and Hopkins [42] highlighted that one advantage of *in-ovo* injection is the earlier stimulation of the immune system. Our study observed a significant difference in IgA levels, a key component of mucosal immunity, with the highest levels in the 0.75 mg Chrysin group. IgA plays a critical role in preventing bacteria and viruses from adhering to epithelial surfaces, a process known as immune exclusion [43]. Chrysin's antioxidant and anti-inflammatory properties may enhance immune responses, specifically mucosal immunity, by promoting higher antibody production [44]. The significant increase in IgA levels in the 0.75 mg Chrysin group suggests that this higher dose could improve mucosal immune function, possibly by modulating cytokine production or enhancing gut barrier integrity [43]. No significant differences were found between groups for IgM and IgG concentrations. The absence of differences in IgM and IgG levels across treatment groups suggests that Chrysin's effects on the immune system may be specific to mucosal immunity rather than systemic humoral immunity. This specificity could be attributed to Chrysin's potential influence on gut-associated lymphoid tissue (GALT), which primarily regulates IgA production [45] . Previous studies on *in ovo* injections to enhance chick immunity have yielded positive results. Gore and Qureshi [46] found that vitamin E injection into the amniotic fluid of 18-day-old embryos enhanced both cellular and humoral immunity. Tufarelli et al.[47] reported that *in ovo* injection of folic acid increased IgM and IgG levels, thereby strengthening the immune system. Goel et al.[8] observed positive effects on immunity with thiamine and pyridoxine injections, while Hassan et al.[30] indicated that *in ovo* injection of various Cu sources did not negatively affect immune parameters. Similarly, Subramaniyan et al.[25] concluded that *in ovo* L-arginine application on day 14 of incubation effectively stimulated the immune response by increasing IgM levels.

In conclusion, this study demonstrates that *in ovo* Chrysin supplementation at optimal doses (0.25 mg and 0.50 mg) enhances body weight, feed efficiency, and mucosal immunity in quails without adversely affecting hatchability. However, the findings also reveal dose-dependent effects, with higher doses (0.75 mg) increasing late embryonic mortality and diminishing growth performance, underscoring the importance of precise dose optimization. While Chrysin showed promising antioxidant and anti-inflammatory benefits, particularly in improving nutrient metabolism and immune defenses, its limited impact on systemic immunity and hatching success highlights the need for further research to refine dosing strategies and *in ovo* injection protocols.

Declarations

Availability of Data and Materials: Materials and data sets from the study are available upon request from the corresponding author (M. Genç).

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Declaration of Generative Artificial Intelligence (AI): The authors declare that the article, tables and figures were not written/created by AI and AI-assisted technologies.

Author Contributions: The conception and design of the study were conducted by MG. MG and UO were responsible for data acquisition, analysis, and interpretation, as well as drafting the manuscript. Biochemical analyses were performed by SK. The critical review and revision of the manuscript were collaboratively undertaken by MG, UO, and SK.

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Research Article

First Cloning, Tissue-specific Expression and Molecular Characterization of RIG-I Gene in Whooper Swan

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Abstract

To investigate the molecular characteristics of the RIG-I gene of whooper swan, RIG-I gene was cloned, sequenced and characterized, and the relative expression levels of the RIG-I gene in five tissues of whooper swans were determined by Real-time Quantitative PCR (qPCR). The RIG-I gene of whooper swan has a 2964 bp CDS that encode a peptide of 987 amino acids, RIG-I is widely expressed in various tissues of the whooper swan, but the expression levels show certain differences. The homology analysis indicated that the RIG-I gene of whooper swan has a more than 90% homology with other *Anseriformes* birds including mallard, swan goose, black swan and mute swan. Phylogenetic analysis showed that RIG-I gene of whooper swan and other birds have grouped in a separate branch. Gene alignment analysis revealed that RIG-I gene of swan has an extra low complexity region compared with that of non-swan birds. The amino acid alignment analysis showed that RIG-I of swan has a specific S62G phosphorylation site mutation compared with that of other birds and ubiquitination/phosphorylation mutation sites (RNF122, CK2, and REUL I) which differ from that of human. This is the first cloning and characterization of RIG-I gene from whooper swan. It may enhance our understanding of the molecular response mechanism of RIG-I against the influenza virus.

Keywords: Cloning, Molecular characterization, RIG-I, Tissue-specific expression, Whooper swan

Introduction

Whooper swan *(Cygnus cygnus),* belonging to the *Anseriformes Anatidae Cygnus*, are widely distributed worldwide waterfowl. They breed in the Palearctic and range from Iceland to the Far East, across Eurasia, and are widely distributed in freshwater regions [1]. Whooper swan, due to their long-distance migratory habits, are often in contact with other birds, which increases their risk of contracting Avian influenza viruses (AIV) [2]. During the H5NX avian influenza pandemic, swans were reported to be infected and die. It makes swan considered as indicators of the H5 subtype of AIV [3,4]. In recent years, H5 avian influenza has evolved into new varieties, which threaten poultry farming and human health [5]. Whooper swan are more sensitive to influenza viruses than other waterfowl [6]. The mechanism behind this phenomenon has attracted great attention from scholars in the field.

Retinoic Acid Inducible Gene-I (RIG-I) is a key intracellular pattern recognition receptor that plays an important role in recognizing and responding to RNA viruses such as avian influenza [7,8]. Barber's study [9] showed that ducks have a natural resistance to avian influenza while chickens are highly susceptible to avian influenza infection. It is strongly associated with ducks having an intact RIG-I gene and chickens lacking it. RIG-I, as a cytoplasmic RNA sensor, is able to respond to infection with influenza viruses, trigger IFN-β production and activate downstream IFN-stimulated antiviral gene expression [10]. Ducks possess a complete RIG-I gene, enabling them to induce IFN production and downstream antigen expression via RIG-I, thus exhibiting natural resistance to AIV [9,10]. On the other hand, Chickens lack the RIG-I gene, although it can express IFN-α by other pathways the absence of RIG-I-mediated IFN-β severely compromises antiviral defense, making them highly susceptible to

AIV [9,11]. Previous experimental studies in Muscovy ducks *(Cairina moschata)* [12], geese [13] and pigeons *(Columba livia)* [14] also confirmed the presence of the RIG-I gene and successfully validated its mediated IFN-β expression and its protective role in antiviral immunity, further supporting the critical immunoprotective functions in different avian populations.

There is currently no research confirming whether the susceptibility of whooper swans to AIV is related to the RIG-I gene, and no study has confirmed whether whooper swans possess the RIG-I gene. Genbank predicted the RIG-I sequences of black swan and mute swan by genome sequencing. However these predictions have not been experimentally validated. In this study, it designs primers based on reference prediction sequences, attempts to amplify the complete gene sequence of the Giant Swan RIG-I, analyzes its molecular characteristics and predicts its spatial structure and function. It will explore the possible relationship between the integrity and function of RIG-I gene and the high susceptibility to avian influenza from the perspective of immunology. It can provide reference and guidance for the effective prevention and control of avian influenza.

Material and Methods

Ethical Statement

This study was approved by the Northeast Forestry University Animal Experiments Local Ethics Committee (Approval no: 2023004).

Experimental Animal and Sample Collection

Samples were collected from the fresh carcass of a whooper swan that succumbed to canine bite injuries despite treatment at the Sanmenxia Wildlife Rescue and Monitoring Center in Henan Province, China. The carcass tested negative for infectious diseases. The lung, liver, spleen, kidney, larynx, and rectum tissue was milled with liquid nitrogen to form homogenates and stored in -80ºC for later use.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from the stored tissue according to the operation instructions of the Eastep® Super Total RNA Extraction Kit (Promega, Madison, America). The purity and concentration of the extracted RNA were determined to use a photometer. CDNA was obtained by reverse transcription of RNA using the PrimeScript™ RT reagent Kit (Takara, Otsu Shiga, Japan).

Sequence Identification and Molecular Cloning

According to the predicted *Cygnus olor* (XM_040540509.1) of RIG-I sequence in Genbank, the primer design software Oligo7 was used to design the amplified full length sequences *(Table 1)*. PCR amplification was performed using the high-fidelity enzyme PrimeSTAR® Max DNA Polymerase (Takara, Otsu Shiga, Japan) with cDNA of Cygnus swan lungs synthesized by the above steps as a template and RIG-I-F/R as a specific primer. PCR products were recovered and purified using Takara MiniBEST Agarose Gel DNA Extraction Kit Ver.4.0 (Takara Bio Inc, Kusatsu, Japan), the purified product was subcloned into the PMDTM19-T vector (Takara, Otsu Shiga, Japan), the recombinant was transformed into *E. coli* Dh5α, and the plasmid was extracted according to the instructions of Plasmid Purification Kit (Midi) (Abnova, Taiwan, China). Finally, the recombinant plasmid was identified by PCR. Three recombinant plasmids, which were identified as positive by PCR, were randomly selected and sent to biotechnology companies for sequencing. The sequencing results were confirmed by BLAST on NCBI *(https://www.ncbi.nlm.nih.gov)* with RIG-I genes from different species [15].

Table 1. *The primer for PCR amplication in this study*

Primer	Primer Sequence (5'-3')	Product Length
RIG-I	F: ATGAGGTTCACGAAGCTGCAAGC R: CTAAAATGGTGGGTACAAGTTGG	2964bp

Bioinformatics Analysis of RIG-I Gene

The homology of the RIG-I gene of whooper swan with that of humans and 16 other animals was compared by Clustau W Method using Meglign software, with sequence information from the NCBI database *(https://www.ncbi.nlm.nih.gov/)*. Using the N-J method of MEGA7.0 software, the phylogenetic relationships and taxonomic positions of RIG-I genes in birds, fish and mammals were observed and analyzed, and used ITOL's online tool *(https://itol.embl.de)* to beautify the modified gene tree [16]. The RIG-I amino acids of whooper swan, humans, pigeons, mallards and swan geese were compared by GeneDoc software. The proteins transcribed by the RIG-I gene of swan were predicted by a genome-wide database search in SMART *(http://smart.embl-heidelberg.de)*, and the hydrophobic profile of RIG-I was predicted by the ProtScale program of ExPASy; used the online tool SignalP *(https://services.healthtech.dtu.dk/service.php?SignalP)* to predict protein signal peptides.

qPCR

The expressions of RIG-I gene in lung, liver, spleen, kidney, larynx, and rectum of whooper swan were detected using relative quantitative PCR. Each tissue sample was tested in triplicate and the average value was taken. Q-PCR was conducted using the TaKaRa SYBR® PrimeScri™ RT-PCR kit (TaKaRa, Beijing, China) according to the manufacturer's instructions [15]. The details of the primer sequences for qPCR are listed in *Table 2*. The cDNA template was from the above experiment.

Table 2. The primer for PCR amplication in this study			
Primer	**Primer Sequence (5'-3')**	**Product Length**	**Reference Sequence**
qRIG-I	F: TGACATCATCGTACTGACACC R: CAGCTGACTTGCAGAGGAGTT	184bp	XM_040540509.1
qGAPDH	F:TTGGCATCGTGGAGGGTCTTATG R:CCCGTTGAGCTCAGGGATGACTT	176bp	XM_040573143.1

Statistical Analysis

With GAPDH gene as the internal reference, the expression level of RIG-I gene in liver was set to 1, the relative expression levels of RIG-1 gene in different tissues were analyzed by $2^{-\Delta\Delta Ct}$ method. Statistical analysis and data plotting were performed using GraphPad Prism 10.1.2 (GraphPad Software Inc.) [17].

Results

Cloning of the RIG-I Gene

Using lung cDNA as a template and RIG-I-F/R as specific primers, the full-length RIG-I was amplified. The PCR amplification products of RIG-I gene fragments of the mute swan were about 2900bp in size, as shown by 1% agarose gel electrophoresis *(Fig 1-A)*. It was consistent with the expected results. The sequencing results showed that the full-length sequence of the cloned RIG-1 gene is 2964 bp, which has high homology with several avian species in NCBI.

Bioinformatics Analysis

The mRNA of RIG-1 in different tissues of the whooper swan was detected by qPCR, with GAPDH as the internal reference. Expression in different tissues was calculated. The qPCR results showed that the RIG-1 gene was expressed in all the tested tissues, with a high level in lung, spleen and kidney, and moderate level in larynx and rectum, but low level in liver *(Fig 1-B)*.

Using Megalign software, the RIG-I gene sequences of whooper swan were compared with those of other birds, mammals and fish *(Fig 2-A)*. The RIG-I gene sequences of whooper swan were found to be 98.9% homologous to those of swan goose *(Anser cygnoides)*, black swan *(Cygnus atratus)* and mute swan *(Cygnus olor)* by comparison. Therefore, the amplified whooper swan RIG-I gene of length 2964bp is matched with swan goose, black swan and mute swan.

The amino acids of RIG-I of whooper swan were compared with those of humans, pigeons, mallard ducks and swan geese by GeneDoc software. The gene sequences

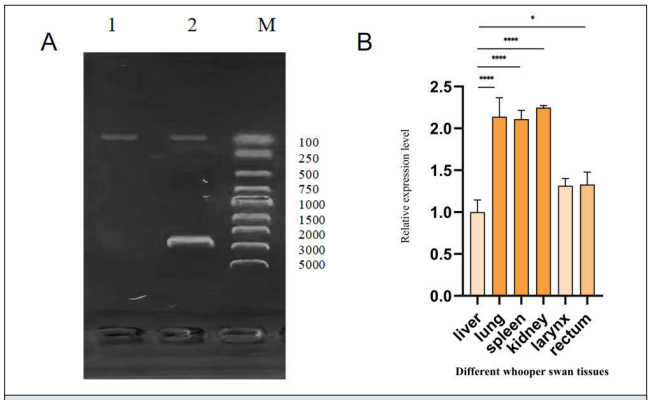

Fig 1. PCR amplification of RIG-I gene and its quantitative distribution in various tissues. **A:** PCR amplification results of RIG-I gene of whooper swan. The first lane is the negative control, the second lane is the amplified fragment, and the M lane is the DL5000 DNA marker, **B:** Relative gene expression patterns of RIG-I in various tissues of swan. The GAPDH was used as internal control. Bars represents the mean ± SD (n=3). * stands for P<0.05, **** stands for P<0.0001. It is considered that P<0.05 is statistically significant

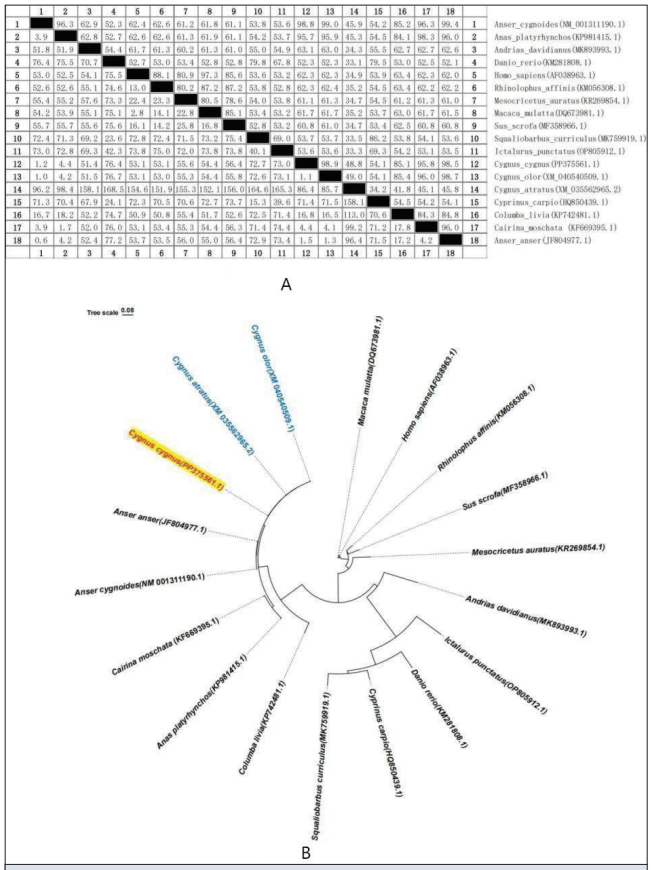

Fig 2. The homology and phylogenetic analysis of RIG-I from whooper swan and other species. **A:** The homology analysis of RIG-I gene from some birds, human, mammals and fishes, **B:** Phylogenetic analysis of RIG-I from some birds, human, mammals and fishes. The blue font represents the predicted RIG-I gene of *Cygnus atratus* and *Cygnus olor*, while the red font and yellow background represent the RIG-I gene of *Cygnus cygnus*

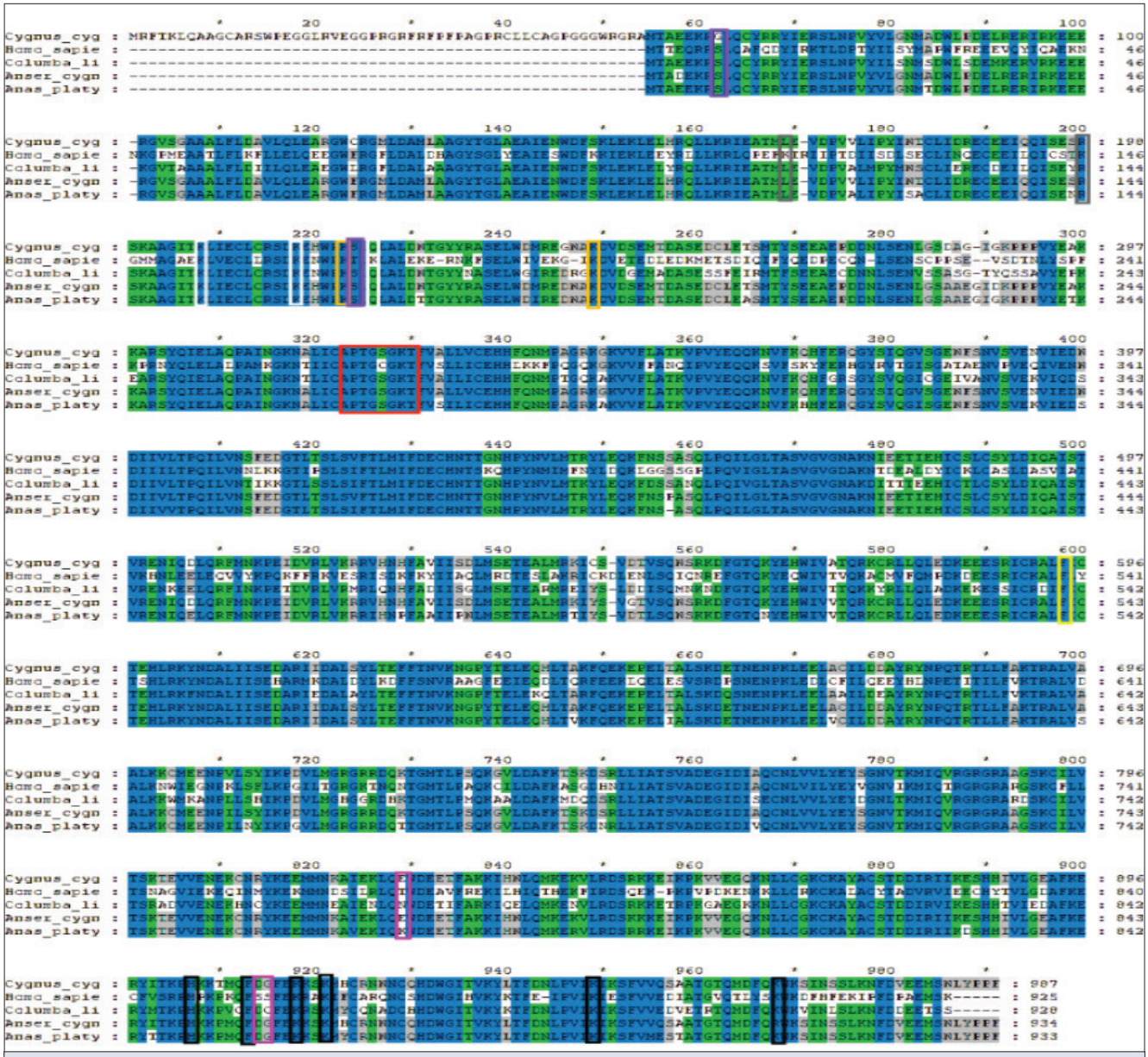

Fig 3. Amino acid alignment of Cygnus Cygnus, Homo sapiens, Columba Livia, Anser cygnoides and Anas platyrhynchos RIG-I. The purple frame represents the phosphorylation site of PKC-α/β. The pink frame denotes the phosphorylation site of CK2. The orange frame indicates the ubiquitination site of TRIM25. The white frame represents the REUL ubiquitination site. The gray frame denotes the ubiquitination site of RNF122. The yellow frame indicates the key residues of the interaction between CARDs and HEL2i. The red frame represents the ATP-binding motif, and the black frame denotes the key residues of RNA ligand binding

of black swan and mute swan were predicted based on the macro-genome sequences, but they were not confirmed by experiments. The starting part of the RIG-I amino acid sequence of whooper swan in *Fig 3* is significantly longer than that of other species. However, the biological significance of this amino acid variant requires further investigation due to limited data.

The amino acid sequence of RIG-I was predicted by SMART, an online tool. The results showed that RIG-I had a classical structure, that is two CARD regions, one DExD/h helicase region and one C-terminal repression domain (RD). However, compared with the traditional RIG-I domain, there is a low complexity region (LCR) at the N-terminal of the swan RIG-I, starting at position 24 and ending at position 39, it may not have a significant effect on the function of the RIG-I gene. There are two CARD domains in the great swan RIG-I protein domain. The first starting at position 55 and ending at position 146, and the second starting at position 152 and ending at position 243. The DEXDc frame RNA enzymatic helicoid structure starts at position 295 and ends at position 505; the RD domain starts at position 862 and ends at position 980, so the swan RIG-I has a more typical RIG-I structure. RIG-I protein was bioinformatics by an online program

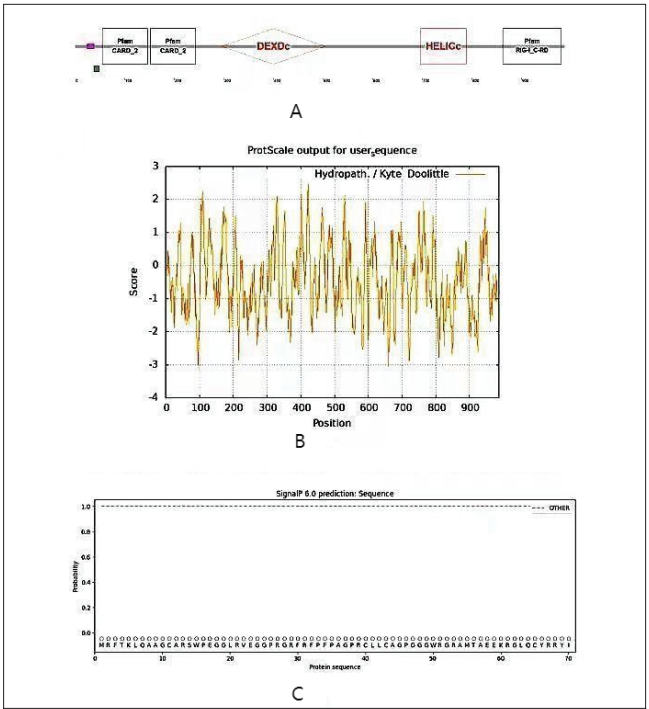

Fig 4. Structure and function prediction diagram of RGI-I proteins of whooper swan. **A:** Whooper swan RIG-I protein domain prediction, **B:** Whooper swan RIG-I protein hydrophobicity prediction, **C:** Whooper sawn RIG-I protein signal peptides prediction

(Fig 4-A). The results indicated that RIG-I protein was a hydrophilic stable acidic protein without signal peptide, so it was not a secretory protein *(Fig 4-B,C)*.

Discussion

The qPCR results of this study indicate that the RIG-I gene in whooper swan exhibits high expression level in the kidney, lung, spleen, rectum and larynx. Unlike duck, muscovy duck and goose where it is highly expressed, RIG-I gene expression in the liver of whooper swans is at a relatively basal level [12,16,18]. The elevated expression levels of RIG-I in the liver of waterfowl, as observed across multiple species, provide compelling evidence for the significant role of RIG-I in the innate immune response to AIV, supporting the possibility of respiratory transmission. And the high expression levels in the larynx and rectum of whooper swan suggest that this species can be infected with AIV via the digestive system [19]. May be the higher RIG-I gene expression level in lung and spleen of the whooper swan may allow it to be infected and die later thus providing a longer period for spreading AIV [20]. However, limited by the small sample size, this study lacked transfection, Western blot analysis and viral infection experiments compared to the studies mentioned. Consequently, the findings of this study can only be interpreted with caution,

In non-avian animals, the RIG-I gene exhibits variations: some animals with deleted RIG-I genes rely on the melanoma differentiation-associated protein 5 (MDA5) gene, a member of the RLR family, to fulfill its role. For instance, the Chinese tree shrew *(Tupaia belangeri chinensis),* which lacks the RIG-I gene, utilizes its MDA5 gene to recognize not only the small nuclear RNA virus originally specifically recognized by RIG-I, but also the Sendai virus of the *Paramyxoviridae* [21,22]. In addition, some animals lack the RIG-I gene-induced IFN pathway. Instead they play a role through other proteins. Also, IFN and MAVS transcripts that help induce IFN have not been detected in lampreys, according to Ma et al.[15] and Lu et al.[23]. Scientists have conducted RIG-I gene amplification experiments on the black flying fox to investigate its role in asymptomatic infections with multiple RNA viruses. These studies aim to understand how RIG-I contributes to the immune response and why black flying fox can carry RNA viruses without showing symptoms, and finally came to the conclusion that the RIG-I gene is intact and has obvious functions in bats [24]. Based on this research background with regard to our experiment, can this explain that the RIG-I gene is not the reason why the whooper swan is susceptible to avian influenza.

The RIG-I gene sequences of the black swan and the mute swan were predicted based on metagenomics, so the sequencing results are for reference only. Compared with *Anser cygnoides*, *Anser anser*, *Anas platyrhynchos*, and *Cairina moschata*, *Columba livia*, RIG-I gene has high homology *(Fig 2-A)*. Compared with mammals, the homology of the RIG-I gene sequence ranged from 61.3% to 62.3% in whooper swan and ranged from 52.5% to 54.4% in fish. It indicated that RIG-I gene had obvious interspecific characteristics. This result is similar to previous reports on the RIG-I gene in mallard ducks [25]. In the evolutionary tree *(Fig 2-B),* the RIG-I gene of *Columba Livia* (GenBank: KP742481.1), which belongs to the same class of birds, was less homologous to the RIG-I gene of whooper swan. Compared with mammals and fishes, there are obvious branches of birds. It can be seen that there are great differences in homology between different species.

The mechanism of RIG-I resistance to avian influenza is that directly interacts with the nucleocapsid of influenza viruses to activate RIG-I. When the PB2 proteins of avian influenza have lower affinity for NP proteins, the exposure of 5' PPP dsRNA to RIG-I is enhanced. Because the residues H904, F910, K915, K918, K946 and K965, which are necessary for the binding of the c-terminal domain of 5' PPP dsRNA, are completely conserved in swan [26] *(Fig 3 black frame)*. RIG-I has the ability to sense avian influenza RNA. RIG-I adopts a 'closed' conformation at resting state, which shifts to an 'open' structure upon binding viral RNA, thereby exposing its CARD domai [27]. The residue F595 required for the tight connection between its

CARD and Hel2i is conserved in order to remain silent *(Fig 3 tellow frame)*. Walker A's ATP-binding motif *(Fig 3 red frame)* is also conserved in the RIG-I gene of swan as a ligand-dependent ATPase. Upon viral infection, RIG-I remains active, and RIG-Is' ubiquitination is induced by TRIM25 can effectively help RIG-I-MAVS interactions [28], ultimately initiating antiviral IFN responses. In birds such as mallard ducks and swan geese, the S8 residues of RIG-I proteins are phosphorylated by protein kinase c-α/β (PKC-α/β), which helps to maintain the inactive state of RIG-I. However, this mechanism in swan is changed, with the S62 residue being replaced by G62. It may have affected the phosphorylation of PKC-α at this site and altered the mode of active regulation of RIG-I. It makes RIG-I of swan less active for IFN induction, but the specific mechanism of effect requires further investigation. Whooper swan S222 (wS222) residues are conserved, suggesting that the pathway might be involved in different residues in different birds *(Fig 3 purple frame)*. Residues K221 and K247, ubiquitylated by TRIM25, are also present in duck RIG-I *(Fig 3 orange frame)*.

PKC-α/β plays an important regulatory role in RIG-I-mediated type I IFN responses. Conventional PKC-α-β-induced RIG-I phosphorylation and TRIM25-mediated RIG-I ubiquitination functionally antagonize each other to tightly regulate RIG-I CARD-mediated antiviral signaling. Although other pathways can produce IFN-α and IFN-β, type I interferon expression after avian influenza infection is largely dependent on RIG-I [29]. Studies have shown that TRIM25 can bind to the CARD domain of RIG-I [30,31], and REUL has a homologous domain pattern and regulates RIG-I in a similar manner. Unlike TRIM25, REUL mediated K154, K164, and K172 residues, and when CK226 residues were mutated, REUL-mediated ubiquitination was attenuated and the ability of RIG-I to induce antiviral signaling was attenuated [32] *(Fig 3 white frame)*. The T770, S854/855 residues in the RIG-I c-terminal domain are critical for phosphorylation regulation of human casein kinase II, but these residues are absent from RIG-I in swan. Thus, there is no process by which cells at rest are phosphorylated by casein kinase II (CK2), and silencing of CK2 enhances RIG-I-mediated antiviral effects compared with normal condition [33] *(Fig 3 pink frame)*. RNF122 interacts with the CARD domain of RIG-I, promoting the K-48 ubiquitin chains at K115 and K146 of the CARD domain and leading to degradation. In birds, both key sites are mutated so that there is no degradation of RIG-I [34] *(Fig 3 gray frame)*.

A significant limitation of this study is the small sample size, stemming from substantial challenges in sourcing suitable specimens. Our original plan required at least three healthy, fresh whooper swan samples to achieve the dual objectives of amplifying the RIG-I gene and investigating its expression profile across various tissues and organs. Procuring such samples, however, proved exceptionally difficult. Since the whooper swan is listed as a national Class II protected animal in China, it is illegal to obtain samples through hunting. During field collection, a significant number of individuals encountered succumbed to infectious diseases, potentially compromising the detection of RIG-I gene tissue expression. Therefore, it cannot be used in this experiment. This sample comes from the vicinity of the Sanmenxia Wildlife Rescue and Monitoring Center. Local residents discovered a stray dog attacking a large swan and immediately reported it to the center. Upon receiving the report, the rescue team swiftly rushed to the scene, but upon arrival, they found that the large swan had unfortunately died. Further analysis at the facility verified the non-detection of significant infectious diseases, including Avian influenza and Newcastle disease, with all other test results coming back negative. The collaborative agreement we signed with multiple national wildlife reserves may potentially allow us to access healthier and fresher specimens in the future. However, achieving this goal in the short term is not feasible. Once we obtain such quality specimens, we will replicate this experimental study to validate the findings.

This study first confirmed that the whooper swan carries a functionally intact RIG-I gene (the sequence has been submitted NCBI, GenBank: PP375561.1). It filled the blank of the lack of experimentally validated RIG-I gene sequences swan species and systematically analyzed its molecular characteristics and functions. Sequence analysis showed that the whooper swan RIG-I had an additional LCR compared with other reported avian RIG-I genes. Notably, based on the genomic prediction, the RIG-I sequences of the black swan *(Cygnus atatus)* and the mute swan *(Cygnus olor)* also contained a similar LCR [12-14,16], however, this finding was excluded from the discussion owing to the absence of experimental validation. Structural variants, known to influence genetic diversity and susceptibility to diseases in various organisms, might similarly impact the whooper swan's vulnerability to AIV. A specific amino acid mutation site S62G was discovered that the mutation might adversely affect the RIG-I-mediated immune response to AIV. Protein structure prediction revealed that the RIG-I of the whooper swan possesses the typical features of RIG-I structural domain composition, indicating that it has the basic molecular architecture as a pattern recognition receptor. However, limited by the status of the whooper swan as a national Class II protected animal in China, this study only obtained a single effective sample, resulting in insufficient sample size. We will actively supplement samples in the follow-up research to further verify the current findings.

Declarations

Availability of Data and Materials: The original data of the paper are available upon request from the corresponding author.

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RESEARCH ARTICLE

Transcriptomics Analysis Identifies Critical Genes Involved in the Infection of Crandell-Reese Feline Kidney Cell Lines by Feline Panleukopenia Virus

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Abstract

Feline panleukopenia virus (FPV) is an extremely contagious pathogen that induces severe vomiting, diarrhea, and dehydration in cats, often resulting in high mortality rates and substantial economic losses. Host responses are crucial for viral entry, replication, assembly, and disease progression, despite so much is not known, especially regarding the interactions between hosts and viruses. Herein, we employed the FPV strain FPV-XJ-04, which was previously isolated and characterized by our team in Xinjiang, China. To perform a comparative transcriptomic analysis of the gene expression profile in Crandell-Reese feline kidney (CRFK) cells following infection with the FPV-XJ-04 strain. Following infection of CRFK cells with the FPV-XJ-04 strain, the differentially expressed genes in CRFK cells were predicted to be involved in several key signaling pathways, including the TNF signaling pathway, IL-17 signaling pathway, cytokine-cytokine receptor interaction, Toll-like receptor signaling pathway, MAPK signaling pathway, and RIG-I-like receptor signaling pathway. In addition, the immune response was significantly enriched in the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Furthermore, we highlight 11 key genes in the TNF signaling pathway that are associated with host defense response to the invasion of FPV-XJ-04 strain. This research provides valuable insights into the gene transcription processes within immune cells, elucidating the pathways critical to the early stages of infection pathogenesis.

Keywords: Feline Panleukopenia Virus, Host-virus interactions, Immune response, Comparative transcriptomic analysis, TNF signaling pathway

INTRODUCTION

Parvoviruses, which belong to the family *Parvoviridae*, constitute a group of small, non-enveloped viruses with single-stranded DNA genomes. These viruses possess a linear DNA genome approximately 4.5-5 kb in length, characterized by hairpin structures formed by inverted terminal repeats (ITRs) at each end of the genome [1,2]. The remaining portion of the viral genome consists of two major open reading frames (ORFs) that encode both non-structural proteins (NS1 and NS2) and structural proteins (VP1 and VP2) within the same mRNA through alternative splicing [3,4]. Parvoviruses are currently widespread globally and capable of naturally infecting a diverse range of hosts [5-8]. Due to their rapid evolution and efficient transmission, the range of potential hosts for parvoviruses is continually expanding, posing significant threats to numerous endangered wild animal species and domestic pets [9].

Carnivore protoparvovirus 1 belongs to the *Protoparvovirus* genus within the *Parvoviridae* family and is characterized as a distinct viral species [1]. Notable members of this species include feline panleukopenia virus (FPV) and canine parvovirus (CPV) [10-12]. FPV is one of the deadliest viral pathogens in pets and can cause diarrhea, vomiting, and feline panleukopenia [13,14]. FPV has a wider host range and higher pathogenicity. In addition to domestic cats, FPV have been reported to infect monkeys [15], tigers [16], lions [17], and linsangs [18], causing significant economic losses. In recent years, FPV has emerged as a significant threat to companion animals, economically valuable species, and

wild fauna in China. Consequently, there is an urgent need for scientific research to curb the dissemination of FPV and to elucidate its molecular pathogenesis.

Host responses are crucial for viral entry, replication, assembly, and pathogenesis [19-21]. Despite significant progress, many aspects remain to be elucidated, especially regarding the intricate interactions between hosts and viruses. The non-structural protein 1 (NS1) of human parvovirus B19 can interact with AP-1 and AP-2 on the TNF-α promoter of the host to regulate the expression of TNF-α [22]; the NS1 protein of B19 can activate relevant factors in the endothelial cell inflammation signaling pathway, thereby affecting the host cell inflammation response [23]. The NS1 protein also plays a significant role in the host cell apoptosis process, and studies have shown that the B19 virus NS1 protein induces red blood cells to arrest in the G1 phase, leading to apoptosis [24]. Other interactions between the virus and the immune system include immune evasion and suppression of potent innate immune responses. In the context of FPV infection, it has been observed that the NS2 protein of FPV interacts with the host cell's TANK-binding kinase 1 (TBK1) molecule, thereby antagonizing the binding of TBK1 to stimulator of interferon genes (STING). This interaction reduces the phosphorylation of downstream STING and interferon regulatory factor 3 (IRF3) molecules, ultimately preventing the activation of IFN-I transcription [25]. Insight into these modulations will further elucidate the pathogenesis of these viruses.

Few studies have investigated cellular host responses to FPV infection, especially using clinical isolates. Transcriptome studies based on next-generation sequencing have the potential to elucidate cellular responses following viral infection, thereby providing further insight into viruses' pathogenesis. Analyzing the transcriptome of infected cells can provide valuable information on virus replication and host-pathogen interactions [26]. Therefore, the present work was conducted to investigate the role of mRNA in the immune defense of host cells against FPV infection. We used RNA-seq to profile mRNA expression in two groups of feline kidney cells: FPV-infected and uninfected, and identified many differentially expressed transcripts. The primary objective of this study was to identify the differentially expressed transcripts and determine mRNA expression patterns during FPV-XJ-04 infection of CRFK cells, elucidating the associated pathways and biological processes.

Material and Methods

Ethical Statement

This study did not involve any human participants, animal experiments, or the use of personal data. Therefore, ethical approval was not required. All procedures were carried out in accordance with the relevant laws and regulations.

Viruses and Cell Culture

Crandell-Reese Feline Kidney (CRFK) cell lines were purchased from Pricella Biotechnology Co., Ltd. (Wuhan, China) and the cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA) containing 5% fetal bovine serum and 1% penicillin-streptomycin at 37ºC with 5% CO_2. FPV strain (FPV-XJ-04) was originally isolated from feces of infected cats in a pet hospital of Xinjiang, China. Initially, the fecal samples were confirmed positive for FPV using a colloidal gold strip test. Subsequently, the fecal material was suspended in cell culture medium, centrifuged, and the resulting supernatant was filtered through a 0.22 μm filter membrane. The filtered supernatant was then inoculated into CRFK cells for blind passage up to the fifth generation, during which cytopathic effects (CPE) became evident. Viral DNA was extracted from individual fecal samples utilizing the TIANamp Virus DNA Kit (TIANGEN, China). The extracted DNA served as the template for detecting FPV using a set of universal primers (FPV-F: 5′-TAACTCCTCTGACTCCGGAC-3′; FPV-R: 5′-ACCACCGTCTGGTTGAACTG-3′). These primers amplify a 750 bp fragment within the FPV genome, specifically covering nucleotides 2,062 to 2,819 [27]. The supernatant from the FPV positive sample was sterilized using a 0.22 μm filter membrane. Subsequently, the filtered supernatant was inoculated into CRFK cells and incubated at 37ºC in a 5% CO_2 atmosphere. The CPE-positive cell cultures were subjected to centrifugation at 28.000 × *g* for 12 min. Subsequently, the samples were negatively stained with 0.5% phosphotungstic acid and examined using transmission electron microscopy (TEM). To further characterize the harvested virus, an indirect immunofluorescence assay (IFA) was performed. In this assay, murine polyclonal antibodies specific to FPV were employed as the primary antibody, while FITC-conjugated goat anti-mouse IgG (H&L) served as the secondary antibody. Finally, the cells were washed, mounted on glass slides, and examined under a fluorescence microscope.

Growth Characterization of the Isolated Virus

To evaluate the viral growth kinetics, CRFK cells were infected with the isolated parvovirus at a multiplicity of infection (MOI) of 0.01. Supernatants from the cell culture medium were harvested at 12 h intervals from 0 to 72 h post-infection (hpi). Viral titers ($TCID_{50}$/mL) were determined using endpoint dilution assays with CRFK cells. Each experiment was performed in triplicate independently, and the results are expressed as the mean ± standard deviation.

RNA-seq and Data Analysis

In the investigation of host transcriptomic responses, CRFK cells were infected with FPV-XJ-04. and CRFK

Table 1. Primers of qRT-PCR used for this work

Primer	Sequence
GAPDH-F	TGGAAAGCCCATCACCATC
GAPDH-R	ACTCCACAACATACTCAGCACCA
TNFAIP3-F	CTACCAACGGGATCATTCAC
TNFAIP3-R	TTCTCGACACCAGTTCAGCT
NFKB1-F	CAATCCAGAAATATTTCAACCA
NFKB1-R	TTCACTAGACGCACCCGG
IL6-F	CTCCTGGTGGTGGCTACT
IL6 -R	CAGAGATTTTGCCGAGGA
IFNB1-F	TTGCCTCAAGGACAGGAT
IFNB1-R	ATCCCGTGCTAGAGGTGC
MAP3K8-F	GTGAAGAGCCAGCGGTTT
MAP3K8-R	ACGGAGGACAACCAAGGC
MAPK8-F	ATGAGCAGAAGCAAGCGT
MAPK8-R	CGGCTCAGCTTCTTGATT
TRAF1-F	GTCTGTAAGCCCAGGAAGCC
TRAF1-R	TTGGGCTCCCCTTGTAGG
TNF-F	CAGGGCTCCGGAAGGTG
TNF-R	TGCAGGCCATGTGGGAG
TRAF3-F	CGCTGAAGCTGCACCCT
TRAF3-R	CTCCGTCTGCTTCGGGT
MAP3K14-F	CAGGCGATGGGCAAGAA
MAP3K14-R	AGATGGCGGCCAGTCCT
IRF1-F	TCCAACCAAATCCCAGG
IRF1-R	CTTTTCCCCTGCTTTGT
NFKBIA-F	GAGCACGCCCAGGACTG
NFKBIA-R	CCGCAGCTCCTTCACCA

cells were cultured in six-well plates (Corning, USA) and divided into two groups, each containing three replicates, for the construction of mRNA libraries. The first group was inoculated with FPV-XJ-04 at a concentration of 1×10^6 $TCID_{50}$, while the second group served as an uninfected control. At 24 h post-infection, total RNA from both groups was isolated using Trizol reagent (Invitrogen, CA, USA) according the manufacturer's instructions. The RNA quality was evaluated via the Bioanalyzer (Agilent, Santa Clara, CA, United States) and sent (1 μg per sample) for mRNA sequencing to Novogene, Inc. (Sacramento, CA, United States). Then, the high-quality clean reads were compared with the specified reference genome by using Bowtie software. The Padj ≤0.05 and the absolute value of $\log_2$ ratio ≥2 were used to identify DEGs. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases were used to analyze the pathways.

Quantitative Real-time PCR

To validate the findings from the RNA-seq experiment, a set of 12 genes associated with the TNF signaling pathway were selected for further analysis through quantitative real-time PCR (qRT-PCR). Total RNA was extracted from both uninfected and FPV-infected CRFK cells, cDNA synthesis was performed using 1.0 μg of the extracted total RNA, in accordance with the protocol provided by the reverse transcription kit manufacturer (RIBOBIO, China). The primers are listed in *Table 1*. The qRT-PCR conditions included a pre-incubation step at 95ºC for 5 min, followed by 40 amplification cycles (95ºC for 30 sec, 58ºC for 30 sec, and 72ºC for 30 sec). Each sample was analyzed in triplicate using a 20 μL reaction mixture containing 2× SYBR Premix Ex Taq II (Takara, USA). The reactions were performed on a Roche Light Cycler 480 II system (Basel, Switzerland), with each reaction containing 100 nM of each primer and 1 μg of cDNA template. All experiments were conducted in triplicate.

Statistical Analyses

When comparisons were made, a Student's *t*-test was performed and *P* value <0.05 was considered statistically significant. The data was presented as "mean ± SD".

Result

Characterization of the FPV-XJ-04 Strain

The PCR analysis using the extracted viral DNA demonstrated a prominent and bright band at 750 bp *(Fig. 1-A)*, indicative of a positive result. To further verify the infection status, fecal samples were inoculated into CRFK cells in an effort to the virus. As shown in *Fig. 1-B*, after five blind passages in CRFK cells, CPE induced by parvovirus became evident, manifesting as cell rounding, pyknosis, disruption of the monolayer, and eventually complete necrosis. By the tenth passage, the viral titer of the nonclonal virus population, as measured by endpoint dilution assay, had increased to 1×10^6 $TCID_{50}$/mL. TEM analysis of negatively stained and purified cell supernatant showed spherical particles with an average diameter of about 20 nm, which is consistent with the characteristic morphology of parvoviruses *(Fig. 1-C)*. The growth kinetics of the resulting clonal virus, named FPV-XJ-04, were subsequently analyzed. The results showed that viral replication initiated steadily and reached its peak at 60 h post-infection *(Fig. 1-D)*.

Identification and Characterization of mRNAs in FPV-XJ-04 Infected CRFK Cells by RNA-seq

To investigate the cellular host responses to FPV-XJ-04 infection, we conducted a transcript profiling experiment using RNA-seq technology. Gene expression profiles from both infected and control samples were compared, with

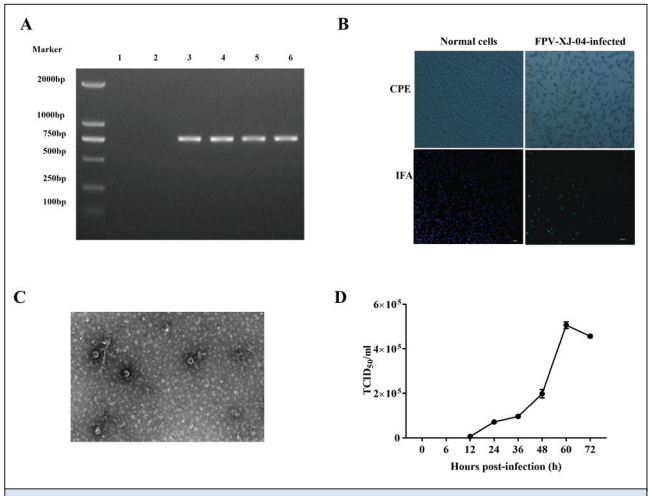

Fig 1. Identification and characterization the FPV-XJ-04 strain. **(A)** PCR products were analyzed by 1.5% agarose gel. Marker, DL 2000 DNA marker; lane 1, 2, nucleotide-free water (negative control), lane 3-6, the FPV-XJ-04 strain, **(B)** The cytopathic effect (CPE) and IFA identification, **(C)** Morphology of FPV-XJ-04 particles exhibited with negative-stained transmission electron microscopy, **(D)** Growth curve of FPV-XJ-04 as measured by end-point dilution. Each datapoint shows averages of three independent replicates, and standard deviations are indicated as error bars

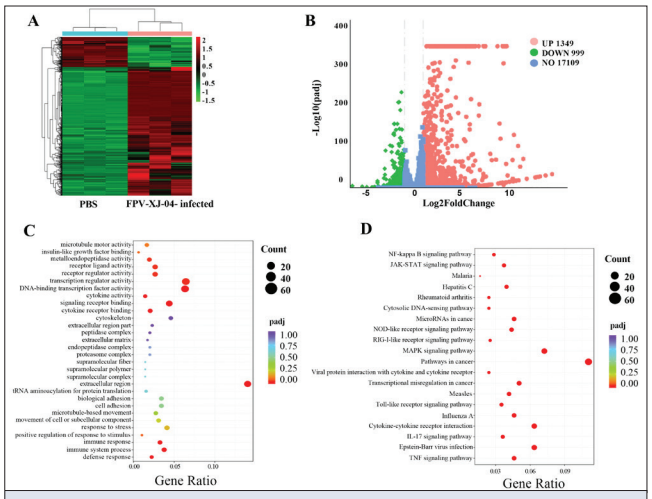

Fig 2. Expression of differentially expressed genes (DEGs) between the CRFK cells infected with FPV-XJ-04 and control group. **(A)** The heatmap shows the expression levels of DEGs between the the CRFK cells infected with FPV-XJ-04 and control group, **(B)** Volcano plot of expressed genes between CRFK cells infected with FPV-XJ-04 and control group. The red, green, and blue denote upregulated, downregulated, and non-regulated genes, respectively, **(C, D)** GO and KEGG analyses of the differentially expressed genes (DEGs) between the CRFK cells infected with FPV-XJ-04 and control group. The rich factor represents the ratio of upregulated genes differentially expressed gene numbers annotated in this pathway term to all gene numbers annotated with this pathway term. A greater rich factor indicates a greater degree of pathway enrichment

expression levels analyzed using the Illumina HiSeq™ 2000 platform. Cluster analysis was applied based on expression patterns to group the samples, revealing potential relationships among them *(Fig. 2-A)*. Our comparative transcriptomic analysis identified 2,348 differentially expressed genes (DEGs) with Padj ≤0.05 and fold change ≥1, of which 1,349 genes were upregulated and 999 genes were downregulated.

Clustering and Functional Enrichment Analysis of Differentially Expressed Genes

The Volcano Plot is presented in *Fig. 2-B*. To investigate the distribution of candidate target genes and elucidate their diverse functions, we employed GO and KEGG pathway analyses to identify the enriched target genes. We performed a GO enrichment analysis to explore the host cell' biological processes in responding to FPV. The defense response, immune system process, positive regulation of response to stimulus, cell adhesion, biological adhesion, cytokine receptor binding, signaling receptor binding, and cytokine activity were the dominant groups in all three DEG sets *(Fig. 2-C)*. Based on KEGG pathway enrichment analysis, most upregulated genes involved TNF signaling pathway, Cytokine-cytokine receptor interaction, IL-17 signaling pathway, Toll-like receptor signaling pathway, NOD-like receptor signaling pathway, RIG-I-like receptor signaling pathway, MAPK signaling pathway, and NF-kappa B signaling pathway *(Fig. 2-D)*. In the GO and KEGG analysis, immune signaling is the main pathway involved in the FPV infection process *(Fig. 2-C,D)*.

Validation of Selected mRNAs

Based on the above results, we hypothesize that immune-related genes and signaling pathways are likely involved in the FPV infection process *(Fig. 3-A,B)*. To validate this hypothesis, total RNA isolated from FPV-infected and uninfected CRFK cells was subjected to qRT-PCR analysis to confirm the differentially expressed mRNA candidates *(Fig. 3-C)*. Previous studies have indicated that the TNF signaling pathway play a central role in virus infection process. The gene expression changes related to

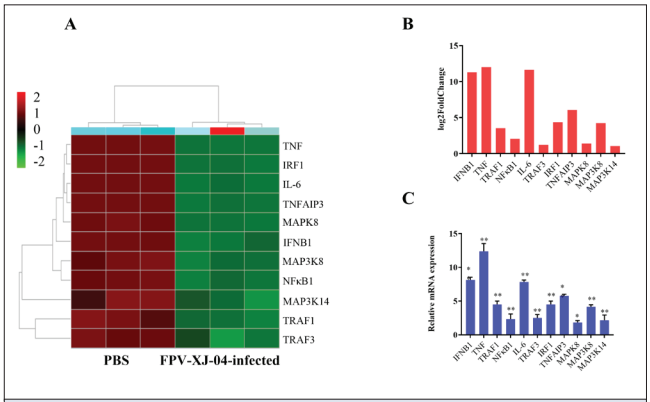

Fig 3. Differentially expressed genes (DEGs) were evaluated by quantitative reverse transcription PCR (qRT-PCR) assays between the CRFK cells infected with FPV-XJ-04 and control group. **(A)** The heatmap shows the expression levels of 11 key genes in the TNF signaling pathway between the CRFK cells infected with FPV-XJ-04 and control group, **(B, C)** The 11 key genes in the TNF signaling pathway expression levels were further detected by qRT-PCR. The results at each time point are expressed as the means ± standard deviations from at least three independent experiments

TNF signaling pathway were assessed to explore whether this pathway can respond to FPV infection process. Specifically, the major upregulated genes involved in the TNF signaling pathway included TNFAIP3, NFKB1, IL6, IFNB1, MAP3K8, MAPK8, TRAF1, TNF, TRAF3, MAP3K14, IRF1. The expressions of these genes using qRT-PCR were in good agreement with the RNA-seq results *(Fig. 3)*.

Discussion

Messenger RNA (mRNA) plays a pivotal role in the regulation of gene expression by participating in the entire process of protein synthesis, thereby serving as a critical link between DNA and proteins. Proper mRNA synthesis and degradation are indispensable for maintaining cellular functions and organismal homeostasis. Moreover, the roles of mRNA in the host's antiviral response have been increasingly elucidated through ongoing research [28]. Recent research indicates that, CRFK cells which are permissive for productive replication of virus, showed induction of a large network of immunological and virally induced pathways, other interaction between viruses and hosts involves a complex interplay between the host's antiviral response and the virus's immune evasion strategies [29].

The isolation and characterization of FPV-XJ-04 were systematically validated through molecular, cytopathic, morphological, and kinetic analyses. The PCR detection of a 750 bp amplicon strongly indicated FPV DNA, targeting conserved regions of the viral genome nucleotides 2,062 to 2,819 [27]. Successful virus isolation in CRFK cells was confirmed by progressive CPE, including cell rounding and necrosis, alongside a rising viral titer, reflecting efficient *in vitro* adaptation. TEM visualization of ~20 nm spherical particles provided definitive morphological evidence consistent with parvoviruses, excluding other pathogens. Growth kinetics show that the virus reaches its replication peak at 60 h after infection, which might be slightly delayed due to unique virus-host interactions or replication kinetics. The gradual titer increase aligns with the lytic nature of autonomous parvoviruses, which depend on host cell cycles.

Furthermore, we identified distinct expression profiles between FPV-XJ-04 infected CRFK cells by RNA sequencing and comparative transcriptomic analysis. RNA-seq analysis identified 2,348 DEGs (1,349 upregulated, 999 downregulated) in FPV-XJ-04-infected cells, reflecting extensive transcriptional reprogramming. Upregulated genes were enriched in immune pathways (e.g., TLR, RIG-I, cytokine signaling), aligning with GO/KEGG findings and indicating a robust antiviral response driven by innate immunity and inflammation. Downregulation of genes may reflect viral evasion via suppression of non-essential processes or metabolic rewiring. The clear clustering of infected samples and control samples highlights the systemic interaction between the host and the pathogen. Future studies should prioritize functional validation of key DEGs (e.g., cytokines, immune receptors) to elucidate their roles in viral control or pathogenesis. To further investigate the biological functions of these genes, the DEGs were mapped to the GO and KEGG pathways. The associated cellular processes discussed below could be classified into several main sections: TNF signaling pathway, IL-17 signaling pathway, and MAPK signaling pathway. We summarized the above analyses and formed a gene change model of FPV-XJ-04 infected CRFK cells.

Transcriptome analysis indicated that FPV infection significantly activated the host's TNF-α and IL-17 signaling pathways. The TNF-α signaling pathway and the IL-17 signaling pathway are associated with multiple immune and inflammatory pathways [30-32]. TNF-α is a major pyrogenic cytokine produced by immune cells during inflammatory responses and the acute phase of infection, and is closely related to the cytokine storm [33]. During viral infections, the expression level of TNF-α is correlated with the severity of the disease. In addition, TNF-α has been reported to stimulate the replication of human polyomavirus in neural cells [34]. Mumps virus infection disrupts the blood-testis barrier by inducing TNF-α [35]. In our research, we found that the genes related to the TNF signaling pathway, including TNFAIP3, NFKB1, IL6, IFNB1, MAP3K8, MAPK8, TRAF1, TNF, TRAF3, MAP3K14, IRF1, and NFKBI, were significantly upregulated after the infection of CRFK cells. Whether the production of TNF-α can stimulate viral replication has not been confirmed, but the induction of TNF-α production by FIPV has been reported [29].

The IL-17 family is considered highly relevant to infectious diseases occurring in epithelial sites [36]. Research on the role of IL-17 in regulating viral infections is also ongoing, where it plays multiple key roles. The cytokine IL-17 may have completely opposite effects in different circumstances. During systemic viral infections, excessive production of IL-17 can lead to liver damage and death. The level of IL-17 during viral infection is closely related to the severity of the disease, and at this time, IL-17 plays a pathological damage role in this disease. This phenomenon has been reported in viruses such as influenza virus, dengue virus, and respiratory syncytial virus [37-39]. It has been reported that IL-17 plays an important role in virus-induced acute lung injury during the H1N1 pandemic. Treating H1N1-infected mice with IL-17 monoclonal antibodies significantly improved the acute lung injury caused by the virus [40]. At the same time, the induction of IL-17 has been reported to antagonize the broad-spectrum antiviral response of monocyte chemoattractant protein-induced protein 1 (MCPIP1) in PRRSV [41]. These results suggest

that FPV infection triggers a host immune storm by activating the classical pro-inflammatory pathway, and its dynamic balance may determine the infection outcome (protective immunity or pathological damage). It is necessary to further analyse its spatiotemporal regulatory mechanism and its relationship with viral replication.

The RNA-seq and qRT-PCR analyses collectively demonstrate significant activation of the MAPK signaling pathway during FPV infection. Key upstream regulators (e.g., MAP3K8, MAPK8) and downstream effectors (e.g., NF-κB, IL-6) within this pathway were markedly upregulated, suggesting its critical role in amplifying pro-inflammatory responses via cytokine production (e.g., TNF, IL-6) and immune cell activation [42]. MAPK signaling likely intersects with TNF and IL-17 pathways to form a synergistic network [43], driving NF-κB-mediated transcriptional activation and exacerbating inflammatory cascades [44]. While this hyperactivation may enhance antiviral defenses, sustained MAPK activity could also contribute to tissue damage or viral immune evasion. Further studies should delineate whether MAPK signaling primarily facilitates host resistance or is exploited by FPV to promote replication, potentially identifying therapeutic targets to modulate this pathway during infection.

Overall, this research shows a series of genes involved in FPV infection and provides a scientific reference for exploring some genes which may aggravate or inhibit virus replication.

Declarations

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

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RESEARCH ARTICLE

Immunomodulatory Potential of Sugar Beet *(Beta vulgaris)* Against Coccidiosis in Broiler Chickens

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Abstract

Recent research reports the immunomodulatory capability of *Beta vulgaris* extract against coccidiosis disease in broiler chickens. Immunomodulatory potential of medicinal plant was evaluated by *in vivo* trial. For this purpose, broiler chicks (n=175) were alienated into five groups. At one week of age chicks all groups were orally infected with parasite (oocysts) of mixed *Eimeria* species. At same age group, A, B and C were cured with Beta vulgaris extract at three doses (100, 200 and 300 mg/kg) of body weight. Group D was treated with Vitamin E and served as positive control and Group E was treated with PBS and served as negative control groups. Cellular Immune response was inquired through four classical assays including Dinitrochlorobenzene (DNCB), PHA-P, CON-A and Carbon Clearance test. Humoral immunity (antibodies levels) was evaluated by hemagglutination test. Results of study showed that *B. vulgaris* extract treated groups showed maximum immune response in terms of increasing both cellular and humoral immunity against *Eimeria* parasite. However, the immunomodulatory response of groups cured with *Beta vulgaris* at 300 mg/kg of body weight was higher ($P<0.05$) as compared to negative control group.

Keywords: Sugar Beet, *Eimeria*, Poultry, Medicinal Plant

INTRODUCTION

The poultry industry is experiencing pressure since parasitic disorders referred to as "hidden enemies" cause chronic and sometimes severe losses without visible signs. The poultry sector is facing difficulties in progress due to parasitic diseases [1]. Among them, poultry coccidiosis is affecting poultry industry at larger scale and each year, huge cost is invested a for prevention, cure and productivity reduction arising from coccidiosis disease in poultry. For coccidiosis in United States, the approximate annual cost has been estimated to be more than USD $ 127 million [2], while in China the cost has been estimated to be over USD $ 73 million [3].

Coccidiosis is caused by *Eimeria* parasite having many species. *Eimeria* species are localized in certain regions of the broiler digestive system [4-6]. Seven *Eimeria* species are known to cause coccidiosis in chickens but *E. tenella* and *E. necatrix* are is the most pathogenic, cataclysmic in the production of broiler chickens and all these species are dissimilar concerning their virulence and every one of

them impacts various areas of the intestines [7,8]. Infection with *Eimeria* disrupts host mucosal cells resulting in disruption of mucosal integrity, cell permeability and loss of nutrients and proteins from the infected cells. This further leads to poor digestion and assimilation of proteins and other nutrients both major influential factors in subclinical and clinical signs of coccidiosis [9].

Some of the factors promoting the occurrence of coccidiosis include a direct cycle whereby the parasite is spread through direct contact, coprophagic transmission, the occurrence of resistant oocysts, non-susceptibility of a different species of *Eimeria*, a high rate of reproduction of oocysts, high stocking density and environmental factors (sporulation) [10]. The largest cost is the subclinical coccidiosis, which made up to three fourths of the overall costs. This is defined by low flock efficiency resulting from high feed consumption and reduced BWG [11]. Since 1939, synthetic anticoccidial drugs and ionophore drugs are used against coccidiosis. However, drug resistance impacts consumers through drug relatives in poultry products and presently it is not dependable for control [12-14].

Fortunately, the immune response generated is long-lived and strong after *Eimeria* infections and therefore vaccination becomes an option for control since treatment requires anticoccidial drugs. However, vaccines are not effective against all *Eimeria* species and poorly managed can reduce flock's performance [15].

Among novel compounds medicinal plants have shown therapeutics effects against different diseases of poultry and are appealing researchers to explore them [16-18]. Among alternatives, botanicals and herbal substances have shown better anticoccidial impacts among these choices [19,20]. Botanicals that are known to be rich in antioxidant compounds including *Camellia sinensis*, *Ageratum conyzoides*, *Vitis vinifera, Sideritis scardica and* Saccharum *officinarum* have been accounted to show excellent anticoccidial and immunomodulatory impacts counter to avian coccidiosis. *Beta vulgaris* is a well-renowned plant that has various antioxidant properties and have medicinal properties [21,22].

Beta vulgaris is well known plant for its therapeutic and medicinal effects in poultry and livestock. While *Beta vulgaris* contains betaine as its main active, other important compounds include flavonoids, alkaloids, terpenoids, steroids, tannins, saponins which have diverse antioxidant and therapeutic potential against disease of animal and public health concern [23]. In view of the possible restorative impacts current research was conducted to investigate its immunomodulatory potential of *Beta vulgaris* against *Eimeria* infection in experimental broiler birds.

Material and Methods

Ethical Statement

This research was initiated with the approval of the Ethics Committee of Agriculture Faisalabad under PSF, Project No. 185, and PARB, Project No. 358 (No. 628/6-08-2013).

Preparation of Plant Extract

Beta vulgaris roots were purchased from local market of Faisalabad and were authenticated by botanist of Department of Botany and extraction of plant material was done following method [24] using Soxhelet Apparatus and then methanolic extract was stored at 4°C for further experimental use.

Collection and Preservation of Parasite

Guts infected with *Eimeria* parasite were collected from various outbreak places in field and afterward were examined in department of Parasitology. *Eimeria* oocysts were isolated and preserved in 2.5% potassium dichromate arrangement involving the standard protocols as described by [25].

Experimental Design

One hundred and seventy five Hubbard day old broiler chicks (Big Bird®) were bought from local hatchery and were raised under well managed system. Adequate ventilation and water were provided. Temperature, during the first week of age, was maintained at 85-90°F; however, it was reduced on weekly basis by 5°F. Light was provided for 24 h throughout the experimental period. Humidity level of environment was 60-70% as required for rearing of birds. Vaccination against Newcastle Disease, Infectious Bronchitis and Infectious Bursal Disease was done as per schedule in broiler chickens. Standard feed excluding anticoccidial additives were offered to birds *Table 1*. Feed and water was provided *ad libitum.*

Table 1. *Composition of feed offered to experimental chicks*

Ingredients	Percent/ Level
Corn	50.00
Rice	12.00
Rice polishing	3.00
Soybean meal	12.00
Canola meal	12.00
Fish meal	3.00
Corn gluten meal 60%	3.00
Molasses	4.00
DCP	1.00
Premix	0.68
DL-methionine	0.12
L–lysine	0.20

For *in vivo* trial, chicks (n=175) assigned to immunomodulatory experiment were subdivided into five equal (n=35) subgroups, i.e., A, B, C, D and E. Of the total 35 infected chicks in each group, 20 and 15 were used for investigations on cell mediated and humoral immunity respectively. At one week of age all groups were orally infected with 50.000 sporulated oocysts of mixed *Eimeria* species. At two weeks of age (14th day), A, B and C were treated with *Beta vulgaris* extract at three doses (100, 200 and 300 mg/kg) of body weight. All doses of plant extracts were dissolved in PBS and administered orally by using soft plastic tube attached with 05 mL sterile syringe. Chicks in subgroups D treated with commercially available preparation of Vitamin E at 87 mg/kg of body weight in diet. Chicks in group D were treated with PBS (1 ml/bird) which was injected in intra-digital space of chicks.

Group D treated with Vitamin E served as positive control and Group E treated with PBS served as negative control groups. The respective treatments were continued for three consecutive days. The experiment was completed in 40 days and all birds were decapitated at the end of the experiment.

Immunological Evaluation

Evaluation of Cell Mediated Immunity: Cellular Immune response was inquired through four classical assays including Dinitrochlorobenzene (DNCB), PHA-P, CON-A and Carbon Clearance tests and their detail is described as below.

Dinitrochlorobenzene Test: It was used to examine the delayed-type hypersensitivity reaction following [26]. Briefly, on day 14 of the experiment, a primary dose (0.1 mL) of 2% DNCB in acetone was applied on 4 cm^2 area on the skin of each of the five chicks followed by a secondary dose on day 21 of the experiment (7 days post primary dose). Skin thickness (mm) was measured using a vernier caliper pre and 24 h post-application of DNCB both after the primary (day 14) and secondary (day 21) exposure to DNCB.

Phytohemagglutinin-P Test: It intradermally was infused in the chick's foot in internal spaces where as a similar treaty was followed for infusion of PBS in left foot (control group). The screw Guage was utilized to estimate the skin depth at various time spans (hours) post PHA-P infusion.

Cocanavalin-A Test: Cell mediated response to CON-A was evaluated by using standard protocols as described by [27]. For this purpose, blood was collected for separation of peripheral blood lymphocytes for Concanvalin-A (CON-A) test on day 14 and 21 of experiment from experimental chicks (n=5).

Carbon Clearance Assay: Carbon clearance indexed was performed in infected chicks by method as reported by [28]. Carbon readers were used in various groups by involving standard protocols. Optical Density (OD) values were measured at 460 nm in ELISA reader.

Evaluation of Humoral Immunity

Microplate Hemagglutination test was utilized for estimation of antibodies by following [29]. Total antibodies Titers (Igs) and immunoglobulins levels of IgG and IgM were also monitored in birds.

Statistical Analysis

ANOVA and DMR tests were used for calculation of statistical significance among different groups using SAS software.

Results

Cellular Immune Response

Higher cellular immune response to PHA-P antigen was seen in chicks treated with *B. vulgaris* (P<0.05) extract as compared to infected group and higher immune response was observed to CON-A antigen (P<0.05) in *(Fig.1)*. Carbon clearance index was same to that of Vitamin E (P>0.05) and was significantly different to infected groups (P<0.05) and cell mediated response was recorded at various time periods of DNCB and was comparable to Vitamin E (P>0.05) in *(Fig. 2)*.

Humoral Immune Response

Prominent antibody response was detected in chick's treated with *B. vulgaris* extract at highest dose and immune response was same to that of Vitamin E (>0.05)

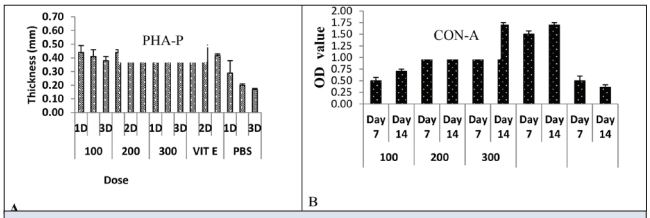

Fig 1. A: Cellular immune response to PHA-P antigen, **B:** Cellular immune response to CON-A antigen. ***VIT E:*** Vitamin E, ***PBS:*** Phosphate-Buffered Saline, ***OD:*** Optical Density

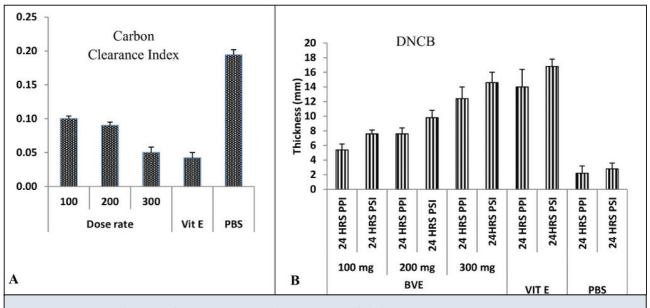

Fig 2. A: Carbon Clearance Index, **B:** Cellular immune response to DNCB. ***VIT E:*** Vitamin E, ***PBS:*** Phosphate-Buffered Saline

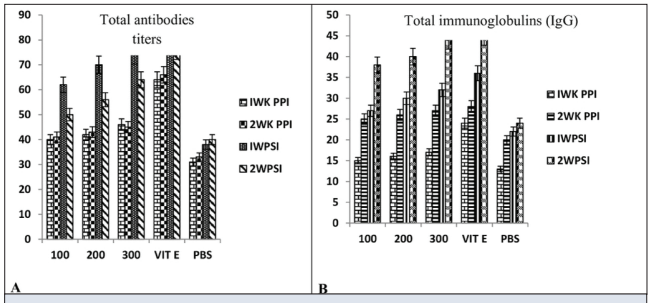

Fig 3. A: Total antibody titers, **B:** Total immunoglobulins (IgG). ***VIT E:*** Vitamin E, ***PBS:*** Phosphate-Buffered Saline

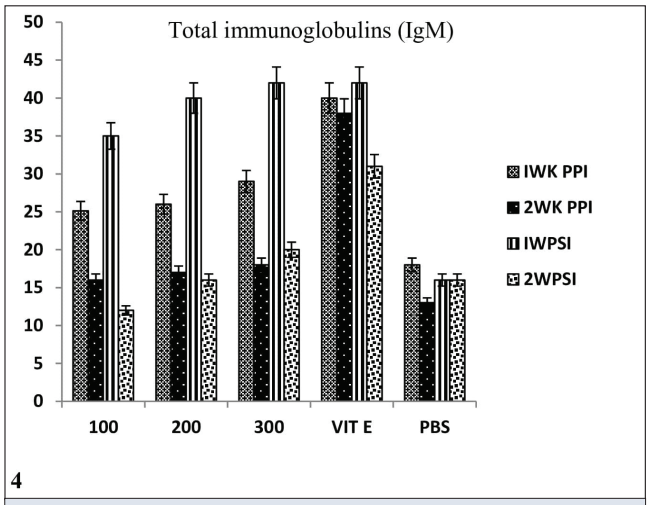

Fig 4. Total immunoglobulins (IgM). ***VIT E:*** Vitamin E, ***PBS:*** Phosphate-Buffered Saline

and elevated Immunoglobulins-G antibody levels were detected in chick's accomplishment the at highest dose of *B. vulgaris* extract and immune response was same to that of Vitamin E in *(Fig. 3)*. Elevated Immunoglobulins-M antibody levels were seen in chick's accomplishment the *B. vulgaris* extract at highest dose and immune response was similar with Vitamin E treated group in *(Fig. 4)*.

Discussion

Botanicals and their compounds have been promising in controlling the pathogenesis of avian coccidiosis as they possess antioxidant and other effective compounds. These compounds provide protection level against coccidiosis and improve cellular and humoral immunity [30,31]. More than 1200 plant species have been reported to possess medicinal and antiprotozoal properties. Plant and their products are included in poultry diets due to growth promoting and natural enhancer impact on bird's immunity [32,33].

Currently research has been focused on use and consumption of herbal product through noting them to be safer approaches in the control of various diseases to reduce drug resistance and toxic effects of drugs. Herbal anticoccidial agents can reduce severity of coccidiosis disease and has positive effect on avian health [34]. Systematic review evidence suggests that medicinal plants have the ability to minimize antibiotic and antiprotozoal inputs in poultry. From results obtained from one study *O. vulgare, C. sativum, A. annua* and *B. pilosa* are suitable plant species for prevention or treatment of bacterial and protozoal infections in poultry. The heterogeneous effect of plants is attributable to variations in dosage and phytochemical contents of the material used on the results of the systematic review. The researchers have already suggested almost 15 years ago a description of used herbal preparations for human clinical trials investigating medicinal plants and similar recommendations should be applied in the further trial's livestock poultry in particular. The lack of patentability for the phytogenic feed additives may be filled by the phytochemical fingerprints alongside that some general descriptions and assessments of the used plant material [35].

In this study, *B. vulgaris* extract exhibited tremendous immunomodulatory activity against *Eimeria* parasite. Similar types of dose dependent trends have also been reported in previous studies on evaluation of immunomodulatory potential of different herbal extracts [36,37]. Plants driven compounds upgrade cellular resistance by expanding by following up on insusceptible cells by their multiplication and furthermore promote antibodies levels [38]. *Carthamus tinctorius* which is generally known as sunflower have displayed to upgrade cell and humoral insusceptibility counter to poultry coccidiosis [39]. *Triticum aestivum* (wheat bran) polysaccharides (arabinoxylans) have promoted immunomodulatory and protective effects to counter to disease due to *Eimeria* parasite in chickens. It additionally produced positive effects on organ weight gain infected birds.

In another study, *Saccharum officinarum* (sugar cane) extract also produced comparative kind of immunomodulatory impact counter to coccidiosis in chickens [40]. In recent study, it reported the immunomodulatory effects of *Carica papaya* extract and results showed that *Carica papaya* improved cellular and humoral immune response in broiler chickens [21].

B. vulgaris (sugar beet) have a role in improving intestinal health and showed excellent anticoccidial effects against experimental infection in chickens as evidenced by reduced oocyst count, lesion score and reduced mortality. It also improved the hematological and serological parameters of birds. *B. vulgaris* is well known for its antioxidant and exhibited immunomodulatory effects in mice [41].

Rosmarinus officinalis and *Thymus vulgaris* are considered as the most used plants in the Algerian pharmacopoeia and contain a great number of phytochemicals against coccidiosis. By the multi-pharmacological effects of their co-products, they can successfully prevent and treat coccidiosis and mitigate the negative impacts on the host's immune response, redox potential, and gut microbiota

due to the *Eimeria* life cycle in many experimental and field trials. The effectiveness of their application in feeds may be different [2].

Furthermore, treatment with the mixture of the medicinal plants can mitigate the impact of coccidiosis in broiler chickens, but this was appreciated in comparison to sulfaclozine. More studies may be required to access the analyzed combination of herbal extracts, to determine the processes by which various compounds have an impact [42]. In another recent research reported the anticoccidial effects of *Illicium verum* (star anise) essential oil in broiler chicks. *Illicium verum* essential oil reduced oocyst count and improved intestinal health of broiler chicks infected with mixed *Eimeria* species [43]. The similar type of Immunomodulatory effects of *Artemisia brevifolia* extract against coccidiosis in broiler chicken. *Artemisia brevifolia* extract improved cellular and humoral immune response in chicks infected with mixed *Eimeria* species [1].

The current research concludes that *Beta vulgaris* has immunomodulatory potential against ccoccidiosis. However, further studies are needed to conduct research and formulate novel drug against *Eimeria* parasite as alternative to synthetic anticoccidial drugs being used in poultry.

Declarations

Availability of Data and Materials: The data will be provided by the main author (A. Abbas) on requirement.

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Competing Interest: There is no dispute of interest between all authors

Generative Artificial Intelligence (AI): No AI tool is used in this manuscript.

Author Contributions: AA anticipated the study; KH, MTA, SS, HS and MMM helped in methodology, work plan, and statistical analysis writeup.

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Research Article

U-Net-Based Approaches for Biometric Identification and Recognition in Cattle

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Abstract

Animal welfare is a factor that directly affects productivity and is one of the cornerstones of sustainable agriculture and animal husbandry practices. Traditional identification methods cause animal stress and create opportunities for theft and fraud. This is because conventional identification methods, unlike biometric methods, do not use the animal's natural features; therefore, the identity can be more easily copied or imitated. To minimize these problems and enhance animal welfare, this study proposes a computer-aided animal identification and recognition system using retina biometrics. In this study: i) Experts manually segmented 80 RGB cattle retinal images. ii) The images were augmented using various angles, generating 540 images. iii) An identification system was developed using the U-Net, SA-UNet, and U-Net++ deep learning models. iv) The performance of the developed identification system was measured for both the original and augmented datasets using the Dice coefficient and IoU. The study's findings show that the identification system's most successful model was U-Net (with a validation accuracy of 97.4%). The findings of this study demonstrate that cattle identification and recognition systems using retinal images were achieved with high accuracy rates. This study investigates retinal recognition and evaluates the performance of deep learning models for retinal identification, while also providing a publicly available expert-annotated ground truth dataset.

Keywords: Biometric identification, Biometric recognition, Cattle retina biometrics, Deep learning segmentation, U-Net architecture

Introduction

Biometric identification in animals, which provides unique identification/recognition of animals by utilizing their physical characteristics, has become an important tool in modern agriculture and livestock sectors [1]. These methods include fingerprint recognition, facial recognition [2], retina scanning [3–5], nose prints [6], body patterns [7], and DNA profiling [8]. These methods give each animal a unique identifier, facilitating easy detection in cases of loss or theft. Biometric identification in animals offers significant benefits not only in cases of loss but also in areas such as health monitoring, reproduction control, and feed consumption tracking [9].

One of the main advantages of biometric identification is that it does not negatively impact animal welfare. Traditional methods have been used for animal identification and recognition up until now. These traditional methods include ear notching, ear tattoos, branding, freeze marking, and ear tagging [10]. Traditional methods can cause stress in animals, increase the risk of infection, and even subject them to physically traumatic procedures.

Ear tagging is still widely used for animal identification and recognition [11]. This method involves attaching unique numbered tags to the animals' ears. However, ear tagging has numerous disadvantages. For instance, the process can cause infections or irritation in the animals' ears. Animals may experience stress, negatively affecting their production efficiency. If the animal moves its head during tagging or an issue arises, it can result in can result in torn ears. Additionally, animals can snag their tags on fences or other objects, leading to ear injuries. Moreover, the ear tagging method has environmental drawbacks. Tags frequently fall off, contributing to plastic waste and environmental pollution. The loss of tags necessitates re-

tagging, which incurs additional costs and subjects the animals to stress again. Tags can also be easily removed or copied, leading to theft and fraud [12]. Consequently, insurance premiums are high, prompting animal owners to avoid insurance. These economic losses associated with ear tagging negatively affect animal owners and the national economy. Therefore, it is crucial to transition to more effective and modern identification methods to eliminate these problems.

Unlike biometric systems, other tracking methods monitor devices rather than animals. Modern technologies like biometric identification provide a safer and more effective way to identify and recognize animals individually. For example, biometric methods such as retina scanning can verify animals' identities using their unique physical characteristics. As with ear tags, these methods can be performed without subjecting animals to external influences and stress. Additionally, since these methods involve scanning body parts, they do not produce waste and thus do not contribute to environmental pollution. Biometric methods cannot be copied, preventing fraud, and there is no risk of loss or deformation over the animal's lifetime. These methods significantly reduce stress levels and protect animal welfare [13–15]. Transitioning to modern identification methods enhances animal welfare, promotes healthier and more efficient livestock practices, reduces the economic burden on animal owners, and supports an environmentally friendly approach, ultimately benefiting the national economy [16]. To promote these methods, necessary infrastructure and training support should be provided, and awareness should be raised within the industry.

In this study, a system for animal identification and recognition from retina biometry was developed using digital image processing methods. When the literature examines studies based on retinal images, it shows a limited number of studies and that these studies generally use ready-made software [17-24]. The retinal vascular pattern is a biometric identifier present from birth, remaining unchanged throughout the animal's life and incapable of being imitated, making it the most secure biometric identification method [25]. This pattern can differ even between twins, clones, and the eyes of the same animal [26]. Obtaining retinal images is painless, easy, reliable, and cost-effective. Other biometric structures, such as fingerprints, faces, palms, and irises, can be altered through plastic surgery, making them less secure than retinas. Numerous researchers have supported the use of retina imaging technology for identifying farm animals [4,19,22,24,27].

To transition to biometric systems, it is essential to first record the biometric data of animals in databases. However, few countries have started identification and recognition studies using biometric markers. Moreover, collected biometric data is kept private. This study investigates retinal recognition and evaluates the performance of deep learning models for retinal identification. Previous studies have focused solely on animal identification without using deep learning methods. In addition to identification, we developed a recognition system in this study. This allows for comparing any given retinal image with all other images using a matching score, determining whether the image belongs to the same animal as the one with the highest match. Data was first collected in Türkiye using retina biometrics and deep learning to identify and recognize cattle. Labeled images are essential for training deep learning methods. Therefore, 80 retina images were annotated by experts to create a ground truth dataset. This number is relatively high, and the dataset manually labeled and frequently used for humans in the literature consists of 40 retinas (DRIVE Dataset). After training with this dataset, the identification performance of U-Net, SA-UNet, and U-Net++ deep learning models was compared. Finally, the recognition system performance of the U-Net model, which showed superior results in cattle identification, was evaluated.

The main contributions of this article are listed below:

- Addressing a gap in literature focused solely on identification by developing a recognition system.
- Demonstrating the effectiveness of deep learning models for retinal identification and recognition.
- To compare the performances of U-Net, SA-UNet, and U-Net++ deep learning models in vessel segmentation.
- To compare the performances of BRISK (Binary Robust Invariant Scalable Keypoints), FAST (Features from Accelerated Segment Test), HARRIS (Harris Corner Detection), SIFT (Scale-Invariant Feature Transform), and SURF (Speeded-Up Robust Features) feature extraction methods.
- To publicly share an expert-annotated ground truth dataset of 80 retinal images.

Material and Methods

Ethical Statement

The study was approved by the Kafkas University Animal Experiments Local Ethics Committee in Turkey (Protocol number: KAÜ-HADYEK/2025-018).

Database Used

The animal material of this study was obtained by taking retinal images from 300 cattle brought to the Faculty of Veterinary Medicine of Kafkas University, Türkiye. The datasets used in this study were collected within the scope of previously published studies [3-5]. The breed, age, sex

and ear tag numbers of the cattle brought to the clinic were recorded. During the data collection phase, the sick animals were first examined in general clinically and recorded if any disease was detected. Then, images were collected in a closed environment using the Optomed Smartscope digital fundus camera, with at least two images from both the right and left eyes. The Optomed Smartscope digital fundus camera was used only to collect retinal images. Deep learning and image processing studies were performed with the models created within the scope of this article. 80 of the 300 cattle collected in this study were manually segmented and used by experts. The reason for selecting 80 images for manual segmentation is that the process is labor-intensive and requires expertise. In the literature, the maximum number of manually segmented retinal images for humans is 40 (DRIVE Database), highlighting the challenge of this task.

Original RGB retina images captured with an Optomed portable fundus device were archived in JPG format at 1536×1152 resolution. 80 RGB fundus images, manually segmented by experts, were resized to 512×512 while preserving the aspect ratio. The manually segmented binary images were saved in PNG format. The segmented image dataset has been publicly shared on Kaggle *(https://www.kaggle.com/datasets/animalbiometry/cattle-retinal-fundus-groundtruth).*

Additionally, augmentation techniques were applied to the images to measure the models' generalization performance. During field capture of retina images from animals, the device's angle of grip could alter the angle of the retina image. Therefore, in this study, the dataset was augmented by rotating 80 original retina images by 0°, 30°, 60°, 90°, 120°, 150°, and 180°. After augmentation, a total of 560 augmented retina images were obtained.

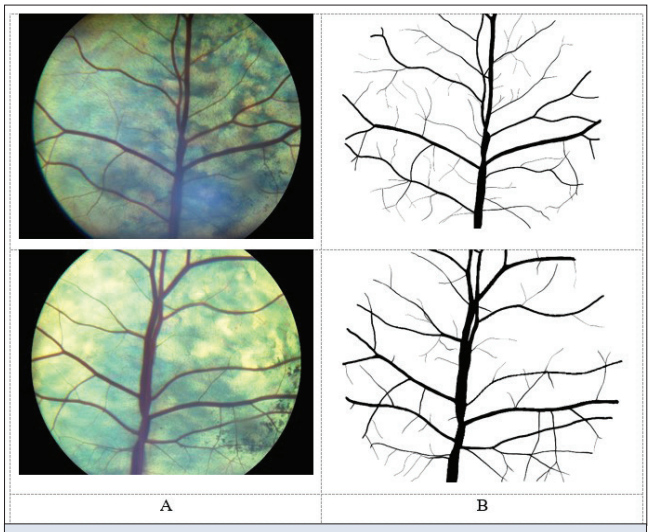

Fig 1. A) Original RGB image, **B)** manually labelled (expert-annotated) image

Fig. 1 presents an example of an RGB retina image and its labelled BW image. During the training phase of deep learning models, both original (n=80) and augmented (n=560) retinal images were utilized. Additionally, an independent set of 1,206 distinct RGB retinal images was employed to evaluate the models' performance.

Retinal Vessel Segmentation And Feature Extraction

In this study, deep learning methods U-Net, SA-UNet, and U-Net++ were used to segment the retinal vascular structure of cattle. After vessel segmentation, features in the images were extracted using BRISK, FAST, HARRIS, SIFT, and SURF methods.

Deep Learning Models

The U-Net, SA-UNet, and U-Net++ deep learning models were utilized for retinal vessel segmentation. Each offers distinct approaches to enhance segmentation performance.

U-Net Model is a convolutional neural network developed for biomedical image segmentation [28]. The fundamental idea behind U-Net is to spatially reduce the image feature map size in the network to store only important features and discard less valuable data, then create a bottleneck to learn significant features and restore them to the original size. The architecture consists of encoder and decoder blocks. The contractive branch (encoder) uses traditional convolution to down-sample the image's representation and produce a compressed feature representation of the input image. The expansive branch (decoder), complementary to the contractive branch, uses up-sampling methods like transpose convolution to ensure the processed output is the same size as the input. In *Fig. 2*, each blue box in the U-Net architecture corresponds to a feature map. Numbers written above the boxes represent channel numbers, and those in the bottom left corners indicate dimensions. White boxes denote copied feature maps, and arrows indicate different operations. The network structure of the U-Net algorithm used for retina vessel segmentation is schematically illustrated in *Fig. 2*.

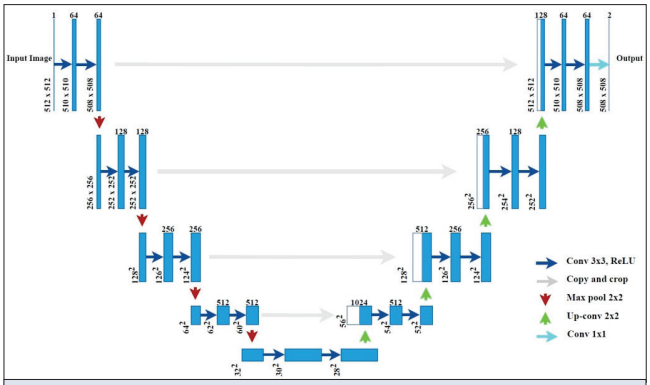

Fig 2. Diagram of U-Net architecture

SA-UNet Model is widely used in medical image segmentation, various variants have been proposed to achieve even better performance. These variants have improved performance but have made the network more complex and less interpretable. To address these issues, Spatial Attention U-Net (SA-UNet) was proposed [29]. In the SA-UNet architecture, convolutional blocks are replaced with structured convolution blocks integrating DropBlock and BN (batch normalization). This enhances the network's representation ability by focusing on vascular features and suppressing insignificant features by adding a few extra parameters. The SA-UNet network structure used for retina vessel segmentation is schematically illustrated in *Fig. 3*. The difference between vascular and non-vascular features in retina fundus images, especially in small and marginal vascular areas, is not distinct.

U-Net++ Model, the encoder captures high-level features from the input image through a series of convolutional and pooling layers. At the same time, the decoder uses

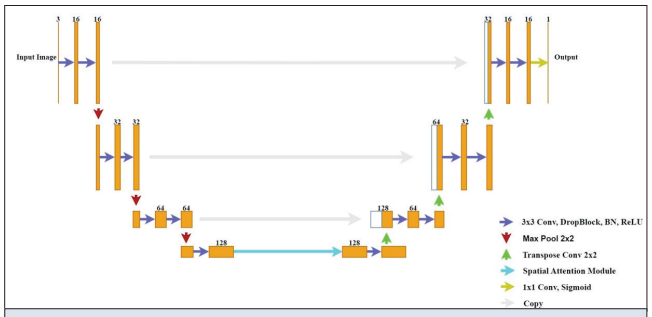

Fig 3. Diagram of SA-UNet architecture

up-sampled representations of these features to generate a dense segmentation map. However, there can be a semantic gap between encoder and decoder features, which may challenge the decoder to reconstruct fine details and produce accurate segmentation. UNet++ addresses this semantic gap by introducing the concept of nested skip pathways. Multiple skip connections are placed between the encoder and decoder blocks at different resolutions. These connections allow the decoder to access and fuse both low-level and high-level features from the encoder, enabling a more comprehensive understanding of the image in finer detail [30]. The UNet++ network structure used for retina vessel segmentation is schematically illustrated in *Fig. 4*.

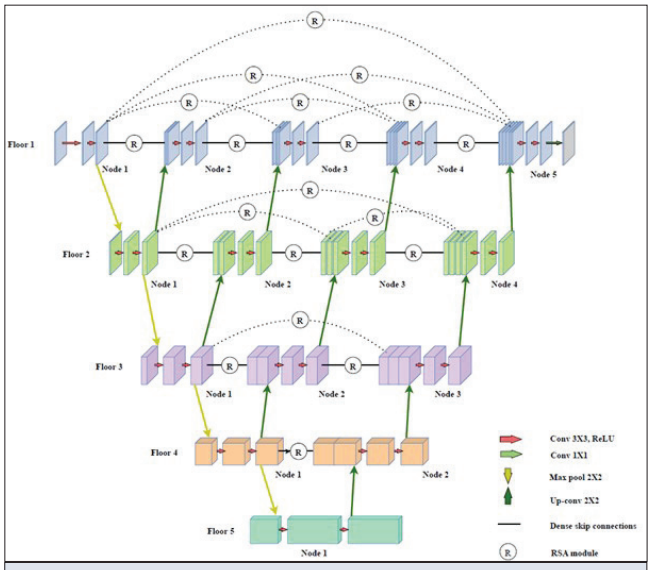

Fig 4. Diagram of UNet++ architecture

Feature Extraction Methods

BRISK, FAST, HARRIS, SIFT, and SURF methods were used for feature extraction in retinal images, and this section provides their descriptions, and the parameters used in the study.

Binary Robust Invariant Scalable Keypoints (BRISK) is a feature extraction algorithm in computer vision and image processing. The processing steps of the BRISK algorithm are as follows:

1. ***Corner Detection:*** BRISK detects specific corner points in the image using a scale space (pyramid structure).
2. ***Orientation Calculation:*** Each key point is assigned to an orientation value based on the bright variations of the surrounding pixels.
3. ***Binary Descriptor Creation:*** Binary descriptors are created using the bright differences of pixel pairs around the key point.
4. ***Matching:*** The binary descriptors are compared using Hamming distance to determine matches.
5. ***Non-Maximum Suppression:*** This method identifies the strongest points to select the most prominent corners.
6. ***Scalability:*** BRISK offers a scalable structure to accurately detect objects of different sizes.

Features from Accelerated Segment Test (FAST) aims to identify prominent distinctive points (corners) in images. Corners are points that contain important information about the geometric structure of an object and are used in many applications, such as object recognition and image matching. The steps of the FAST algorithm are as follows:

1. ***Corner Candidate Detection:*** The algorithm detects corner candidates by comparing a specific pixel with its surrounding pixels. The pixels are evaluated based on the bright differences along a circular contour.
2. ***Circle Definition:*** A circle with a radius of 16 pixels is defined around each corner candidate. The pixels

within this circle are classified as brighter, darker, or similar in brightness compared to the central pixel.

3. ***Threshold Value Check:*** The pixels on the circle must differ from the central pixel by a certain threshold value. If at least 12 pixels are found to be either brighter or darker than the center pixel, that pixel is considered a corner.
4. ***Accelerated Check:*** The algorithm does not evaluate all 16 pixels in the circle to determine the presence of a corner. Instead, it initially examines the diagonal pixels (e.g., pixels 1, 5, 9, and 13). The evaluation process is terminated if these four pixels fail to satisfy the corner criteria.
5. ***Non-Maximum Suppression:*** Among the detected corners, the strongest ones are selected for feature extraction using the non-maximum suppression method.

The HARRIS method is a gradient-based corner detection algorithm. The steps for extracting features are as follows:

1. ***Gradient Calculation:*** Compute the gradients in the x and y directions around each pixel using Sobel or other edge detection operators to find the rate of brightness change.
2. ***Harris Matrix Formation:*** Using the gradients, construct a structure called the Harris matrix, which helps determine if a pixel is a corner.
3. ***R Score Calculation:*** Calculate an "R" score for each pixel to classify it as a corner, edge, or flat region.
4. ***Corner Detection:*** If the R score exceeds a certain threshold, the pixel is marked as a corner; positive R indicates a corner, while negative R indicates an edge or flat area.
5. ***Non-Maximum Suppression:*** Filter the corners with high R scores using non-maximum suppression to identify and extract the strongest corners as features.

The introduced Scale Invariant Feature Transform (SIFT) method is an image descriptor for image-based matching proposed by David G. Lowe in 2004. The descriptor is used for numerous purposes in computer vision related to point-matching different views of a 3D scene and view-based object recognition. The SIFT descriptor is invariant with translations, rotations, and scaling transformations in the image space and is invariant with perspective transformations and illumination changes [31].

The SIFT algorithm consists of four steps [32]:

1. ***Recognition of key points with scale space:*** Points of interest (key points) are detected by blurring images at different scales with a Gauss filter and creating different images (DoG).
2. ***Improvement of key points according to contrast and edge thresholds:*** The detected points of interest are positioned more precisely, and points with low contrast or located at the edges are eliminated.
3. ***Assigning the direction of each key point:*** Each key point is assigned a direction by calculating the gradient directions around it. This ensures that the features remain stable against rotations.
4. ***Creating feature definitions:*** Using the gradient magnitude and direction information around each key point, feature vectors are created. These vectors are used for comparison and matching.

SIFT is used to detect points of interest from a grey-level image, giving a summative description of local image structures in a local neighborhood around each point of interest. This descriptor is used to match corresponding points of interest between different images. SIFT is used for studies such as object categorization, texture classification, image alignment, and biometrics [33].

The Speeded Up Robust Features (SURF) method is an algorithm used for detection and recognition in the fields of image processing and computer vision [34]. SURF is a scale-invariant feature detector based on the Hessian matrix. Instead of using a different metric to select position and scale, a Hessian detector is used for both. The Hessian matrix is a square matrix of second-order partial derivatives of a numerical field and is roughly analogous to using a series of box-type filters. The main interest of the SURF approach is that it quickly computes operators using box filters, thus enabling real-time applications such as tracking and object recognition [35].

The SURF algorithm consists of four main parts:

- Integral image generation
- Hessian detector
- Descriptive orientation assignment
- Creating an identifier

The integral image $I_{\sum}$ is calculated with Equation 1.

$$I_{\sum}(x,y) = \sum_{i=0}^{i\leq x}\sum_{j=0}^{j\leq y} I(x,y) \quad (1)$$

Here I is the input image. The time required for the calculation is invariant to change in size. The Hessian matrix is given in Equation 2.

$$H(x,\sigma) = \begin{bmatrix} L_{xx}(x,\sigma) & L_{xy}(x,\sigma) \\ L_{xy}(x,\sigma) & L_{yy}(x,\sigma) \end{bmatrix} \quad (2)$$

L_{xx}, is the convolution of the second-order Gaussian derivative with the image at x = (x, y). L_{yy} *ve* L_{xy} are treated similarly. The determinant of this matrix is used to find points of interest. Each point is compared to eight

points on the local scale, nine points on the upper scale, and nine points on the lower scale.

Table 1 provides the parameters used in the study to obtain features from retinal vessels using the BRISK, FAST, HARRIS, SIFT, and SURF methods.

Performance Measures

This study used accuracy, validation, and loss metrics to measure the training performances of U-Net, SA-UNet, and U-Net++ models. The 5-CV technique was used when training the models [4] FAR, FRR, precision, recall, and accuracy metrics were used to measure the recognition performance of the learning models, and the matching score approach was used to measure the identification performance of the models.

Table 1. *Model parameters of feature extraction methods*

Method	Parameter/Value
BRISK	thresh = 0 octaves = 3 patternScale = 1.0f
FAST	nonmaxSuppression = False threshold = 10
HARRIS	maxCorners = 1000 qualityLevel = 0.01 minDistance = 10 blockSize = 5 k = 4 mask = None
SIFT	nfeatures = 3000 nOctaveLayers = 30 contrastThreshold = 0.04 edgeThreshold = 1000 sigma = 1.6
SURF	hessianThreshold = 100 nOctaves = 4 OctaveLayers = 30 Extended = False upright = False

Accuracy is the ratio of correctly matched retinal images to the total number of retinal images, calculated as in Equation 3. This metric shows how accurately the model makes predictions during training.

$$\text{Accuracy} = \frac{\#correctly\ matched\ retina\ images}{\#total\ retina\ images} \quad (3)$$

Validation is the evaluation made on images that have not been used in the training data set before. It shows the model's success on new data (that it has not seen before). This doesn't happen based on the data the model sees during training, so predicting how well the model can perform on real-world data is important. Its formulation is the same as accuracy; only the data set changes.

Loss: the BCEDiceLoss function was used to calculate losses in cattle identification. BCEDiceLoss consists of the combination of Binary Cross Entropy (BCE) and Dice loss. Binary cross-entropy loss (BCE) is used to evaluate the probability that a pixel belongs to a particular class. On the other hand, Dice loss is mainly used in segmentation tasks and is known for determining object boundaries more effectively. Dice loss evaluates performance by measuring the agreement between pixels predicted by the model and actual labels. BCEDiceLoss combines the advantages of these two functions, allowing the model to increase pixel-wise accuracy and detect object boundaries more precisely. Thanks to this integration, the model's overall performance is improved, and the cattle retinal identification process is aimed at being more efficient and accurate. BCEDiceLoss function is calculated as in Equation 4.

$$\text{BCEDiceLoss}=\text{BCELoss}+(1-\text{DiceLoss}) \quad (4)$$

The purpose of the recognition system is to check whether the cattle whose retina has been scanned are in the database. According to the optimum threshold value determined here, whether the animal is in the database or not is questioned. The study defined the value at which the FAR metric was minimized as the optimum threshold value. According to the determined threshold value, the recognition performance of U-Net, SA-UNet, and U-Net++ models was measured with FAR, FRR, precision, recall, and accuracy metrics.

False Acceptance Rate (FAR) is a metric that expresses the rate at which cattle not in the database are mistakenly accepted as being in the database when checking whether the cattle whose retinas have been scanned are in the database. This ratio is used to evaluate the reliability and accuracy of the recognition system. The FAR formula is given in Equation 5.

$$FAR = \frac{FP}{FP + TN} \quad (5)$$

False Rejection Rate (FRR) is a metric that expresses the rate at which cattle in the database are mistakenly rejected as if they were not in the database while checking whether the cattle whose retina was scanned are in the database. This ratio is used to evaluate the accuracy and reliability of the recognition system. FRR is calculated as the rate at which the system cannot recognize correctly registered cattle, as in Equation 6.

$$FRR = \frac{FN}{FN + TP} \quad (6)$$

Precision refers to the ratio of cattle recognized by the system as existing in the database, among the cattle actually in the database, when checking whether the cattle whose retinas have been scanned are in the database. In other words, it is the ratio of the system's true positive

predictions to the total positive predictions and is calculated as in Equation 7.

$$Precision = \frac{TP}{TP + FP} \quad (7)$$

Recall refers to how many of the cattle in the database are correctly recognized by the system when checking whether the cattle whose retinas have been scanned are in the database. It is the ratio of true positive predictions to total true positives and is calculated as in Equation 8.

$$Recall = \frac{TP}{TP + FN} \quad (8)$$

The primary purpose of the identification system is to compare the retinal image of the tested cattle with all cattle retina images in the database to obtain a match score between retina pairs. The match score gives us the percentage of how many distinctive feature points of the two compared images overlap. The identification process is successful if the images with the highest match score belong to the same cattle. In this case, the identity of the cattle the system tests are determined by finding the closest match in the database. The match score is calculated using the formula in Equation 9.

$$Matching\ Score = \sqrt{\frac{(matchingPoints)^2}{(pointsImg1)*(pointsImg2)}} \times 100 \quad (9)$$

MatchingPoints represents the number of matching feature points between the reference and test images. pointImg1 indicates the total feature points in the reference image, and pointImg2 indicates the total feature points in the test image.

Results

This study used U-Net, SA-UNet, and UNet++ deep learning models for retinal biometric identification and recognition. Animal identification and animal recognition are distinct and equally important processes. Animal recognition focuses on determining whether an unknown animal exists in the database. In contrast, animal identification informs the user which animal matches the unknown animal in the database. To increase the efficiency of livestock farming, particularly in large-scale animal farms, it is crucial to implement identification and recognition processes. During the training phase of the models applied for identification and recognition, the parameters listed in *Table 2* were used. These parameters were determined based on experimental studies.

The 5-fold cross-validation method was used when training the models [36]. Each part was used for testing, while the remaining was reserved for training. The model's accuracy was generalized by taking the average of these five folds. Average Dice Similarity Coefficient (Dice) and Intersection Over Union (IoU) were used to quantitatively evaluate the performance of the methods and compare them with others. In *Table 3*, IoU and dice are calculated for two data sets (original and augmented) of each model and presented next to the ± deviation value.

Table 2. Training parameters of U-Net, SA-UNet, and UNet++ models

Parameter	Value
Optimizers	Adam
Loss	BCEDiceLoss
Size	512 x 512
K_Folds	5
Batch_size	2
Epochs	300
Learning Rate	0.001
LR Scheduler	ReduceLROnPlateau
Scaler	GradScaler

Table 3 compares different models' performance using Dice and IoU (Intersection over Union) metrics. Dice Score is a metric that measures the similarity of two clusters. Values closer to 1 indicate that the model shows better segmentation performance. IoU (Jaccard Similarity) measures segmentation success by evaluating the intersection and union ratio. High IoU values suggest that the model distinguishes the target object better. As can be seen from *Table 3*, when the data augmentation method is used, an improvement is observed in the Dice and IoU values of all models. In particular, the IoU and Dice scores of the U-Net and SA-UNet models showed the highest performance.

Table 3. Quantitative results of the models for the animal identification system (mean ± SD)

Dataset	Model	Dice	IoU
Original	U-Net	0.977±0.001	0.954±0.001
	SA-UNet	0.976±0.001	0.954±0.001
	UNet++	0.976±.001	0.953±0.002
Augmented	U-Net	**0.983±.000**	**0.966±0.001**
	SA-UNet	**0.983±0.000**	**0.966±0.001**
	UNet++	0.982±.001	0.965±0.001

As seen in *Table 3*, U-Net and SA-UNet models are the most successful models in retinal vessel segmentation, with a success rate of 0.983±0.000 from the Dice method and 0.966±0.001 from the IoU method. The U-Net++ model showed a very close but lower performance than the other models. When the performances of these models were compared in terms of accuracy, verification, and loss metrics, the results in *Table 4* were obtained.

Table 4. *Comparison of performances of U-Net, SA-UNet, and U-Net++ models in identification*

Dataset	Model	Training Acc (%)	Test Acc (%)	Loss (%)
Original	U-Net	96.9±0.2	96.6±0.2	6.9±0.3
	SA-UNet	96.9±0.1	96.6±0.1	6.9±0.3
	U-Net++	97.0±0.1	96.6±0.1	6.9±0.1
Augmented	U-Net	**97.8±0.1**	**97.4±0.0**	**5.1±0.1**
	SA-UNet	97.8±0.1	97.4±0.1	5.1±0.1
	U-Net++	97.7±0.1	97.3±0.1	5.2±0.1

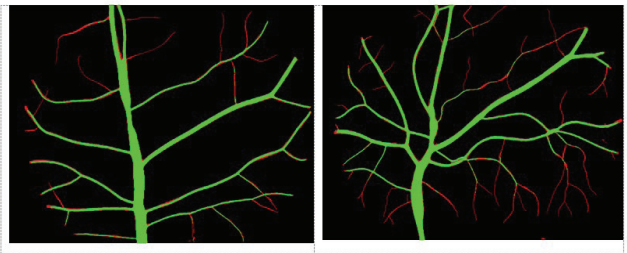

Fig 5. Ground truth U-Net matching result **A)** sample 1, **B)** sample 2

Table 4 compares the performance of U-Net, SA-UNet and U-Net++ models during training. Training accuracy represents the model's accuracy on the training data, while validation accuracy indicates its performance on the validation data. The loss function reflects the model's error rate, with lower values indicating better optimization. When data augmentation is applied, an increase in both training and validation accuracy is observed across all models, while the loss function values decrease. This demonstrates that data augmentation enhances the model's learning capacity and generalization ability.

In conclusion, when considering *Table 3* and *Table 4* together, the U-Net model is the most successful in vessel segmentation, achieving a validation accuracy of 0.974±0.000.

Fig. 5 presents the intersection of the vessel patterns segmented by the U-Net model with the manually segmented vessel patterns for the most successful model. In these retina images belonging to two different animals, the red indicates manually annotated vessels, while the green shows the sections matched by the U-Net model.

The developed computer-aided cattle identification and recognition system aims to prevent theft and fraud while ensuring animal welfare. The feasibility of such systems continues to be explored, as research in this area is ongoing. The findings obtained in this study demonstrate that the identification and recognition system can be successfully implemented. In this study, after evaluating the training performances of the models for identification in *Table 3* and *Table 4*, a recognition system based on U-Net was developed. The augmented dataset was used in the recognition system because higher success was achieved with this dataset. The advantage of this dataset is its higher generalization capability due to the inclusion of images at different angles. The performance of five different feature extraction methods (BRISK, FAST, HARRIS, SIFT, and SURF) used in the developed U-Net-based recognition system was measured, and the results are presented in

Table 5. *Performance of the animal recognition system for the augmented dataset*

Method	FAR (%)	FRR (%)	Precision (%)	Recall (%)	Accuracy (%)	Threshold	Exe.Time (s)
BRISK	18.52	17.49	97.84	82.51	82.42	30.50	1.80
FAST	10.34	12.84	98.75	87.16	87.40	22.60	4.80
HARRIS	11.03	11.40	98.33	88.60	88.64	36.90	0.24
SIFT	20.51	20.91	99.14	79.09	79.10	33.40	6.00
SURF	30.77	30.76	99.03	69.24	69.24	25.50	216.00

Table 6. *Confusion matrix of the recognition system (for 1206 test retina images)*

BRISK			
Actual Label	(-)	88	20
	(+)	192	906
		(-)	(+)
		Predicted Label	

FAST			
Actual Label	(-)	104	12
	(+)	140	950
		(-)	(+)
		Predicted Label	

HARRIS			
Actual Label	(-)	121	16
	(+)	129	940
		(-)	(+)
		Predicted Label	

SIFT			
Actual Label	(-)	31	8
	(+)	244	923
		(-)	(+)
		Predicted Label	

SURF			
Actual Label	(-)	18	8
	(+)	363	817
		(-)	(+)
		Predicted Label	

Table 5. In addition, the execution times of the methods are also shown in *Table 5*. As can be seen from *Table 5*, the fastest model is HARRIS with 0.24 sec.

As shown in *Table 5*, feature extraction from the retinal vessels was performed using the BRISK, FAST, HARRIS, SIFT, and SURF models. The threshold values in *Table 5* were determined experimentally and represent the optimal values that maximize the system's performance. These values indicate the degree of match between the retinal features of the scanned animal and those in the database. Specifically, suppose a value smaller than the threshold shown in *Table 5* is obtained when matching an animal's retina with the database. In that case, it indicates that the animal is not present in the database. For instance, in the model where features were extracted using the SIFT method segmented by the U-Net technique, a threshold level above 33.4 suggests that the scanned animal is present in the database, meaning it has been previously identified.

BRISK exhibits relatively high false acceptance (FAR: 0.1852) and false rejection rates (FRR: 0.1749). However, its precision is high at 0.9784, suggesting strong performance in minimizing false positives. Despite this, with a recall of 0.8251 and accuracy of 0.8242, BRISK demonstrates average overall performance compared to other methods.

FAST presents one of the lowest false acceptance (FAR: 0.1034) and false rejection (FRR: 0.1284) rates. Its precision (0.9875) and recall (0.8716) are high, and with an accuracy of 0.8740, FAST outperforms BRISK, indicating superior recognition accuracy.

HARRIS also shows low FAR (0.1103) and FRR (0.1140), along with high precision (0.9833) and recall (0.8860). These metrics demonstrate that the method effectively minimizes false positive and false negative results, achieving an overall accuracy of 0.8864.

SIFT, while having slightly higher false acceptance (FAR: 0.2051) and false rejection (FRR: 0.2091) rates than other methods, achieves high precision at 0.9914, implying that false positives are rare. Nevertheless, its recall (0.7909) and accuracy (0.7910) remain average.

SURF displays the highest FAR (0.3077) and FRR (0.3076) values, indicating significant false acceptance and rejection levels. Although SURF's precision is excellent at 0.9903, its recall (0.6924) and accuracy (0.6924) are lower than the other methods.

Table 6 presents the confusion matrices obtained. The recognition process was performed using the matching scores given in Equation 9. *Table 6* shows correct and incorrect predictions for features obtained using the BRISK, FAST, HARRIS, SIFT, and SURF methods with these scores. In the confusion matrix, the (+) label shows the correctly identified ones, while the (-) label shows the incorrectly identified ones. *Table 6* shows that the number of animals correctly identified in the database is relatively high. However, the rate of incorrectly identifying a foreign animal not in the database is also higher. Overcoming this issue is believed to be achievable by increasing the number of animals in the database as much as possible. Additionally, having a more significant number and variety of retina images for the animals in the database will help address this issue.

In conclusion, the HARRIS method is the most successful model due to its low false acceptance and rejection rates and high precision and recall values. While FAST also demonstrates strong performance, HARRIS achieves

the highest accuracy and recall, making it the most effective method overall. SIFT, although showing excellent precision, has relatively lower recall and accuracy. BRISK and SURF exhibit lower overall success rates. Therefore, HARRIS emerges as the best-performing method in this study. These results revealed that the U-Net+HARRIS based recognition system can be used effectively.

Discussion

This study used deep learning methods for animal identification and recognition from retinal images. Our findings demonstrated high accuracy rates, suggesting that the developed system is suitable for practical applications in animal biometric identification.

Previous studies have also emphasized the potential of retinal images as a unique biometric identifier in livestock. For example, Barry et al.[17] and Rojas-Olivares et al.[24] reported that retinal imaging technology is effective in distinguishing individual sheep and lambs, respectively. Similarly, Gionfriddo et al.[18] demonstrated the feasibility of using retinal images for identifying individual dogs. These studies primarily relied on traditional image analysis or proprietary software, whereas our study utilized deep learning models, achieving significantly higher segmentation and identification performance.

In terms of segmentation, our U-Net model achieved a Dice score of 0.983±0.000 and an IoU score of 0.966±0.001 on the augmented dataset, indicating excellent vessel segmentation performance. Comparable research by Mustafi et al. [20] using retinal images for goat identification reported moderate segmentation accuracy without deep learning enhancements, highlighting the performance advantage of our approach. Regarding the recognition system, while previous studies such as Allen et al.[22] mainly evaluated retinal images through manual or semi-automatic feature matching, our deep learning-based system combined automatic vessel segmentation with feature extraction techniques (BRISK, FAST, HARRIS, SIFT, SURF), resulting in a recognition accuracy of 88.64% with the HARRIS method. This is a significant improvement over the conventional methods reported in earlier works [17,19,22].

One limitation of our study is the manual segmentation of retina images, which, although reviewed for accuracy, can introduce subjective variability. Similar concerns were noted in earlier works where manual annotation was used [17,24]. Future studies can benefit from larger datasets and the application of semi-automatic or fully automatic annotation methods. Another noteworthy contribution is the public release of our annotated dataset and source code, which contrasts with prior studies where data and methods were often proprietary [3-5,22]. This openness is expected to accelerate multidisciplinary research in animal biometrics.

The differences between this study and the existing literature are as follows:

- All studies have focused on identification, meaning they check whether the animal is in the database. In this study, we developed both identification and recognition systems.
- Almost all studies in the literature have performed recognition using the software embedded in retinal imaging devices. In this study, we implemented the steps of digital image processing individually.
- The retinal images collected in previous studies are private, while the dataset in this study is publicly available on Kaggle.
- In our previous work, we only developed an identification system using image preprocessing methods, while this study demonstrates that deep learning models are more successful.

The proposed biometric identification and recognition system has a high potential for industrial and commercial use. In the livestock sector, especially in large-scale farms and meat production facilities, animal identification and tracking are critical requirements. While traditional methods rely on physical identification tools such as ear tags or microchips, biometric systems offer a more reliable and tamper-proof alternative.

The proposed system can make important data such as vaccination history, health status and genetic information of animals accessible quickly and accurately. This can increase efficiency by improving disease management and lineage tracking processes. At the same time, it can provide great convenience in animal trade and pre-slaughter health checks and traceability requirements. Considering the increasingly stringent traceability and animal welfare standards of the European Union and other international markets, it is also possible for this system to create a competitive advantage in global trade. In addition, biometric identification has great potential in improving quality control processes in meat and dairy production, increasing food safety, and providing consumers with more transparent information. In today's world where interest in sustainable agricultural practices is increasing, adopting such technologies can contribute to the digitalization of the livestock sector and accelerate its integration into modern agricultural systems. Therefore, the proposed biometric identification and recognition system has an important practical application in terms of efficiency, security, and traceability in the livestock sector, beyond being just an academic research topic.

In conclusion, a deep learning-based computer-aided

animal identification and recognition system was developed using retinal images. A computer-aided identification and recognition system can significantly contribute to the agriculture and livestock sector, enhancing animal welfare. According to the study's findings, the U-Net deep learning-based system achieved an identification accuracy of 97.4%. The system's identification accuracy pertains to determining whether an animal whose retina has been scanned has been previously identified. The recognition accuracy of the developed system was 88.64%, with a precision of 98.33%. The lower accuracy value of biometric recognition than identification can be explained as follows.

Biometric identification involves determining the specific identity of an animal among all animals in the database, enabling the tracking of its vaccination, health, and lineage records. In contrast, biometric recognition simply determines whether an animal exists in the database without specifying its identity. Biometric recognition is the process of determining whether an animal is in the database. Large herds, especially in animals such as cattle that are raised in different places and can be sold before being slaughtered, can be difficult to trace the origins. For this reason, biometric recognition can easily determine which animals belong to you. However, false matches or misses may occur since the system only answers "yes" or "no" in this process. When working with large data sets, errors can occur due to false negatives (not found in the database) or false positives (assumed to be present even though not in the database). The error rate can increase, especially when animals with similar retinal patterns are involved. Tracking animal movements increases efficiency and contributes to preserving meat quality by reducing the risk of disease spread.

Publicly available ground truth datasets of manually segmented animal retina images are scarce, posing challenges for multidisciplinary research in animal husbandry. This study contributes to the increase in multidisciplinary research, enabling the development of more successful identification and recognition systems.

Declarations

Availability of Data and Materials: The dataset used in the study is publicly available at https://www.kaggle.com/datasets/animalbiometry/cattle-retinal-fundus-groundtruth. The source codes are available at https://github.com/muhammedakyuzlu/retinal-vessel-detection-identification-unet-variants

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Ethical Approval: The study has been approved by the Institutional Animal Care and Use Committee of Kafkas University (KAÜ-HADYEK/2025-018).

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

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LETTER TO EDITOR

Acute Gangrenous Mastitis Observed Following Diarrhea in the Postpartum Period in a Queen

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Dear Editor

We would like to present a rare case of acute gangrenous mastitis in a cat that developed after treatment of diarrhea in the postpartum period.

A 2-year-old British Shorthair cat was brought to Afyon Kocatepe University Animal Hospital with complaints of discontinuing breastfeeding for about 2 days and black discoloration in the left abdominal mammary gland for 3 days. In patient's history, it was learned that the cat gave birth for the first time two weeks ago and treated for postpartum diarrhea after birth. In the clinical examination, it was determined that there was loss of appetite, vomiting, weakness. Black necrotic flap on the skin, wound and pus discharge in the abdominal mammary region were observed *(Fig. 1-a)*. The cat's body temperature was measured at 39.4ºC. A complete blood count and biochemistry panel results were as in *Table 1*. A wound swab was taken and sent to the laboratory for microbiological analysis and antibiotic susceptibility testing. Due to its antiseptic properties, 1 g. of ethacridine lactate powder (Rivanol® 1 g., Şifa, İzmir, Türkiye) was dissolved in 1 liter of water and applied on the wound for 3 weeks. After cleaning the wound area, centella asiatica (Madecassol® 1% cream, Bayer Consumer Care AG, Switzerland) and nitrofurazone (Furacin® 0.2% pomade, Sanofi, Istanbul, Türkiye) were applied to the wound area twice daily. "Blind" antibiotic treatment was given without delay until the antibiogram results was obtained. For this purpose, Enrofloxacin (5% Baytril®, Bayer AG, Leverkusen, Germany) was administered intramuscularly at a dose of 5 mg/kg body weight once a day for 5 days. In addition, Cefuroxime (Cefaks®, 750 mg, DEVA Holding A.Ş., Istanbul, Türkiye) was given intravenously at a dose of 25 mg/kg body weight twice a day for 5 days. Metoclopramide (Metpamid®, 5 mg/mL, Sifar, Istanbul, Türkiye) (antiemetic agent and dopamine D2 antagonist) was administered intramuscularly at

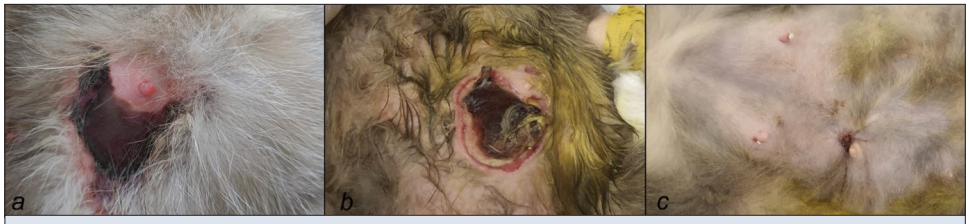

Fig 1. a- Pre-treatment image of the left caudo-abdominal mammary gland (day zero), **b-** Mammary gland in the third day of treatment, **c-** Mammary gland on the twenty-third day treatment

Table 1. *Hematological and serum biochemical parameter values at the beginning of treatment and on the second day treatment*

Parameters	Parameter Values at the Beginning of Treatment	Parameter Values on the Second Day of Treatment	Reference Values
WBC (10^3µL)	54.6	26.6	6.0-17.0
LYM (%)	50.2	55.9	12.0-30.0
MID (%)	3.4	0.6	2.0-4.0
GRA (%)	46.4	43.5	62.0-87.0
LYM (10^3µL)	27.38	14.87	1.0-4.8
MID (10^3µL)	1.83	0.17	0.2-1.5
GRA (10^3µL)	25.3	11.6	3.0-12.0
HB (g/dL)	10.9	9.0	12.0-18.0
HCT (%)	28.1	24.2	37.0-55.0
PLT(10^3µL)	167.0	240.0	200.0-500.0
Urea (mg/dL)	95	NS	9.8-35
ALP (IU/L)	33.0	NS	11.0-49.0
ALT (IU/L)	38.5	NS	10.0-130.0
AST (IU/L)	56.4	NS	17.0-48.0
Albumin (g/dL)	2.7	NS	2.5-4.0
Globulin (g/dL)	4.0	NS	2.3-5.3
Albumin/Globulin	0.7	NS	0.8-1.5
BUN (mg/dL)	44.4	NS	17.0-35.0
Creatinine (mg/dL)	0.8	NS	0.5-1.8
BUN/Creatinine	57.7	NS	4.0-35.0
Calcium (mg/dL)	9.0	NS	8.0-11.2

NS: No sample

a total dose of 3 mg once a day for 5 days. Meloxicam (Meloxicam®, baVET, İstanbul, Türkiye) was applied subcutaneously at a dose of 0.2 mg/kg body weight once a day for 3 days. Five mL of solution containing electrolytes, vitamins (especially B), amino acids and dextrose (Duphalyte®, Zoetis Manufacturing& Research Spain, S.L) was given subcutaneously for 3 days. Sodium chloride 0.9% solution (40 mL/kg body weight/day) was used for the treatment of fluid loss. The Elizabethan collar was used to prevent the animal from licking the wound area. According to microbiological analysis results, *Escherichia coli* was identified from the wound swab by VITEK 2 Compact (BioMérieux, France). The antibiotic resistance of isolate to various antimicrobial agents was tested on Mueller Hinton agar (MHA; Oxoid Limited, Hampshire, UK) using Kirby-Bauer disc diffusion method according to CLSI (2020) [1]. The most effective antibiotic was determined to be enrofloxacin followed by amoxicillin/clavulanic acid, lincomycin/spectinomycin, trimethoprim/sulfamethoxazole and amikacin. According to these results, it was determined that effective antibiotic groups were selected at the beginning of treatment. After

mastitis, the cat made a full recovery on the twenty-third day. Mammary gland images on the third and twenty-third days of treatment are shown in *Fig. 1-b,c* respectively.

A variety of bacterial isolates have been reported in published reports of acute gangrenous mastitis in cats [2-6]. In our case, *E. coli* was isolated from the udder of a cat after treatment of diarrhea in postpartum period, suggesting that mastitis may have been caused by this agent. Only three articles have provided detailed information about the treatment protocol [2,4,5]. There is not enough knowledge about which treatment procedure (mastectomy [5] or open wound therapy [2,4]) should be applied and which parameters are required for the medical treatment procedure. In our case, medical treatment was used. It is thought that the medical treatment mentioned above can be used as an alternative to surgical treatment.

This is a reported case of acute gangrenous mastitis occurring after treatment of diarrhea in postpartum period in a cat. Diarrhea in the postpartum period may cause acute gangrenous mastitis. Lactating cats with postpartum diarrhea should be monitored for acute gangrenous mastitis. Improvement in hematological parameters after initiation of treatment should be considered as a correct response to medical treatment.

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Even if the journal ceases to be published for any reason, the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) will continue to protect the journal content in the long term and provide convenient access to users. Electronic services of Kafkas University Information Technologies Department will be used for the journal to maintain this responsibility.

RESPONSIBILITIES OF SUBJECT EDITORS

Subject editors do reviews and evaluations in accordance with the main publication goals and policies of the journal and in line with the criteria that will contribute to the development of the journal.

Author information is kept confidential in articles sent to the subject editor for preliminary evaluation by the editor.

Subject editors thoroughly examine the sections of the introduction, materials and methods, results, discussion and conclusion, in terms of journal publication policies, scope, originality and research ethics. Subject editor submits its decision (rejection, revision or peer-review) after evaluation to the chief editor in a reasoned report.

Subject editor may request additional information and documents related to the study from the authors, when necessary.

In multidisciplinary studies, the article can be submitted for the evaluation of multiple subject editors.

RESPONSIBILITIES OF REFEREES

Double-blinded peer-review procedure is applied in Kafkas Universitesi Veteriner Fakultesi Dergisi in order to evaluate the articles submitted to the journal in accordance with the principle of impartiality and in objective criteria; that is, referees and writers do not know about each other.

The referees submit their opinions and reports to the editor-in-chief to ensure the control and suitability of a submitted article, its scientific content, scientific consistency and compliance with the principles of the journal. When a referee makes a decision "reject" about an article, he/she prepares the reasons for the decision in accordance with the scientific norms and presents it to the editor.

The referee(s) also gives the authors the opportunity to improve the content of the article. Accordingly, the revisions requested from the authors should be of a quality that explains/questions specific issues rather than general statements.

Referees appointed for the evaluation of the articles agree that the articles are confidential documents and will not share any information about these documents with third parties, except for the editors participating in the evaluation.

Referees should place their criticism on scientific infrastructure and write their explanations based on scientific evidence. All comments made by the referees to improve the articles should be clear and direct, and should be written away from disturbing the feelings of the author. Insulting and derogatory statements should be avoided.

If a referee has an interest relationship with the author(s) on one or more issues, he/she must report the situation to the editor and ask his/her to withdraw from the referee position. The same is also applicable when the authors illegally obtain information about the referees of the article and try to influence them.

The editor-in-chief can share the comments and reports from the referees with the editors/associate editors and the relevant subject editor, as necessary, to ensure that the decision on the article is optimal. If necessary, the editor may share the critical decision and its grounds that a referee has sent about the article with the other referee(s) and present them to their attention.

Referee(s) may request revision many times for the article they evaluated.

The content of the referee reports is checked and evaluated by editor-in-chief/editors/associate editors. The final decision belongs to the editorial.

RESPONSIBILITIES OF AUTHOR(S)

It is not tolerable for the author (s) to send an article, which has been already sent to another journal, to Kafkas Universitesi Veteriner Fakultesi Dergisi within the scope of "which accepts" or "which publishes first" approach. If this is detected, the article is rejected at any stage of the evaluation. As a possible result of these actions, in the process following the previous acceptance of the article sent to another journal, the withdrawal request with this excuse that the authors submit for this article, the evaluation process of which is going on in our journal, is evaluated by the editors and associate editors of the journal and disciplinary action on the grounds of ethical violations about those responsible is started. This unethical action is also informed to the journal editor (if known) who accepted the article.

It is essential that the articles to be sent to Kafkas Universitesi Veteriner Fakultesi Dergisi include studies that have up-to-date, original and important clinical/practical results and prepared in accordance with the journal's writing rules.

Authors should choose the references they use during the writing of the article in accordance with the ethical principles and cite them according to the rules.

The authors are obliged to revise the article in line with the issues conveyed to them during the initial evaluation, preliminary evaluation and peer-review phases of the article and to explain the changes they made/did not make sequentially in the "response to editor" and "response to reviewer comments" sections.

If information, documents or data regarding to the study are requested during the evaluation process, the corresponding author is obliged to submit them to the editorial.

Authors should know and take into account the issues listed in the "General Ethical Principles" section regarding scientific research and authors.

The authors do not have the right to simultaneously submit multiple articles to Kafkas Universitesi Veteriner Fakultesi Dergisi. It is more appropriate to submit them with acceptable time intervals for the journal's policy.

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INSTRUCTION FOR AUTHORS

1- Kafkas Universitesi Veteriner Fakultesi Dergisi (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly (E-ISSN: 1309-2251). We follow a double-blind peer-review process, and therefore the authors should remove their name and any acknowledgment from the manuscript before submission. Author names, affiliations, present/permanent address etc. should be given on the title page only.

The journal publishes full-length research papers, short communications, preliminary scientific reports, case reports, observations, letters to the editor, and reviews. The scope of the journal includes all aspects of veterinary medicine and animal science.

Kafkas Universitesi Veteriner Fakultesi Dergisi is an Open Access journal, which means that all content is freely available without charge to the user or his/her institution. Users are allowed to read, download, copy, distribute, print, search, or link to the full texts of the articles, or use them for any other lawful purpose, without asking prior permission from the publisher or the author. This is in accordance with the BOAI definition of Open Access.

2- The official language of our journal is English.

3- The manuscripts submitted for publication should be prepared in the format of Times New Roman style, font size 12, A4 paper size, 1.5 line spacing, and 2.5 cm margins of all edges. The legend or caption of all illustrations such as figure and table and their appropriate position should be indicated in the text. Refer to tables and figures in the main text by their numbers. Also figure legends explanations should be given at the end of the text.

The figures should be at least 300 dpi resolution.

The manuscript and other files (figure etc.) should be submitted by using online manuscript submission system at the address of http://vetdergi.kafkas.edu.tr/

During the submission process, the authors should upload the figures of the manuscript to the online manuscript submission system. If the manuscript is accepted for publication, the Copyright Agreement Form signed by all the authors should be sent to the editorial office.

4- The authors should indicate the name of the institute approves the necessary ethical commission report and the serial number of the approval in the material and methods section. If necessary, the editorial board may also request the official document of the ethical commission report. In case reports, a sentence stating that "informed consent" was received from the owner should be added to the main document. If an ethical problem is detected (not reporting project information, lack of ethical committee information, conflict of interest, etc.), the editorial board may reject the manuscript at any stage of the evaluation process.

5- Authors should know and take into account the "Generative Artificial Intelligence (AI)" and other matters listed in the **"Ethical Principles and Publication Policy"** section regarding scientific research and authors.

6- Types of Manuscripts

Original (full-length) manuscripts are original and proper scientific papers based on sufficient scientific investigations, observations and experiments.

Manuscripts consist of the title, abstract and keywords, introduction, material and methods, results, discussion, and references and it should not exceed 12 pages including text. The number of references should not exceed 50. The page limit does not include tables and illustrations. Abstract should contain 200±20 words.

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

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

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

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

Reviews are original manuscripts that gather the literature on the current and significant subject along with the commentary and findings of the author on a particular subject (It is essential that the author/s have international scientific publications on this subject). The title and summary of this manuscript should be prepared as described for the full-length original articles and the remaining sections should be followed by introduction, text (with appropriate titles), conclusion, and references.

"Invited review" articles requested from authors who have experience and recognition in international publishing in a particular field are primarily published in our journal.

Review articles submitted to our journal must be prepared in accordance with any of the three categories listed below.

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

Systematic reviews include the search for original studies published in that field on a specific topic, the evaluation of validity, synthesis and interpretation within a systematic methodology.

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

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

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

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

Example: Yang L, Liu B, Yan X, Zhang L, Gao F, Liu Z: Expression of ISG15 in bone marrow during early pregnancy in ewes. *Kafkas Univ Vet Fak Derg*, 23 (5): 767-772, 2017. DOI: 10.9775/kvfd.2017.17726

If the reference is a book, it should follow surnames and initial letters of the authors, title of the book, edition number, page numbers, name and location of publisher and year of publication. If a chapter in a book with an editor and several authors is used, names of chapter authors, name of chapter, editors, name of the book, edition number, page numbers, name and location of publisher and year of publication and the formatting should be performed as shown in the example below.

Example: Mcllwraith CW: Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed., 339-447, Lea and Febiger, Philadelphia, 1988.

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

In the references can be reached online only, the web address and connection date should be added at the end of the reference information. The generally accepted scientific writing instructions must comply with the other references. Abbreviations, such as "et al" and "and friends" should not be used in the list of the references.

Follow the link below for EndNote Style of Kafkas Universitesi Veteriner Fakultesi Dergisi;
https://researchsoftware.com/downloads/journal-faculty-veterinary-medicine-kafkas-university

9- Latin expression such as species names of bacteria, virus, parasite, and fungus and anatomical terms should be written in italic character, keeping their original forms.

10- The editorial board has the right to perform necessary modifications and a reduction in the manuscript submitted for publication and to express recommendations to the authors. The manuscripts sent to authors for correction should be returned to the editorial office within a month. After pre-evaluation and agreement of the submitted manuscripts by the editorial board, the article can only be published after the approval of the field editor and referee/s specialized in the particular field.

11- All responsibilities from published articles merely belong to the authors. According to the ethical policy of our journal, plagiarism/self-plagiarism will not be tolerated. All manuscripts received are checking by plagiarism checker software, which compares the content of the manuscript with a broad database of academic publications.

12- The editorship may request the language editing of the manuscript submitted to the journal. If the article is accepted, it will not be published without language editing. Before publication, a declaration and/or certificate stating that proofreading is done by a registered company will be requested from the corresponding author.

13- No fee is charged at any stage in Kafkas Üniversitesi Veteriner Fakültesi Dergisi (No APC/APF)

SUBMISSION CHECKLIST

Please use below list to carry out a final check of your submission before you send it to the journal for review. Ensure that the following items are present in your submission:

- Cover Letter

• Importance and acceptability of the submitted work for the journal have been discussed (Please avoid repeating information that is already present in the abstract and introduction).

• Other information has been added that should be known by the editorial board (e.g.; the manuscript or any part of it has not been published previously or is not under consideration for publication elsewhere.

- Title Page

• Title, Running Title (should be a brief version of the title of your paper, no exceed 50 characters)

• The author's name, institutional affiliation, Open Researcher and Contributor ID (ORCID)

• Congress-symposium, project, thesis etc. information of the manuscript (if any)

• Corresponding author's address, phone, fax, and e-mail information

- Manuscript

• Title, abstract, keywords and main text

• All figures (include relevant captions)

• All tables (including titles, description, footnotes)

• Ensure all figure and table citations in the text match the files provided

- Declarations

• Availability of Data and Materials

• Acknowledgements

• Funding Support

• Competing Interests

• Generative Artificial Intelligence (AI)

• Authors' Contributions

Further Considerations

• Journal policies detailed in this guide have been reviewed

• The manuscript has been "spell checked" and "grammar checked"

• Relevant declarations of interest have been made

• Statement of Author Contributions added to the text

• Acknowledgment and conflicts of interest statement provided