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## REVIEW ARTICLE

# Emergence and Spread of Antimicrobial Resistance in Bacterial Food-Borne Pathogens

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## Abstract

The emergence of antimicrobial resistance (AMR) in bacterial food-borne pathogens is a real concern for public health and food security worldwide. *Salmonella*, *Escherichia coli*, *Campylobacter*, *Listeria monocytogenes*, and *Shigella* pathogens are becoming resistant to antimicrobials commonly used to treat infections, reducing the effectiveness of treatment and increasing the threat of fatal disease. Risk factors associated with this crisis include excess and inappropriate use of antimicrobials in both human and veterinary medicine, extensive use in food animal production to promote growth and prevent illness, poor hygienic standards in the food production chain, and the resurgence of antimicrobial resistance genes in environmental reservoirs. Antimicrobial resistance in bacteria affecting human beings, animals, and ecosystems represents a critical One Health challenge. Its implications are severe and include greater morbidity and mortality, longer stays in hospitals, and economic burden on health care and agriculture. The fight against AMR requires the rationalized use of antibiotics, more regulation, good agricultural practices, and sustainable options, including probiotics, phytochemicals, bacteriophages, and vaccines. Priorities should be directed toward new antimicrobials, alternative treatments, and combined surveillance in the future. This review article has described the key elements of AMR in food-borne pathogens.

**Keywords:** Foodborne pathogens, Antimicrobial resistance, One health, Alternative therapies

## INTRODUCTION

Food-borne bacterial pathogens represent a significant global public health issue because they are able to contaminate food and water, as well as cause acute illness and contribute to large-scale outbreaks [1]. They are mainly spread via poorly stored foodstuffs of animal and plant sources, undercooked, or contaminated food items, and their effects go far beyond acute morbidity to long-term effects and enormous economic burdens [2]. Some of the most important pathogens include *Salmonella* spp., *E. coli* (particularly Shiga toxin-producing strains such as O157:H7), *Campylobacter* spp., *L. monocytogenes*, and *Shigella* spp. [3]. These bacteria not only lead to gastroenteritis, systemic infection, and long-term health issues, but it is also becoming harder to handle, not only because of their ability to acquire and transmit antimicrobial resistance (AMR). The morbidity and mortality of food-borne disease are overwhelming. The World Health Organization (WHO) estimates that

over 600 million individuals in the world are affected annually by food-borne diseases, with a death toll of about 420,000, and children under the age of five years are particularly vulnerable [4]. A significant part of this burden is due to bacterial pathogens, especially in low- and middle-income countries where inadequate food safety measures, lack of healthcare services/ affordable healthcare, and surveillance mechanisms contribute to this burden. These infections produce high economic costs due to the expenses of healthcare, productivity loss, and product recalls, as well as restrictive trade, which is increasing because of globalization, climate change, and international food trade [5]. Such realities point to why such a One Health approach, which connects human, animal, and environmental health, is critical.

Adding to this difficulty is the development of antimicrobial resistance in food-borne pathogens that has become one of the most significant global health threats of the 21<sup>st</sup> century [6,7]. Food-producing animals have



broad use of antibiotics in treatment, prevention, and even to promote growth, which has a selective pressure on resistant strains that can easily transfer to humans through the food chain, direct contact, or environmental sources. Pathogens that cause food-borne diseases are genetically flexible and can rapidly acquire resistance genes through horizontal gene transfer and therefore exacerbate the issue [8]. Consequently, resistant pathogens, like fluoroquinolone-resistant *Campylobacter* and multidrug-resistant *Salmonella*, and extended-spectrum  $\beta$ -lactamase (ESBL)-producing *E. coli*, are getting harder to treat, sometimes necessitating expensive or toxic alternatives. The resultant effect is an increased burden of long-term illness, increased hospitalization, and mortality [9].

Therefore, AMR in food-borne bacterial pathogens poses a microbiological challenge, a global health and food security, and an economic crisis [10]. To solve this problem, a holistic and multi-disciplinary strategy is needed that incorporates medicine, veterinary science, agriculture, and environmental management in order to reduce resistance throughout the food chain [11]. This review thus identifies the rise and prevalence of antimicrobial resistance in food-borne bacterial pathogens, their resistance mechanisms, modes of transmission, public health consequences, and mitigation measures.

## MAJOR BACTERIAL FOOD-BORNE PATHOGENS OF CONCERN

Bacterial pathogens are globally prevalent, frequently associated with food-borne outbreaks, and have a significant impact on food safety and public health. *Salmonella*, *Escherichia coli*, *Campylobacter*, *Listeria monocytogenes*, and *Shigella* are among the leading causes of food-borne illnesses worldwide, contributing to considerable morbidity, mortality, and economic losses. Their ability to contaminate a wide range of food products, persist along the food production chain, and increasingly exhibit antimicrobial resistance further underscores their public health importance (Fig. 1) [3].

***Salmonella* spp.** remains one of the leading causes of food-borne illnesses globally, transmitted through contaminated poultry, eggs, meat, and dairy products, as well as fresh produce [12]. Non-typhoidal *Salmonella* (NTS) typically causes gastroenteritis with diarrhea, fever, and abdominal cramps, but can also result in invasive infections in vulnerable populations. Typhoidal *Salmonella*, such as *S. Typhi* and *S. Paratyphi*, are restricted to humans and cause systemic enteric fever [13-15]. The growing concern lies in antimicrobial resistance, with NTS showing multidrug resistance to ampicillin, chloramphenicol, and TMP-SMX, alongside increasing resistance to fluoroquinolones and third-generation cephalosporins

due to extended-spectrum  $\beta$ -lactamases (ESBLs) like *bla*CTX-M. Alarming, extensively drug-resistant (XDR) *S. Typhi* strains, particularly reported from Pakistan, are resistant to almost all commonly used antibiotics except azithromycin and carbapenems, complicating treatment options [16]. This makes *Salmonella* not only a significant cause of morbidity and mortality but also a priority pathogen in the fight against antimicrobial resistance.

***Escherichia coli***, while a normal commensal of the human gut, includes several pathogenic strains that are major food-borne threats [17,18]. Shiga toxin-producing *E. coli* (STEC), particularly O157:H7, causes hemorrhagic colitis and life-threatening hemolytic uremic syndrome (HUS), often linked to undercooked beef, unpasteurized milk, and leafy vegetables [18,19]. Enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), and enteroaggregative *E. coli* (EAEC) are important causes of travelers' and childhood diarrhea, especially in developing countries. Resistance among pathogenic *E. coli* is a rising issue, with many strains acquiring ESBL genes (*bla*CTX-M) and plasmid-mediated colistin resistance genes (*mcr*-1) that spread rapidly through the food chain, especially via poultry and meat [20]. Infections with multidrug-resistant *E. coli* are increasingly difficult to treat, and in the case of STEC, antibiotics are avoided altogether because they can trigger toxin release and worsen disease [21]. This dual challenge of virulence and resistance makes *E. coli* a significant global concern [22,23].

***Campylobacter* spp.** mainly, *C. jejuni* and *C. coli* are the most frequently reported bacterial causes of human gastroenteritis worldwide. Infection occurs through undercooked poultry, unpasteurized milk, or contaminated water, with very low infectious doses sufficient to cause disease [24]. Symptoms include fever, abdominal pain, and bloody diarrhea, with serious post-infection complications such as Guillain-Barré syndrome. Resistance in *Campylobacter* is particularly troubling: fluoroquinolone resistance, caused by mutations in the *gyrA* gene, is now widespread, limiting treatment options [25]. Tetracycline resistance via the *tet*(O) gene is also common, while macrolide resistance, though lower, is emerging. Since macrolides (e.g., azithromycin) are the preferred treatment for severe cases, the rise of resistance threatens to undermine the last reliable therapeutic option. The close link between antimicrobial use in poultry and resistance in *Campylobacter* highlights the need for strict regulation and surveillance in food production systems [25].

***Listeria monocytogenes*** is an opportunistic food-borne pathogen of high concern due to its ability to cause listeriosis, a severe invasive infection in pregnant women, newborns, the elderly, and immunocompromised individuals [26]. Unlike many other pathogens, *Listeria* can survive and grow at refrigeration temperatures and persist in food-processing environments through biofilm

formation [27]. Outbreaks are commonly linked to ready-to-eat foods, deli meats, unpasteurized cheeses, and smoked fish. Clinical manifestations include septicemia, meningitis, and adverse pregnancy outcomes such as stillbirth or neonatal sepsis [28]. Although *Listeria* remains largely susceptible to first-line antibiotics such as ampicillin combined with gentamicin, resistance has occasionally been reported to tetracyclines and macrolides through genes like *tet(M)* and *erm(B)*. Of greater concern is its resistance to disinfectants and sanitizers, which enables persistence in food-processing plants, increasing the risk of contamination and outbreaks [29]. Preventing *Listeria* infections, therefore, relies heavily on strict hygiene measures and food safety regulations rather than therapeutic interventions.

**Shigella spp.** Highest infectivity is seen with *Shigella* spp. (*S. sonnei* and *S. flexneri*) which can cause disease after a few organisms within the body. They easily spread via contaminated foods and drinks and via person-to-person contact, and so are especially prevalent in places with poor sanitation [30]. Shigellosis clinically manifests as dysentery with fever, cramping abdominal pains, and bloody diarrhea. Treatment can be very much required in order to reduce the morbidity and avert the spread; however, resistance is quick to develop. Ciprofloxacin, TMP-SMX, and azithromycin-resistant strains are now widespread, and in recent years, extensively drug-resistant (XDR) *S. sonnei* has been reported across the world, providing few effective oral treatment options [31]. This tendency presents a significant public health problem because *Shigella* epidemics can spread rapidly, and the emergence of resistant bacteria adds further limitations to treatment choices in both communal and medical institutions. These major bacterial foodborne pathogens are shown in Fig. 1.

### Emerging Food-Borne Bacterial Pathogens

Emerging food-borne bacterial pathogens are also of

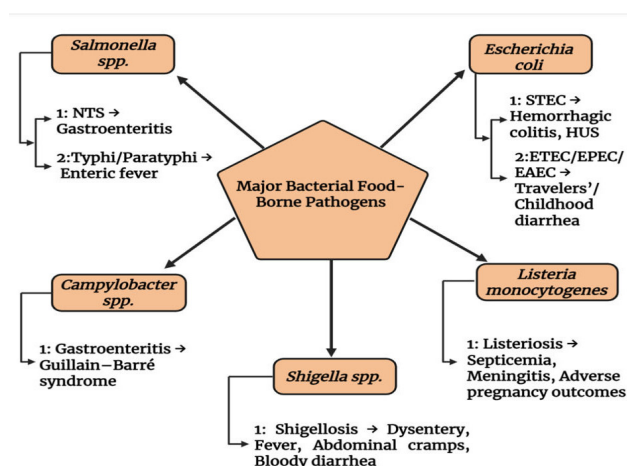


Fig 1. Major bacterial food-borne pathogens of concern

concern. *Vibrio parahaemolyticus* and *V. vulnificus*, which are linked with the consumption of seafood, are of increasing concern because they are spreading as a consequence of climate change [32]. *Yersinia enterocolitica*, which is mainly spread by pork, has been found to cause outbreaks regionally, and the bacteria are resistance to fluoroquinolones and cephalosporins [33]. *Cronobacter sakazakii* is a pathogen that is present in powdered infant formula and is a specific hazard to neonates, resulting in meningitis and septicemia with high mortality rates [34]. They are less frequent, but mention of these causative organisms illustrates how dynamic food-borne diseases are and how new hazards are being detected, commonly in conjunction with frontline antibiotic resistance. These emerging food-borne bacterial pathogens are explained in the following section:

**Vibrio parahaemolyticus:** *V. parahaemolyticus* is a naturally occurring halophilic, Gram-negative bacterium in estuarine and marine habitats is *Vibrio parahaemolyticus*. One of the most common causes of gastroenteritis linked to seafood is eating raw or undercooked shellfish, including clams, crabs, and oysters [35]. The thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) toxins of *V. parahaemolyticus* are primarily responsible for its pathogenicity; they damage intestinal epithelial cells and cause fever, cramping in the abdomen, watery diarrhea, and nausea. Infection rates in temperate places have increased because of the expansion of their ecological niche brought on by climate change, particularly ocean warming [36].

**Vibrio vulnificus:** Another halophilic marine bacterium linked to serious wound and foodborne illnesses is *Vibrio vulnificus*. It can infect shellfish, especially oysters, and is prevalent in warm coastal waters. Ingestion or contact with tainted seawater through exposed wounds can result in infection [37]. Known for its high death rate, *V. vulnificus* frequently causes necrotizing fasciitis and septicemia, particularly in people with diabetes, liver illness, or immunocompromised conditions. The production of cytotoxins, metalloproteases, and capsular polysaccharides that aid in tissue destruction and immune evasion is linked to the organism's virulence [38]. Because of the growing worldwide seafood trade and rising sea surface temperatures, its increasing prevalence is becoming a significant problem.

**Yersinia enterocolitica:** A chronic concern with chilled goods, especially milk, pig products, and ready-to-eat items, is *Yersinia enterocolitica*, a psychrotrophic, Gram-negative bacterium that can survive and grow at refrigeration temperatures [39]. It results in yersiniosis, which can mimic appendicitis and is characterized by fever, enterocolitis, and mesenteric lymphadenitis. A plasmid-encoded type III secretion system (T3SS) and outer membrane proteins that promote adhesion, invasion, and resistance to host

immune systems are examples of virulence factors <sup>[40]</sup>. The organism is a major concern for food sectors that use lengthy cold chains because of its capacity to survive at low temperatures and withstand cold storage.

***Cronobacter sakazakii*:** An opportunistic bacterium primarily associated with powdered infant formula, *Cronobacter sakazakii* (previously known as *Enterobacter sakazakii*), poses a significant risk to newborns and babies <sup>[41]</sup>. This Gram-negative bacterium can cause serious infections like necrotizing enterocolitis, sepsis, and newborn meningitis. Its survival in dry food matrices and resistance to desiccation help explain why it persists in production settings. To increase its survival during processing and storage, *C. sakazakii* creates biofilms and has stress tolerance mechanisms <sup>[42]</sup>. Its importance to public health is highlighted by the severity of infection consequences, even if its prevalence is minimal.

## MECHANISMS OF ANTIMICROBIAL RESISTANCE IN FOOD-BORNE PATHOGENS

There are now a variety of mechanisms through which food-borne bacterial pathogens overcome the effects of antimicrobial agents, and this makes their treatment and control measures complicated <sup>[43,44]</sup>. These are drug inactivation via enzymes, efflux pumps, and decreasing permeability, target change or shielding, and horizontal gene transfer (Fig. 2) <sup>[18]</sup>. Importantly, the relative contribution of these mechanisms varies among major food-borne pathogens and explains the distinct resistance patterns observed in organisms such as *Salmonella*, *Campylobacter*, *Escherichia coli*, and *Shigella*. Cumulatively, these mechanisms of adaptation not only contribute to bacterial survival on exposure to antimicrobial stress but also promote the distribution and perpetuation of resistance throughout ecosystems <sup>[45]</sup>.

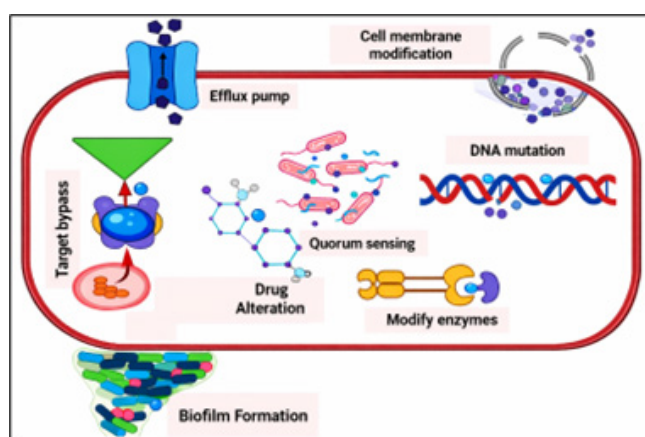


Fig 2. Phenotypic mechanism of antibiotic resistance in foodborne bacterial pathogens

### Enzymatic Inactivation of Drugs

The enzymatic alteration or deactivation of antimicrobial compounds is one of the most common AMR mechanisms of food-borne pathogens. Numerous bacteria will make enzymes, e.g.,  $\beta$ -lactamases that cleave the  $\beta$ -lactam ring in penicillin and cephalosporins, making them inactive. Extended-spectrum beta-lactamases (ESBLs) are the most common cause of  $\beta$ -lactam resistance in *E. coli* and *Salmonella* spp. <sup>[46]</sup>, and are particularly relevant to the emergence of multidrug-resistant and extensively drug-resistant (XDR) *S. Typhi*, where ESBLs and carbapenemases severely limit therapeutic options. ESBLs cause resistance to large numbers of  $\beta$ -lactams (including third-generation cephalosporins). On the same note, carbapenemases destroy carbapenems, which are called antibiotics of last resort. Aminoglycoside-modifying enzymes (AMEs) physically alter aminoglycosides by adding phosphate, acetate, or adenosine groups, hence reducing their affinity to the ribosomal sites <sup>[47]</sup>. These enzymatic alterations highly limit the treatment measures and the rise of multidrug resistance in food-borne bacteria.

### Efflux Pumps and Reduced Permeability

Efflux systems and changes in membrane permeability are another primary defense strategy employed by food-borne pathogens. Efflux pumps are membrane-bound proteins that actively transport antimicrobials to the extracellular space, reducing the drug concentrations to below efficacious levels <sup>[48]</sup>. For example, the efflux pump AcrAB-TolC in *E. coli* and *Salmonella* confers resistance to several classes of pharmaceutical antibiotics, including fluoroquinolones, tetracyclines, and chloramphenicol <sup>[49]</sup>. The CmeABC efflux pump is very important in *Campylobacter* spp. in resistance to macrolides and fluoroquinolones. Meanwhile, decreased expression or structural modulation of outer membrane porins reduces antibiotic uptake <sup>[50]</sup>. Collectively, these mechanisms promote multidrug resistance and enable the bacteria to persist even against a varied collection of antimicrobial substances.

### Target Modification and Protection

The mechanism of action of an antimicrobial agent is usually its binding to a particular target in bacteria, and mutation or variation of that target may cause resistance <sup>[45]</sup>. *Campylobacter* and *Salmonella* have point mutations in their *gyrA* gene that change the DNA gyrase, resulting in fluoroquinolone resistance <sup>[18]</sup>. Similarly, mutation in the ribosomal RNA or ribosomal proteins may lead to macrolide, tetracycline, and aminoglycoside resistance (Fig. 2) <sup>[51]</sup>. The other example is for *Streptococcus pneumoniae*, in which mutations in penicillin-binding proteins decrease  $\beta$ -lactam binding affinity (this again is rarer in the prototypical food-borne pathogen). Bacteria

will also employ protective proteins, including *tet(M)*, that protect the ribosomal targets against tetracycline. Such alterations help bacteria to grow and survive beyond antimicrobials [52].

### Horizontal Gene Transfer

Horizontal gene transfer (HGT) is a key driver in the spread of AMR in food-borne pathogens, contributing to the rapid uptake of resistance determinants that build up in other bacteria.  $\beta$ -lactam, aminoglycoside, and sulfonamide resistance genes can be found on plasmids, which transfer between bacteria easily in a cross-species and cross-genus manner since they are extrachromosomal elements of DNA [53]. Transposons are mobile pieces of genetic material that incorporate resistance genes into the DNA of chromosomes or plasmids and enable their transmission. Integrons encode multidrug resistance and integrate gene cassettes encoding resistance determinants [8]. As another example, integrons in the *Shigella* and *Salmonella* are often carry resistance genes to sulfonamides, aminoglycosides, and trimethoprim [54]. In *Salmonella* and *Shigella*, integrons frequently harbor resistance genes against sulfonamides, aminoglycosides, and trimethoprim, contributing to the emergence of multidrug-resistant and XDR lineages. Environments within the food chain, livestock production systems, and environmental reservoirs facilitate horizontal gene transfer (HGT), serving as reservoirs for the emergence and dissemination of novel multidrug-resistant food-borne pathogens that can be transmitted between animals and humans [53].

## DRIVERS OF AMR EMERGENCE IN FOOD-BORNE BACTERIA

The emergence of antimicrobial resistance (AMR) of food-borne pathogens is not a random process, but its rapid development is a direct consequence of the selective pressures and interdependent activities in human health, agriculture, food production, and the environment (Table 1) [55]. Mounting evidence points to several important drivers that promote the existence and proliferation of resistant bacteria throughout the continuum of the farm-to-fork. This has been a significant global public health hazard [56].

### Overuse and Misuse of Antibiotics in Human Medicine

Inappropriate prescription of antibiotics in medical care among human beings is one of the major contributors to AMR in human health. Such are the use of antibiotics to treat viral diseases, over-the-counter self-medication, inadequate treatment with antibiotics, and excessive use of broad-spectrum agents, which exert potent selective pressure on resistance [57]. These selective conditions, such

as food-borne pathogens like *Salmonella* and *Shigella*, can cause recurrent infections in humans, leading to repeated exposure to antibiotics, thereby letting the resistant strains multiply. Also, multidrug-resistant strains of enteric pathogens acquired in the hospital setting may be the cause of hospital-acquired infections whose transmission into the community could be accomplished by host carriers and through contaminated sewage streams [58].

### Antibiotic Use in Food Animals

One of the most significant causes of food-borne bacterial resistance is the extensive use of antimicrobials in the food production of livestock. Antibiotics are often administered not only for treatment (therapy) but also for disease prevention (prophylaxis) and growth promotion. Continuous low-dose exposure creates an ideal environment for the selection of resistant bacteria in the gut flora of animals [59]. These resistant pathogens, such as *E. coli*, *Salmonella*, and *Campylobacter*, can then contaminate meat, milk, eggs, and other animal-derived foods [60]. For instance, the widespread use of tetracyclines and fluoroquinolones in poultry and cattle farming has been strongly linked to resistance in *Campylobacter* and *E. coli*. Moreover, resistant strains can spread to farm workers and surrounding communities, creating a One Health problem that connects animal, human, and environmental health (Table 1) [61].

### Transmission Pathways of AMR Food-Borne Pathogens

**Farm-to-Fork Continuum:** The farm-to-fork continuum describes the journey of food from primary production to consumption, and at every stage, resistant bacteria can enter or proliferate. On farms, resistant pathogens originating from livestock or contaminated feed can contaminate meat, milk, or eggs [78]. In aquaculture and produce farming, resistant bacteria from fertilizers, and irrigation water may persist on seafood, fruits, and vegetables. Once these foods enter the processing chain, opportunities for amplification and cross-contamination increase, making the entire continuum a major route for AMR dissemination [79].

**Food Processing and Handling Practices:** In slaughterhouses, dairies, and food processing plants, lapses in hygiene and sanitation allow resistant pathogens to contaminate products. For example, improper separation of clean and contaminated carcasses during meat processing may spread resistant *Salmonella* [80]. Similarly, inadequate pasteurization or unsanitary handling of milk and dairy products can allow resistant *L. monocytogenes* or *E. coli* strains to persist. Resistant bacteria can also survive in biofilms that form on food-contact surfaces, making eradication difficult and contributing to long-term contamination [81].

**Table 1.** Drivers of antimicrobial resistance in food-borne bacteria

| Driver of AMR                                     | Key Practices/Source                       | Examples of Commonly Used Antibiotics        | Major Pathogens Involved                                  | Mechanism of Resistance Spread                                | Public Health Implications                       | References |
|---------------------------------------------------|--------------------------------------------|----------------------------------------------|-----------------------------------------------------------|---------------------------------------------------------------|--------------------------------------------------|------------|
| Antibiotic Use in Food Animals (Therapy)          | Treatment of sick animals with antibiotics | Penicillins, tetracyclines, fluoroquinolones | <i>E. coli</i> , <i>Salmonella</i> , <i>Campylobacter</i> | Selection pressure enriches resistant strains in gut flora    | Infections in humans become harder to treat      | [62]       |
| Antibiotic Use in Food Animals (Prophylaxis)      | Preventive use in herds/flocks             | Sulfonamides, macrolides                     | <i>E. coli</i> , <i>Salmonella</i>                        | Maintains background resistance even without active infection | Reduces drug effectiveness in clinical medicine  | [62]       |
| Antibiotic Use in Food Animals (Growth Promotion) | Low-dose continuous feed additives         | Tetracyclines, streptomycin                  | <i>Enterococcus</i> spp., <i>E. coli</i>                  | Promotes selection of multidrug-resistant gut bacteria        | Transmission to humans via meat, eggs, milk      | [63]       |
| Poultry Farming                                   | Antibiotics in broiler & layer systems     | Fluoroquinolones, tetracyclines              | <i>Campylobacter jejuni</i> , <i>E. coli</i>              | Resistant pathogens contaminate meat/eggs                     | Foodborne outbreaks with drug-resistant strains  | [64, 65]   |
| Cattle & Dairy Farming                            | Antibiotics for mastitis, growth           | $\beta$ -lactams, tetracyclines              | <i>Salmonella</i> spp., <i>L. monocytogenes</i>           | Resistant bacteria in milk & meat                             | Milk-borne resistant infections                  | [66]       |
| Aquaculture                                       | Antibiotics in fish/shrimp farms           | Oxytetracycline, sulfonamides                | <i>Vibrio</i> spp., <i>Aeromonas</i> spp.                 | Resistance genes spread via water                             | Marine resistome expansion                       | [67]       |
| Slaughterhouse Contamination                      | Cross-contamination during slaughter       | N/A                                          | <i>Salmonella</i> , <i>Campylobacter</i>                  | Spread from gut contents to carcasses                         | Multidrug-resistant pathogens enter food chain   | [68]       |
| Dairy Processing                                  | Post-pasteurization contamination          | N/A                                          | <i>L. monocytogenes</i>                                   | Survival and recontamination in dairy products                | Risk of outbreaks via cheese/milk                | [69]       |
| Produce Contamination                             | Irrigation with contaminated water         | N/A                                          | <i>E. coli</i> O157:H7, <i>Salmonella</i>                 | Resistant strains spread from manure/water                    | Fresh produce as AMR carriers                    | [70]       |
| Food Handlers                                     | Poor hygiene in food processing            | N/A                                          | <i>Shigella</i> spp., <i>E. coli</i>                      | Transfer via human contact                                    | Foodborne outbreaks in retail/household settings | [71, 72]   |
| Global Food Trade                                 | Export/import of contaminated foods        | N/A                                          | <i>Salmonella</i> , <i>Campylobacter</i>                  | Rapid transboundary movement                                  | Globalization of resistant pathogens             | [73]       |
| Environmental Release (Manure Use)                | Animal manure as fertilizer                | Residual antibiotics, resistant bacteria     | <i>E. coli</i> , <i>Salmonella</i>                        | Resistance genes move to soil microbes & crops                | Human exposure via raw produce                   | [74]       |
| Environmental Release (Wastewater)                | Hospital/industrial effluents              | Multiple antibiotic residues                 | Environmental bacteria & pathogens                        | Horizontal gene transfer in water bodies                      | Creates “hotspots” of AMR                        | [75]       |
| Soil Resistome                                    | Long-term accumulation of resistance genes | N/A                                          | <i>Pseudomonas</i> , <i>Enterobacteriaceae</i>            | Natural gene exchange via plasmids, transposons               | Source of novel AMR traits                       | [76]       |
| Aquatic Ecosystems                                | Runoff from farms, aquaculture             | Tetracyclines, quinolones                    | <i>Vibrio</i> , <i>Aeromonas</i> , <i>Enterococcus</i>    | Resistance genes persist in sediments & rivers                | Zoonotic spillover to humans and wildlife        | [77]       |

**Cross-Contamination in Retail and Household Settings:**

During handling and preparation of food products in markets or at home, cross-contamination is a major hazard factor. Raw meat may transfer resistant bacteria to fresh vegetables or ready-to-eat foodstuffs by using a common cutting board, a knife, or a storage vessel [82]. Hygienic cooking or refrigeration is also relevant in

terms of bacterial survival and transmission [83]. Even low-level resistant strains can infect people, in case they colonize especially children, older people, or people with compromised immunity.

**International Trade and Travel:** Globalization also boosts the movement of resistant pathogens across borders. Seafood, fresh produce, and meat trade may bring foreign

bacterial species whose resistant nature was not a common phenomenon <sup>[84]</sup>. Similarly, international travel exposes individuals to resistant strains present in food or water, which can then spread upon their return home. This unregulated and swift movement facilitates the internationalization of AMR transmission, and it explains the need to work more internationally to combine surveillance systems <sup>[85]</sup>.

## PUBLIC HEALTH IMPLICATIONS OF AMR IN FOOD-BORNE PATHOGENS

The existence of antimicrobial resistance (AMR) in foodborne pathogens poses a significant threat to the population, whose impacts extend beyond personal infection, affecting healthcare systems, food safety, and global economies <sup>[18,86,87]</sup>. Antibiotic resistance in pathogens not only complicates treatment outcomes but also increases the risk of transmission of pathogens with serious consequences to society <sup>[60]</sup>.

### Increased Morbidity and Mortality

Diseases resulting from antimicrobial-resistant food-borne pathogens are often associated with increased morbidity and mortality, as opposed to those affected by susceptible pathogens <sup>[65,88]</sup>. Antibiotic-resistant *Salmonella*, *E. coli*, and *L. monocytogenes* infections frequently lead to long-term illnesses, increased complications, and increased incidences of hospitalizations. There is a disproportionately increased risk in the vulnerable group, e.g. those are children, the elderly, and the immunocompromised, with some of the infections developing into life-threatening ailments, e.g., septicemia or meningitis <sup>[89]</sup>.

### Limited Treatment Options

The limitation of effective treatment options is one of the most relevant immediate effects of AMR. The first-line antibiotics have included fluoroquinolones and third-generation cephalosporins, to which there has been increased resistance in food-borne pathogens. In some cases, pathogens exhibit multi-drug resistance (MDR), rendering conventional therapies ineffective and forcing reliance on last-resort antibiotics such as carbapenems or colistin <sup>[90]</sup>. This not only complicates treatment regimens but also increases the risk of adverse side effects and treatment failure.

### Economic Burden on Healthcare and the Food Industry

The economic implications of resistant food-borne pathogens are twofold:

**Healthcare Systems:** Resistant infections increase the length of hospital stays, diagnostic costs, and treatment expenses, straining already burdened healthcare systems.

The need for more expensive or prolonged therapies further amplifies costs <sup>[91]</sup>.

**Food Industry:** Outbreaks of resistant pathogens can lead to mass recalls, trade restrictions, reputational damage, and loss of consumer trust. Also, the cost of the operations is greatly increasing due to regulatory interventions, testing requirements, and stricter biosecurity measures. Taken together, AMR exerts an economic dual burden on the food production industry and on public health <sup>[92]</sup>.

**Zoonotic and Pandemic Potential:** Food-borne pathogens are zoonotic in nature and are transmitted between animals and humans either through food, direct contact, or via environmental contamination. With the introduction of resistance characteristics, the threats grow exponentially, since these pathogens can evolve far and wide both in species and geographical distributions <sup>[87]</sup>. Globalization of food trade and human movements can lead to localized outbreaks becoming a worldwide menace. The development of pandemic-potential strains, e.g., multidrug-resistant *Salmonella* or *E. coli*, shows the ability of AMR food-borne pathogens to cross borders and become global crises, particularly in those environments with low surveillance and limited healthcare resources <sup>[93]</sup>.

## STRATEGIES TO MITIGATE AMR IN FOOD-BORNE PATHOGENS

The alarming rise of antimicrobial resistance (AMR) in food-borne pathogens requires multifaceted mitigation strategies that combine human, animal, and environmental health interventions under a One Health approach <sup>[94]</sup>. Preventing the spread of resistant strains and reducing antibiotic dependence are essential to safeguarding public health, food security, and global economies (*Table 2*) <sup>[95,96]</sup>.

## FUTURE DIRECTIONS

Tackling the growing problem of antimicrobial resistance (AMR) in food-borne pathogens requires practical, innovative, and globally coordinated solutions. With resistant strains of *Salmonella*, *E. coli*, and *Campylobacter* becoming more common, the search for safe and effective alternatives to conventional antibiotics is more important than ever. Promising options include bacteriophages that specifically attack resistant bacteria, antimicrobial peptides produced by natural immune defenses, nanotechnology-based drug delivery systems designed to reduce toxicity while improving precision, and the use of probiotics and plant-derived compounds to strengthen host immunity and block pathogen colonization. Vaccination in food animals, such as poultry, against *Salmonella*, also offers a way to lower infection rates and reduce the need for antibiotics. At the same time, modern tools like genomics, proteomics, and metagenomics can speed up the discovery

**Table 2.** Strategies to mitigate AMR in food-borne pathogens under One Health

| Strategy                    | Specific Action                             | Target Sector                         | Expected Impact                               | Examples/ Tools                          | References    |
|-----------------------------|---------------------------------------------|---------------------------------------|-----------------------------------------------|------------------------------------------|---------------|
| Rational Antibiotic Use     | Prescribe only when clinically indicated    | Human Medicine                        | Reduce unnecessary exposure to antimicrobials | Prescription guidelines                  | [97]          |
|                             | Correct dosing and full treatment adherence | Human Medicine                        | Minimize resistance selection                 | Clinical stewardship programs            | [97]          |
|                             | Avoid self-medication                       | Human Medicine                        | Prevent misuse and resistance                 | Public health education                  | [98]          |
| Veterinary Stewardship      | Use only under veterinary supervision       | Veterinary Medicine                   | Ensure responsible use                        | Prescription-only model                  | [99]          |
|                             | Ban use for growth promotion                | Veterinary Medicine                   | Reduce selective pressure                     | EU/WHO bans, alternative feed strategies | [99]          |
|                             | Limit prophylactic use                      | Veterinary Medicine                   | Prevent resistance buildup                    | Targeted metaphylaxis                    | [99]          |
| Good Agricultural Practices | Improve farm biosecurity and sanitation     | Agriculture                           | Reduce pathogen circulation                   | Hygiene protocols                        | [100]         |
| Food Safety Practices       | Prevent cross-contamination                 | Food Chain                            | Limit spread of resistant bacteria            | HACCP implementation                     | [101]         |
| Food Handling               | Proper cooking and storage                  | Consumers                             | Inactivate resistant bacteria                 | Food safety campaigns                    | [102]         |
| Alternatives to Antibiotics | Phytochemicals and essential oils           | Veterinary/ Agriculture               | Suppress resistant pathogens                  | Plant-based feed additives               | [103,104]     |
|                             | Probiotics and prebiotics                   | Veterinary/ Agriculture               | Enhance gut health and immunity               | <i>Lactobacillus</i> , inulin            | [103,105,106] |
|                             | Vaccines against pathogens                  | Veterinary                            | Reduce infection incidence                    | <i>Salmonella</i> vaccines in poultry    | [103]         |
| Policy & Regulation         | Enforce restrictions on antibiotic sales    | Governance                            | Minimize OTC misuse                           | Prescription-only policies               | [107]         |
|                             | Establish integrated surveillance networks  | One Health (Human–Animal–Environment) | Early detection of resistance                 | WHO-GLASS, OIE-WAHIS                     | [107]         |
| Global Cooperation          | Harmonize regulations and outbreak response | International                         | Prevent cross-border AMR threats              | Tripartite collaboration (WHO–FAO–OIE)   | [108]         |

of resistance genes, new therapeutic targets, and early outbreak signals. The One Health approach is central, recognizing that resistance spreads across humans, animals, and the environment, and highlighting the need for strong surveillance in farms, hospitals, food production systems, and even wastewater. Success will depend on collaboration between researchers, veterinarians, farmers, policymakers, and global organizations, with special attention to low- and middle-income countries where resources are limited. Sustainable, fair, and coordinated efforts will be key to protecting both food safety and public health.

## CONCLUSION

A major health concern across the globe is antimicrobial resistance among food-borne pathogens, e.g., *Salmonella*,

*E. coli*, *Campylobacter*, *L. monocytogenes*, and *Shigella*. This crisis is spurred by the misuse of antibiotics in humans and animals, their use in large amounts in food production, poor hygiene practices, and environmental reservoirs of resistance genes. The effects are far-reaching, including sickness and death, extended hospital stays, a reduction in costs incurred in health care, and lost profits in the economy. AMR, as a classical example of One Health, connects the health of humans, animals, and the environment. It should be handled by more responsible usage of antibiotics, tighter actions, and safer food processing and farming. Other potential options, like probiotics, bacteriophages, vaccines, and phytochemicals, are promising as additional ways to mitigate the use of antibiotics. Consequently, the COVID-19 pandemic has exacerbated the spread of AMR, which requires immediate international cooperation and interdisciplinary action.

With antimicrobial protection, we are securing the future of effective treatment, robust food systems, and population health.

## DECLARATIONS

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

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## REVIEW ARTICLE

# Evidence-Based Tiered Framework for Feline Pain Management: A Systematic Review

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**Abstract**

This systematic review developed an evidence-based framework for feline pain management. The systematic search of Scopus, ScienceDirect, and PubMed from 1980 to October 2023 found 42 eligible studies that explored pharmacological and non-pharmacological approaches to feline pain management. The outcome measures included analgesic efficacy, rescue analgesia, and adverse effects. Rescue analgesia was a key endpoint in multimodal therapies and was mostly used in severe pain models. Multimodal therapies were mainly employed in complex cases/procedures with severe pain potentials, resulting in higher rescue rate (16.6%) compared to monotherapy in routine procedures/neuter (5.29%). Pre-emptive analgesics were always associated with the best outcomes. The UNESP-Botucatu multidimensional scale was one of the most frequently used and validated pain assessment tools. The adverse effects were usually easy to manage, with the most frequent being opioid-related dysphoria and NSAID-related gastrointestinal effects. Following these results, we propose a tiered framework: a base of NSAIDs and local anaesthetics (Tier E1), supplemented with opioids for severe pain (Tier E2) and adjunctive stress-reducers (Tier E3). This is a pre-emptive, structured and severity-specific method necessary to ameliorate the cycle of treating pain insufficiently, thereby improving the welfare of cats, as well as staff safety and strengthening the veterinary practice.

**Keywords:** Feline pain management, Multimodal analgesia, Pre-emptive analgesia, Systematic review, Tiered framework

## INTRODUCTION

Pain affects the quality of life and well-being in cats, with the potential of delaying recovery and causing enduring physiological and behavioural alterations<sup>[1]</sup>. Cats' pain and stress are often overlooked, given their inability to express feelings. Despite all the progress in veterinary medicine, studies have shown that pain management in cats is still under-addressed compared to dogs<sup>[1]</sup>. A survey by Adams and Munoz revealed that 6.7% of cats were given analgesics after surgery compared to 16% of dogs undergoing similar procedures, including ovariohysterectomy and orchiectomy<sup>[1]</sup>. Cats are generally undertreated with

analgesics due to various factors, such as the absence of appropriate analgesic agents or the inability to detect pain through their distinctive behavioural patterns<sup>[2,3]</sup>.

Unmanaged pain in cats poses a major safety risk. Defensive aggression is directly provoked by fear and discomfort, which significantly raises the possibility of being bitten or scratched by handlers, veterinarians, and owners<sup>[4-6]</sup>. These injuries are directly and physically harmful and may introduce the risk of contracting zoonotic diseases. Trust in the veterinary team is lost when owners feel that their cat is not receiving the attention it deserves. These events have a detrimental effect on client satisfaction, indirectly through negative word-of-mouth and decreasing the



willingness to pursue future care, including necessary follow-up visits and vaccination<sup>[7]</sup>. As a result, distrust in pain-mitigating approaches and quality of care could have a direct financial implication on pets' long-term health<sup>[8]</sup>. Meanwhile, effective care leads to high client satisfaction, which is directly proportional to better practice and financial performance. Thus, proficient pain management is a critical factor in economic viability.

Pain recognition in cats is complicated by their inherent behavioural patterns, and the challenge in developing effective screening and measurement instruments. Despite the availability of some validated pain assessment scales, only 10% of surveyed veterinary clinics use these tools for postoperative pain measurement in cats<sup>[9]</sup>. Moreover, the complex nature of validated pain assessment tools poses a hindrance to their regular use in clinics<sup>[10]</sup>. This problem is further exacerbated by the lack of pain management education in veterinary schools, gender-specific pain perception, and the limited variety of appropriate analgesics<sup>[11,12]</sup>. Several drugs used in other animals are either not licensed or off label for cats, and veterinarians are unwilling to prescribe these medications due to fears of side effects and lack of specific usage guidelines<sup>[13,14]</sup>. The undertreatment of pain in cats is also influenced by factors such as transportation stress and anxiety when managed in a new environment, confinement in a carrier and inappropriate handling<sup>[15]</sup>. These factors may cause aggressive behaviour that complicates the treatment<sup>[16,17]</sup>. Cats that had negative experiences with veterinarians are more likely to feel anxious during their next appointments<sup>[18,19]</sup>. Neglecting pain results in insufficient utilisation of analgesics and less effective pain management<sup>[20]</sup>.

Effective pain management is essential in veterinary practice given its significance to animal welfare, human safety and economic efficiency. A stress and pain-free cat is easier to manage, creates confidence in the owner, and improves adherence to follow-up appointments. Therefore, a detailed review of the current evidence on pain management modalities in cats is pertinent to support clinical practice and advance feline welfare. By synthesising the available evidence, this review presents a clear and actionable framework that will enable clinicians to effectively and confidently manage feline pain. The implementation of evidence-based protocols is key to ending the vicious circle of undertreatment, thereby improving feline welfare and the relationships with clients, guaranteeing staff safety, and protecting the economic well-being of veterinary facilities. This systematic review aims to: (i) stratify the efficacy of analgesic interventions based on pain severity, (ii) analyse the effects of multimodal therapy and timing on pain-related outcomes, (iii) describe the available pain assessment tools, and (iv) to integrate the findings on efficacy, safety and global availability into a practical tiered framework for clinical decision making.

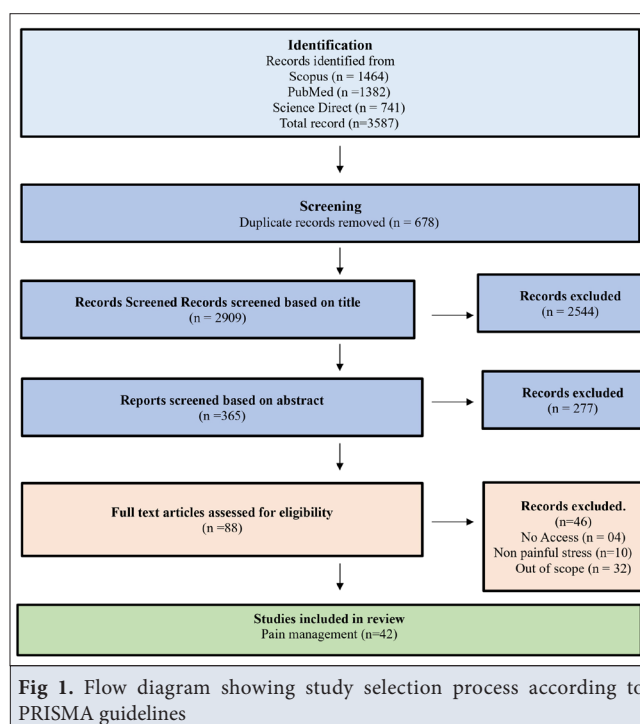
## MATERIAL AND METHODS

### Search Methods

Articles published between 1980 to October 24<sup>th</sup>, 2023, were searched across three digital databases: Scopus, ScienceDirect and PubMed. This time frame was considered to elucidate the different advanced methods and improvements in pain management in the last four decades. A methodical exploration was conducted utilising a set of predetermined terms by using the PICO tool. The keywords and search strings used in all the databases were “cat OR cats OR feline) AND (stress OR pain) AND “management”. The search was restricted to articles written in English. Duplicates found across the various databases were eliminated during the article selection process (*Fig. 1*). All the retrieved data were imported into a Microsoft Excel sheet for data cleaning, sorting and storage.

### Study Selection

**Inclusion and Exclusion Criteria:** According to the PICO tool, this review focused on domestic cats (P) experiencing pain and stress due to some surgical, medical procedures or were in a clinical or experimental setting. We investigated a range of analgesic interventions (I), including opioids, NSAIDs, local blocks, acupuncture and their combination. These were compared with placebo, no treatment, or an alternative analgesia regimen (C). The primary outcome was analgesia efficacy, assessed using the need for rescue analgesia, stress-related measures, and adverse effects associated with each intervention (O).



Studies involving the treatment of pain in client-owned or research cats with a specific surgical or medical condition were included. Meanwhile, studies that only investigated non-painful stress or anxiety (e.g. associated with transportation, hospitalisation, or unfamiliar environments) were excluded from the qualitative synthesis.

Research articles that lacked clear assessments or measurements of pain, as well as review articles, opinion pieces, editorials, case reports or case series, and commentaries were excluded. During the abstract and full-text screening, studies with significant methodological flaws were also excluded. This included studies with small sample sizes (<6 animals per group) and those employing methods that were not related to the research question.

The data screening and extraction were performed independently by two reviewers UK and MSB. Any disagreements arising between the two reviewers were resolved through a discussion leading to a consensus.

**Data Extraction and Synthesis:** Articles fulfilling the inclusion criteria were read thoroughly. Data were extracted systematically using a pre-established, standardised approach. Extracted data included: author name, year of publication, research objectives, pain condition (orthopaedic, ovariohysterectomy, osteoarthritis, etc.), intervention details (analgesic drugs, dosage, route of administration), pain assessment tools, comparator group, outcomes (primary and secondary),

rescue analgesia (incidence and indications), adverse effects, and main findings.

A narrative and descriptive approach was used for the data synthesis and qualitative analysis. Studies were initially categorised as surgical and non-surgical. Subsequently, to enable a clinically relevant analysis, the pain in these categories was grouped by severity (severe, moderate, mild), the invasiveness of the procedure, duration of pain and potential for distress. This severity ranking was utilised to organise the synthesis of findings and guide clinical recommendations. In studies related to pain due to surgical conditions, the number of cats that required rescue analgesia in each study was recorded (where available). The data were further analysed based on the intervention strategy (e.g., multimodal vs. single therapy), drug class, and timing of administration, which assisted in identifying the most effective strategies for optimising pain control. Geographical visualisations were generated using Python mapping libraries with base map data sourced from OpenStreetMap. A formal meta-analysis was not possible given the large degree of heterogeneity between the studies, particularly in terms of study designs, interventions and outcome measures.

## RESULTS

### Study Characteristics

A total of 42 studies met the inclusion criteria for the systematic review. Twenty-five studies were classified

**Table 1.** Descriptive characteristics of the studies involving interventions on pain severity in surgical and non-surgical procedures

| Study                           | Year | Objective             | Condition (n)                      | Treatment                                                                                                                                                                                                | Dosage                                                                                                          | Timing           | Duration | Pain Assessment                                                            | Outcome                                            |
|---------------------------------|------|-----------------------|------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------|------------------|----------|----------------------------------------------------------------------------|----------------------------------------------------|
| King et al. <sup>[34]</sup>     | 2016 | Evaluate the efficacy | Onychectomy/ OVH/ Castration (358) | Robenacoxib <sup>①</sup>                                                                                                                                                                                 | 2 mg/kg SQ                                                                                                      | Pre-op           | 3 days   | Palpometer, behaviour view from distance/social interaction, posture score | Lower rescue analgesia                             |
| Speranza et al. <sup>[40]</sup> | 2015 | Compare analgesia     | Orthopedic surgery (147)           | G1: Robenacoxib <sup>①</sup><br>G2: Meloxicam <sup>②</sup>                                                                                                                                               | G1: 2 mg/kg SQ + 1-2.4 mg/kg PO<br>G2: 0.3 mg/kg SQ                                                             | Pre-op & post-op | 9 days   | Global investigator/ owner scale, Cortisol level                           | Non inferior to meloxicam                          |
| Thomson et al. <sup>[39]</sup>  | 2013 | Evaluate the efficacy | Corneal ulceration (17)            | Morphine <sup>③</sup>                                                                                                                                                                                    | 1 ocular drop 50 µL                                                                                             | Pre-op           | Once     | Aesthesiometer                                                             | Not effective for corneal pain                     |
| Rufiange et al. <sup>[21]</sup> | 2022 | Compare analgesia     | OVH (27)                           | OSA: Ketamine <sup>①</sup> + midazolam <sup>③</sup> + dexmedetomidine <sup>②</sup> + buprenorphine <sup>①</sup><br>meloxicam <sup>②</sup> ,<br>Bupivacaine <sup>③</sup> IP<br>OFA: without buprenorphine | OSA: 4 mg/kg + 0.25 mg/kg + 40 µg/kg + 20 µg/kg IM + 0.2 mg/kg SQ<br>OFA: same no buprenorphine                 | Pre-op           | Once     | FGS, DIVAS                                                                 | Buprenorphine eliminated need for rescue analgesia |
| Corrêa et al. <sup>[22]</sup>   | 2021 | Compare analgesia     | OVH (70)                           | MG: Maropitant <sup>③</sup><br>LG: Lidocaine <sup>③</sup><br>KG: Ketamine <sup>①</sup>                                                                                                                   | MG: 1 mg/kg IV + CRI 1.67 µg/kg/min<br>LG: 1.5 mg/kg IV + CRI 50 µg/kg/min<br>KG: 1 mg/kg IV + CRI 10 µg/kg/min | Pre- & intra-op  | Once     | VAS, UNESP Batucatu scale                                                  | Individual CRI effective, combination not superior |

Table 1. Continue

| Study                               | Year | Objective           | Condition (n) | Treatment                                                                                                                                                  | Dosage                                                                                     | Timing         | Duration | Pain Assessment                                    | Outcome                                                                                   |
|-------------------------------------|------|---------------------|---------------|------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------|----------------|----------|----------------------------------------------------|-------------------------------------------------------------------------------------------|
| Pereira et al. <sup>[25]</sup>      | 2021 | Compare analgesia   | OVH (30)      | D25: Dipyrone <sup>③</sup><br>D12.5: Dipyrone<br>M: Meloxicam <sup>②</sup>                                                                                 | D25: 25 mg/kg IV & q24h PO<br>D12.5: 12.5 mg/kg IV & q24h PO<br>M: 0.1 mg/kg IV & q24h PO  | Pre & post-op  | 3-6 days | VAS, CSU-FAPS, Glasgow pain scales                 | All protocol were equally effective. No statical difference in pain score                 |
| Corrêa et al. <sup>[26]</sup>       | 2019 | Compare analgesia   | OVH (30)      | GM30: Maropitant <sup>③</sup><br>GM100: Maropitant                                                                                                         | GM30: 1 mg/kg & CRI 30 µg/kg/h<br>GM100: 1 mg/kg & CRI 100 µg/kg/h                         | Pre & intra-op | Once     |                                                    | 100 ug/kg/hr decreased rescue analgesia                                                   |
| Vicente & Bergström <sup>[28]</sup> | 2018 | Compare analgesia   | OVH (75)      | GL: Lidocaine <sup>③</sup><br>GLB: Lidocaine <sup>③</sup> + Bupivacaine <sup>③</sup>                                                                       | GL: 1.5 mg/kg<br>GLB: 1 mg/kg + 1 mg/kg                                                    | Pre-op         | Once     | HR, MAP                                            | Incisional local block significantly improved the intraoperative analgesia vs control     |
| Ribeiro et al. <sup>[29]</sup>      | 2017 | Evaluate efficacy   | OVH (20)      | Dry needle simulation <sup>③</sup> + ketamine <sup>①</sup> +, midazolam <sup>③</sup> + tramadol <sup>③</sup>                                               | D points simulated 20 min preop to end of surgery + 5 mg/kg + 0.5 mg/kg + 2 mg/kg IM       | Pre & intra-op | Once     | UNESP Batucatu scale, VAS, Behaviour score         | YNSA decreased need for rescue analgesia                                                  |
| Marques et al. <sup>[30]</sup>      | 2015 | Evaluate efficacy   | OVH (20)      | Infrared laser acupuncture <sup>③</sup> + ketamine <sup>①</sup> +, midazolam <sup>③</sup> + tramadol <sup>③</sup><br>Control: same drugs, no acupuncture   | Laser acupuncture +5mg/kg+0.5mg/kg +2mg/kg IM                                              | Pre-op         | Once     | MCPS, DIVAS                                        | Laser acupuncture lowered the incidence of rescue analgesia.                              |
| Fudge et al. <sup>[31]</sup>        | 2021 | Compare analgesia   | OVH (151)     | BUP: Bupivacaine <sup>③</sup><br>BLE: Bupivacaine <sup>③</sup> + lidocaine <sup>③</sup> + epinephrine<br>DEX: Dexamethasone<br>MEL: Meloxicam <sup>②</sup> | BUP: 1 mg/kg<br>BLE: 1 mg/kg + 2 mg/kg + 0.005 mg/kg<br>DEX: 0.125 mg/kg<br>MEL: 0.2 mg/kg | Intra-op       | Once     | NRS                                                | MEL had significantly lower pain scores at 3h than the BLE.                               |
| Machado et al. <sup>[32]</sup>      | 2018 | Compare dose        | OVH (27)      | Remifentanil <sup>③</sup>                                                                                                                                  | CRI 0.1/0.2/0.4 µg/kg/min                                                                  | Intra-op       | Once     | UNESP Batucatu scale                               | 0.4 µg/kg/min effective; no rescue analgesia needed                                       |
| Guerrero et al. <sup>[33]</sup>     | 2014 | Comparing analgesia | OVH (21)      | G1: Alfaxalone <sup>②</sup> + Meloxicam <sup>②</sup><br>MK: Ketamine <sup>①</sup> + Medetomidine <sup>②</sup>                                              | G1: 5 mg/kg IV & bolus 2 mg/kg<br>MK: 5 mg/kg IV & bolus 2 mg/kg + 30 µg/kg IM             | Pre & intra-op | Once     | CPS, VAS, MWT                                      | ketamine-medetomidine provide better analgesia than alfaxalone.                           |
| Teixeira et al. <sup>[24]</sup>     | 2020 | Compare analgesia   | OVH (28)      | Dipyrone <sup>③</sup> + Tramadol <sup>③</sup>                                                                                                              | 25 mg/kg + 2 mg/kg                                                                         | Post-op        | 5 days   | UNESP Batucatu scale, Glasgow pain scales, VAS, BG | No additive benefit of dipyrone with tramadol                                             |
| Heo et al. <sup>[27]</sup>          | 2018 | Compare analgesia   | OVH (18)      | A: Meloxicam <sup>②</sup><br>B: Meloxicam <sup>②</sup> + Buprenorphine <sup>④</sup>                                                                        | A: 0.3 mg/kg SQ<br>B: 0.3 mg/kg SQ + 20 µg/h patch                                         | Pre & post-op  | Once     | Cortisol level, 4A-VET pain scale, VAS             | Meloxicam + buprenorphine patch combination significantly reduces pain vs meloxicam alone |

Table 1. Continue

| Study                               | Year | Objective             | Condition (n)                         | Treatment                                                                        | Dosage                                                | Timing           | Duration  | Pain Assessment                                                 | Outcome                                                         |
|-------------------------------------|------|-----------------------|---------------------------------------|----------------------------------------------------------------------------------|-------------------------------------------------------|------------------|-----------|-----------------------------------------------------------------|-----------------------------------------------------------------|
| Quarterone et al. <sup>[23]</sup>   | 2017 | Compare analgesia     | OVH, Orchiectomy (24)                 | Meloxicam <sup>②</sup> + Fentanyl <sup>②</sup>                                   | 0.1 mg PO + 2 µg/kg IV                                | Pre-op           | Once      | UNESP Batucatu scale                                            | Insufficient for OVH; adequate for orchiectomy                  |
| Skouropoulou et al. <sup>[36]</sup> | 2018 | Evaluate the efficacy | Ovariectomy (20)                      | TAP: bupivacaine <sup>③</sup> + 2% lidocaine <sup>③</sup><br>Control: Saline     | TAP: 1 mg /kg + 1.5 ml                                | Pre-op           | Once      | SDS                                                             | TAP effective; no rescue analgesia vs control 100% rescue       |
| Cicirelli et al. <sup>[35]</sup>    | 2022 | Evaluate efficacy     | Orchiectomy/castration (60)           | Ropivacaine <sup>③</sup><br>Control: NaCl                                        | 1 mg/kg                                               | Pre-op           | Once      | UNESP Batucatu scale, HR, BP, RR                                | Ropivacaine superior; lower pain scores vs control              |
| Taylor et al. <sup>[37]</sup>       | 2010 | Compare analgesia     | Neutering (153)                       | G1: Buprenorphine <sup>①</sup><br>G2: Butorphanol <sup>②</sup>                   | G1: 10-20 µg/kg<br>G2: 0.4 mg/kg                      | Pre-op           | Once      | SDS                                                             | Pain scores significantly lower in buprenorphine vs butorphanol |
| Coelho et al. <sup>[38]</sup>       | 2023 | Evaluate the efficacy | Feline Chronic gingivitis (FCGS) (22) | CBD <sup>③</sup>                                                                 | 4 mg/cat bid                                          | Preop & post op  | 15 days   | COPS C/E, SDAI, HR, BP, WL                                      | CBD treated cats more comfortable and active.                   |
| Adrian et al. <sup>[41]</sup>       | 2021 | Evaluate efficacy     | DJD pain (109)                        | RR: Robenacoxib <sup>①</sup><br>PP: Placebo                                      | RR: 1 mg/kg PO                                        | Daily            | 6 weeks   | FMPI, CSOM, AMs Owner assessment                                | Improvement after 6 weeks, and not 3 weeks based on activity.   |
| Gruen et al. <sup>[42]</sup>        | 2021 | Evaluate efficacy     | DJD associated chronic pain (126)     | Frunevetmab <sup>③</sup>                                                         | 1-2.8 mg/kg SQ & IV                                   | Repeated 28 days | 56 days   | AMs, CSOM FMPI, Owner Global Assessment, Veterinary Assessments | Effective at day 42-56; no difference between SQ vs IV routes   |
| Monteiro et al. <sup>[43]</sup>     | 2015 | Compare analgesia     | Osteoarthritis (15)                   | GM: Meloxicam <sup>②</sup><br>TM: Meloxicam <sup>②</sup> + Tramadol <sup>③</sup> | M. 0.05 mg/kg sid<br>TM: 0.05 mg/kg sid + 3 mg/kg bid | Daily            | 25 days   | Peak vertical force, RMTS, Motor activity measure               | No added benefit of tramadol to meloxicam                       |
| Gunew et al. <sup>[44]</sup>        | 2008 | Evaluate efficacy     | OA (92)                               | Meloxicam <sup>②</sup>                                                           | 0.01 - 0.03 mg/kg drops                               | Daily            | 12 months | Simple discontinuous scales                                     | Safe and palatable for long-term osteoarthritis management      |
| Gearing et al. <sup>[45]</sup>      | 2016 | Evaluate efficacy     | Kaolin-induced inflammatory pain (30) | Felinized anti-NGF mAb <sup>③</sup>                                              | 2 mg/kg NV-02 SQ                                      | Pre-emptive      | Once      | Discontinuous scoring system                                    | Significantly reduced lameness                                  |

Studies were categorised based on pain severity. The superscripts number following analgesics agent indicates their regulatory and evidence status. Regulatory status <sup>①</sup> Approved for and evidence supported, <sup>②</sup> Extra label and evidence supported, <sup>③</sup> Limited evidence, not recommended or investigational

4A-VET: 4A-Vet Pain Scale, CBD: Cannabidiol, CRI: Constant Rate Infusion, CSU-FAPS: Colorado State University Feline Acute Pain Scale, DIVAS: Dynamic Interactive Visual Analogue Scale, DJD: Degenerative Joint Disease, FCGS: Feline Chronic Gingivostomatitis, FGS: Feline Grimace Scale, FMPI: Feline Musculoskeletal Pain Index, MWT: Mechanical Wound Threshold, OTMS: Oral Transmucosal, RMTS: Response to Mechanical Temporal Summation, SDS: Simple Descriptive Scale, TAP: Transversus Abdominis Plane, VAS: Visual Analogue Scale, YNSA: Yamamoto New Scalp Acupuncture

based on the severity of the procedure and synthesised in Table 1, while 13 studies on mechanical and thermal threshold were summarised in Table 2. The remaining four studies which focussed specifically on the validation of pain assessment tools, are reviewed in the text below.

Of the 42 articles, 38 studies reported data on the adverse

effects of various pain treatment modalities. All the studies were grouped based on context (surgical, non-surgical, experimental) and further classified by the severity of the procedure (minimal, mild, moderate and severe). Information on the animals' age, sex and bodyweight was not provided in all the studies. Global distribution of studies

**Table 2.** Healthy Pharmacologic Challenge (Thermal and Mechanical Threshold)

| Study                              | Year | Objective                                                      | Condition (n)                  | Intervention                                                                                           | Dosage                                                                                                                                                                                           | Timing             | Pain Assessment | Outcome                                                                                                      |
|------------------------------------|------|----------------------------------------------------------------|--------------------------------|--------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------|-----------------|--------------------------------------------------------------------------------------------------------------|
| Ambros & Duke <sup>[49]</sup>      | 2013 | Determine thermal and mechanical antinociceptive effects       | Healthy-Analgesic Testing (24) | Ketamine low vs high dose                                                                              | LD 0.5 mg/kg + CRI 5 µg/kg/min vs 0.5 mg/kg + CRI 23 µg/kg/min                                                                                                                                   | Pre-stimulus +CRI  | TT, MT          | High dose of ketamine increases thermal and mechanical threshold vs low dose                                 |
| Simon et al. <sup>[50]</sup>       | 2016 | Evaluate antinociceptive effects opioids combination           | Healthy-Analgesic Testing (6)  | H-Sal: Hydromorphone<br>H-Bupre: Hydromorphone + buprenorphine<br>H-Butor: Hydromorphone + butorphanol | H-Sal: 0.1 mg/kg IV<br>H-Bupre: 0.1 + 0.02 mg/kg IV<br>H-Butor: 0.1 mg/kg + 0.2 mg/kg IV                                                                                                         | Pre-stimulus       | TT              | Hydromorphone + buprenorphine longer duration (2-3 h)<br>Butorphanol decreased hydromorphone antinociception |
| Wegner & Robertson <sup>[52]</sup> | 2007 | Dose-related thermal antinociceptive effects of hydromorphone  | Healthy-Analgesic Testing (21) | Hydromorphone                                                                                          | 0.025, 0.05, 0.1 mg/kg IV                                                                                                                                                                        | Pre-stimulus       | TT              | 0.1 mg/kg dose provided most potent analgesia                                                                |
| Ferreira et al. <sup>[51]</sup>    | 2011 | Compare IV vs OTM methadone                                    | Healthy-Analgesic Testing (8)  | IV group: Methadone<br>OTM group: Methadone                                                            | IV group: 0.3 mg/kg IV<br>OTM group: 0.6 mg/kg                                                                                                                                                   | Pre-stimulus       | MT              | OTM; significant antinociception from 10 min to 6 h; longer duration than IV                                 |
| Doodnaught et al. <sup>[53]</sup>  | 2017 | Evaluate oral tapentadol analgesic effect                      | Healthy-Analgesic Testing (6)  | BUP: Buprenorphine<br>Low TAP: Tapentadol<br>High TAP: Tapentadol                                      | BUP: 0.02 mg/kg IM<br>Low TAP: 25 mg PO<br>High TAP: 50 mg/kg PO                                                                                                                                 | Pre-stimulus       | TT              | Higher dose tapentadol: prolonged effect for 2 h like buprenorphine; no significant TT increases vs placebo  |
| Pypendop et al. <sup>[54]</sup>    | 2016 | Determine the antinociceptive effects of IV/ buccal opioids    | Healthy-Analgesic Testing (6)  | Morphine, Methadone<br>Hydromorphone<br>Oxymorphone                                                    | Morphine: 0.2 mg/kg IV or 0.5 mg/kg buccal<br>Methadone: 0.3 mg/kg IV or 0.75 mg/kg buccal<br>Hydromorphone: 0.1 mg/kg IV or 0.25 mg/kg buccal<br>Oxymorphone: 0.1 mg/kg IV or 0.25 mg/kg buccal | Pre-stimulus       | TT              | Significant thermal antinociception: IV hydromorphone/ methadone, and buccal methadone only                  |
| Carrozzo et al. <sup>[55]</sup>    | 2018 | Determine the effects of fentanyl CRI on thermal thresholds.   | Healthy-Analgesic Testing (6)  | F3: Fentanyl low dose<br>F5: Fentanyl high dose                                                        | F3: 3 µg/kg + CRI 3 µg/kg/h<br>F5: 5 µg/kg + CRI 5 µg/kg/h                                                                                                                                       | Pre-stimulus + CRI | TT              | 5 µg/kg/h increased TT during infusion.                                                                      |
| Farnworth et al. <sup>[58]</sup>   | 2015 | Assess thermal CO <sub>2</sub> laser as analgesia measure tool | Healthy-Analgesic Testing (60) | Saline Morphine, Buprenorphine, Medetomidine, Tramadol, Ketoprofen                                     | Saline: 0.2 mL<br>Morphine: 0.5mg/kg<br>Buprenorphine: 20 µg/kg<br>Medetomidine: 2 µg/kg<br>Tramadol: 2 mg/kg<br>Ketoprofen: 2 mg/kg                                                             | Pre-stimulus       | TT              | Morphine and Tramadol significantly increased response time; others non-significant                          |
| Steagall et al. <sup>[57]</sup>    | 2007 | Evaluate a prototype pressure stimulus device                  | Healthy-Analgesic Testing (8)  | Carprofen, Buprenorphine<br>Normal saline                                                              | Carprofen: 4 mg/kg,<br>Buprenorphine: 0.01 mg/kg<br>Normal saline 0.3 mL SQ                                                                                                                      | Pre-stimulus       | TT, MT          | Buprenorphine: longer duration than carprofen (up to 8 h)                                                    |
| Dixon et al. <sup>[56]</sup>       | 2007 | Development of a pressure nociceptive threshold testing device | Healthy-Analgesic Testing (11) | Butorphanol                                                                                            | 0.4 mg/kg SQ                                                                                                                                                                                     | Pre-stimulus       | MT              | Increased pressure threshold from the baseline                                                               |

**Table 2.** Continue

| Study                         | Year | Objective                                             | Condition (n)                  | Intervention                                                         | Dosage                                                                                                                        | Timing       | Pain Assessment              | Outcome                                                                                               |
|-------------------------------|------|-------------------------------------------------------|--------------------------------|----------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------|--------------|------------------------------|-------------------------------------------------------------------------------------------------------|
| Briggs <sup>[48]</sup>        | 1998 | To determine the antinociceptive effects              | Healthy-Analgesic Testing (8)  | Oxymorphone, Butorphanol (individual and combination) ± acepromazine | Oxymorphone: 0.025-0.20 mg/kg IV<br>Butorphanol: 0.025-0.20 mg/kg IV<br>Combinations at 0.1 mg/kg total<br>ACE: 0.05 mg/kg IV | Pre-stimulus | MT (rectal balloon catheter) | Oxymorphone + butorphanol combinations superior to alone; adding ACE further enhanced antinociception |
| Taylor et al. <sup>[46]</sup> | 2007 | Evaluate TT testing for NSAID analgesia investigation | Healthy-Analgesic Testing (26) | Ketoprofen                                                           | 2 mg/kg SQ                                                                                                                    | Pre-stimulus | TT                           | Insufficient sensitivity for NSAID analgesia study                                                    |
| Taylor et al. <sup>[47]</sup> | 2007 | Evaluate MT for NSAID analgesia testing               | Healthy-Analgesic Testing (8)  | Group C: Carprofen<br>Group B: Buprenorphine                         | Group C: 4 mg/kg SQ<br>Group B: 0.01 mg/kg SQ                                                                                 | Pre-stimulus | MT                           | Carprofen and buprenorphine, prevented inflammatory hyperalgesia                                      |

*Summary of the studies that applied thermal and mechanical threshold in healthy cats to evaluate the pharmacodynamics of the analgesic agents*

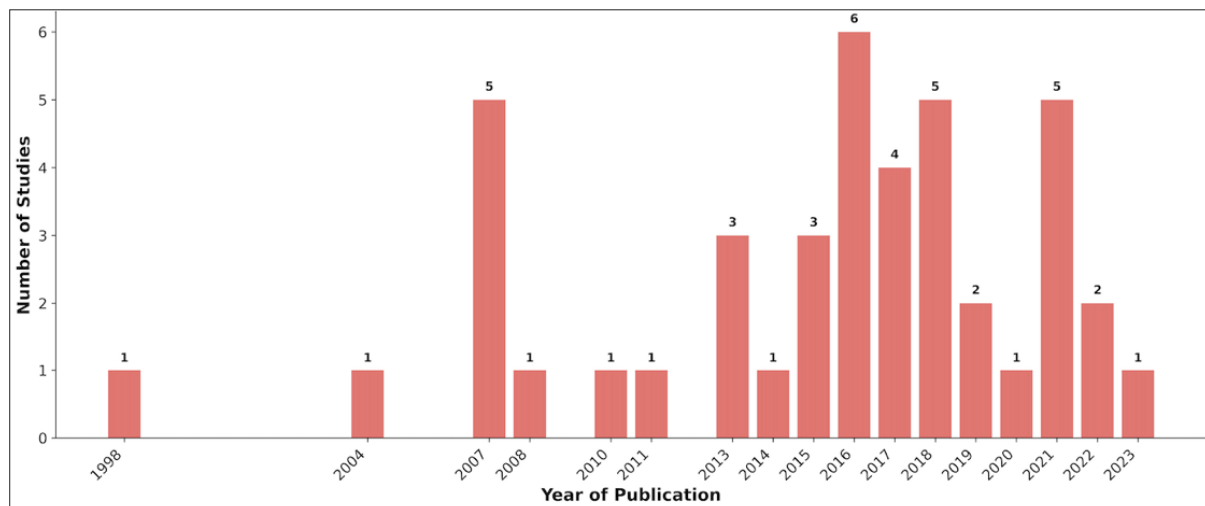


**Fig 2.** Year-wise distribution of studies on pain included in this systematic review, showing publication trends from 1998 to 2023

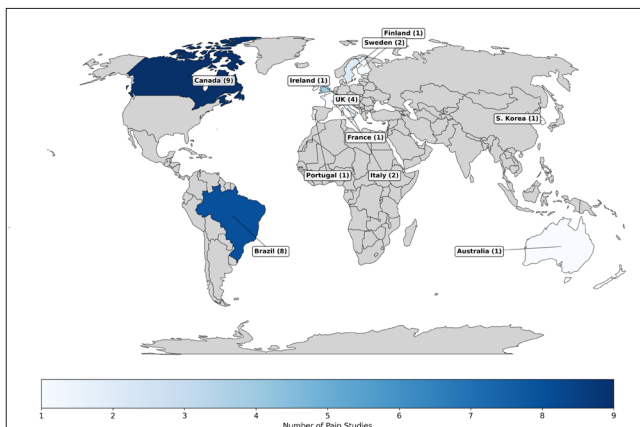


**Fig 3.** Global distribution of studies on pain in cats included in the systematic literature review. The map displays the number of studies per country using a color gradient (darker colors indicate more studies). Base map data from OpenStreetMap

and year of publication are shown in [Fig. 2](#) and [Fig. 3](#).

### Surgical Versus Non-Surgical Procedures

Overall, 20 studies were related to pain arising from surgical procedures such as ovariohysterectomy, ovariectomy, orchiectomy<sup>[21-37]</sup>, dental problems<sup>[38]</sup>, ocular issues<sup>[39]</sup> and orthopaedics<sup>[40]</sup>. The identified pain-related studies for non-surgical conditions primarily investigated on chronic degenerative disease and osteoarthritis<sup>[41-44]</sup>, as well as kaolin-induced inflammatory pain model<sup>[45]</sup>. [Table 1](#) depicts the severity of the interventions and their regulatory status for these studies. [Table 2](#) summarises the research conducted on healthy cats. These studies focused on pain due to kaolin injection<sup>[46, 47]</sup>, noxious visceral pain stimuli<sup>[48]</sup> and evoked thermal and mechanical threshold model for different drugs comparison<sup>[49-58]</sup>.

The remaining four studies<sup>[59-62]</sup> focused specifically on validation of pain assessment tools. Among the included studies, the UNESP-Batucatu multidimensional composite pain scale was the most frequently applied validated tool, demonstrating significant efficacy in surgical condition. Other commonly employed outcomes included the Visual Analogue Scale, physiological parameters, mechanical and thermal threshold, which were predominantly used in the experimental setting (*Table 1, Table 2*).

For severe pain models like orthopaedic surgeries, results strongly recommend the use of multimodal analgesia approaches. King et al.<sup>[34]</sup> and Speranza et al.<sup>[40]</sup> concluded that the use of preoperative NSAIDs such as robenacoxib significantly reduced the need for rescue analgesia compared to a placebo. Furthermore, Speranza et al.<sup>[40]</sup> found robenacoxib to be non-inferior to meloxicam for postoperative pain control.

Most of the included studies focused on moderate pain models such as ovariohysterectomy. A multimodal analgesia approach involving the opioid-sparing protocol, combined with dexmedetomidine, ketamine, and local anaesthesia, was effective in managing moderate pain. However, the addition of buprenorphine provided stronger analgesic effects, thereby further reducing the rescue rate<sup>[21]</sup>. A few studies highlighted the importance of a local anaesthesia. For example, a Transversus Abdominis Plane (TAP) block<sup>[36]</sup> and incisional block with buprenorphine and lidocaine<sup>[28]</sup>, both yielded good intra- and postoperative analgesic effects. New emerging techniques, along with local anaesthesia like laser and scalp acupuncture, demonstrated good potential in reducing the need for rescue analgesia<sup>[29,30]</sup>. Unlike the other effective options, dipyrone's efficacy was limited. Results demonstrated little to no advantage over placebo and other analgesics like meloxicam and tramadol<sup>[21,22]</sup>.

For moderate chronic pain conditions such as osteoarthritis, degenerative joint disease (DJD) and feline chronic gingivitis (FCGS), long-term management protocols were evaluated. NSAIDs were the cornerstone of these protocols. Both meloxicam and robenacoxib improved comfort and activity level, demonstrating effectiveness for long-term use<sup>[41,44]</sup>. A single injection of frunevetmab (anti-nerve growth factor) was also effective in ameliorating DJD pain for several weeks<sup>[42]</sup>. In FCGS, cannabidiol (CBD) improved outcomes, including activity and comfort, compared to placebo<sup>[38]</sup>.

Several studies investigated the use of thermal threshold (TT) and mechanical threshold (MT) devices, along with their subtypes, to characterise analgesic pharmacodynamics in healthy cats (*Table 2*).

Butorphanol, hydromorphone, buprenorphine and methadone significantly increased the withdrawal

**Table 3.** Frequency of analgesic modalities reported across all 38 studies

| Type of analgesia              | Studies | Percentage (%) |
|--------------------------------|---------|----------------|
| Opioids                        | 12      | 31.6           |
| NSAIDs                         | 5       | 13.2           |
| Opioids and NSAIDs combination | 6       | 15.8           |
| Local Analgesics               | 7       | 18.4           |
| Acupuncture                    | 2       | 5.3            |
| Anti-Nerve Growth Factor (NGF) | 2       | 5.3            |
| Miscellaneous                  | 4       | 10.5           |
| Total                          | 38      | 100.0          |

Miscellaneous category includes the following single-agent therapies: cannabidiol, NMDA antagonists, alpha-2 adrenergic agonists, and neurokinin-1 receptor antagonists

threshold<sup>[50,52,57]</sup>. In contrast the model showed little to no efficacy for tapentadol, reflecting its underperformance and poor performance in clinical trials<sup>[53]</sup>. The types of analgesia used in all 38 studies are summarised in *Table 3*.

### Proportion of Cats Subjected to Rescue Analgesia and the Effectiveness of Different Drug Protocols

A total of 924 cats were recruited in studies reporting rescue analgesia following pain intervention. Descriptively, 18.9% of the cats required rescue analgesia, especially those in the control group (61.4%) compared to cats in the treatment group (38.9%). Specifically, 10.2% and 41.6% of cats in the treatment and control groups received rescue analgesia.

The occurrence of rescue analgesia was 16.6% (95% CI 12.7-21.3) in multimodal arms, 5.3% in individual therapy arms (95% CI 3.5-8.0) and 41.6% in control arms (95% CI 35.8-47.7) as described in *Table 4*.

The frequency of rescue analgesia requirement by category arms was highest for NSAIDs (12.9%; 95% CI 9.9-16.7), followed by opioids (3.1%; 95% CI 1.4-6.6), opioids and NMDA agonists (26.2%; 95% CI 15.3-41.1), acupuncture (5.0%; 95% CI 0.9-23.6) and miscellaneous

**Table 4.** Rescue analgesia by drug protocol

| Category           | Rescue Required (n) | Total Cats (N) | Rescue Rate (%) | 95% CI (%) |
|--------------------|---------------------|----------------|-----------------|------------|
| Multimodal therapy | 48                  | 289            | 16.6%           | 12.7-21.3  |
| Individual therapy | 20                  | 378            | 5.3%            | 3.5-8.0    |
| Control/ Placebo   | 107                 | 257            | 41.6%           | 35.8-47.7  |
| Total              | 175                 | 924            |                 |            |

Rescue analgesia rates categorised by analgesic protocol. A lower percentage indicates better outcomes. The 95% confidence interval (CI) was calculated by the Wilson method. n: number of cats required rescue analgesia, N: total number of cats in that category

**Table 5.** Comparison of rescue analgesia among the different pharmacological classes and therapeutic categories.

| Analgesic Category       | Studies | Rescue Required (n) | Total Cats (N) | Rescue Rate (%) | 95 % CI (%) |
|--------------------------|---------|---------------------|----------------|-----------------|-------------|
| NSAIDs                   | 4       | 48                  | 371            | 12.9            | 9.9-16.7    |
| Opioids                  | 3       | 6                   | 193            | 3.1             | 1.4-6.6     |
| Opioids+NMDA antagonists | 2       | 11                  | 42             | 26.2            | 15.3-41.1   |
| Acupuncture              | 2       | 1                   | 20             | 5.0             | 0.9-23.6    |
| Miscellaneous (CBD, LA)  | 2       | 2                   | 41             | 4.9             | 1.4-16.1    |
| Total                    | 13      | 68                  | 667            |                 |             |

Rescue analgesia rates categorised by different pharmacological classes and therapeutic categories. A lower percentage indicates better outcomes. The 95% confidence interval (CI) was calculated by using the Wilson method. n: number of cats required rescue analgesia, N: total number of cats in that category

**Table 6.** Comparison of rescue analgesia between the timing of drug administration

| Timing of Administration | Studies | Rescue Required (n) | Total Cats (N) | Rescue Rate (%) | 95% CI (%) |
|--------------------------|---------|---------------------|----------------|-----------------|------------|
| Pre-operative            | 6       | 11                  | 238            | 4.62            | 2.6-8.1    |
| Postoperative            | 2       | 10                  | 51             | 19.61           | 11.0-32.5  |
| Pre + post-operative     | 3       | 38                  | 331            | 11.48           | 8.5-15.4   |
| Pre + Intra-operative    | 2       | 9                   | 47             | 19.15           | 10.4-32.5  |
| Total                    | 13      | 68                  | 667            | 10.19           |            |

Comparison of rescue analgesia rates between the timing of analgesic and the time of administration. A lower percentage indicates better outcomes. The 95% confidence interval (CI) was calculated by using the Wilson method. n: number of cats required rescue analgesia, N: total number of cats in that category. Timing refers to primary analgesia administration rather than the rescue analgesia

(4.9 %; 95% CI 1.4-16.4) as described in [Table 5](#).

The occurrence of rescue analgesia based on the timing of drug administration arms is depicted in [Table 6](#). The highest incidence was observed postoperatively (19.6%; 95% CI 11.0-32.5), followed by pre- and intraoperative (19.2%; 95% CI 10.4-32.5), and pre- and postoperative (11.5%; 95% CI 8.5-15.4). The lowest incidence was recorded preoperative alone (4.6%; 95% CI 2.6-8.1).

### Side Effects of Different Drugs Used for Feline Pain Management

Adverse effects associated with analgesic interventions

were reported in 20 studies. The frequency and nature of these effects were varied by drug class, as summarised in [Table 7](#). The most reported side effects were gastrointestinal symptoms (e.g. vomiting, diarrhoea) associated with NSAIDs and behavioural symptoms (e.g. euphoria, dysphoria, mydriasis) with opioids. Other significant effects included salivation with CBD and dipyrone, and neurological signs in cats administered robenacoxib.

### Synthesis of Evidence and Clinical Framework

To establish a practical framework for clinical practice, data regarding efficacy ([Table 1](#), [Table 2](#), [Table 3](#)), rescue analgesia ([Table 4](#), [Table 5](#), [Table 6](#)) and safety ([Table 7](#))

**Table 7.** Side effects related to different analgesics

| Compound                                                                                          | Dose & Route                                                             | Treated Cats | Cats with Side Effects | Side Effect                                                                           | Reference                       |
|---------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------|--------------|------------------------|---------------------------------------------------------------------------------------|---------------------------------|
| Cannabidiol (CBD)                                                                                 | 4 mg/kg PO                                                               | 22           | 08                     | Salivation, licking, headshaking (5); diarrhoea (1); vomiting (2)                     | Coelho et al. <sup>[38]</sup>   |
| Ketamine                                                                                          | LD 0.5 mg + CRI 5-23 µg/kg/min IV                                        | 16           | 16                     | Mydriasis                                                                             | Ambros & Duke <sup>[49]</sup>   |
| Ketamine + Midazolam + Dexmedetomidine + Buprenorphine + Meloxicam + Bupivacaine (OSA multimodal) | 4 mg/kg + 0.25mg/kg + 40 µg/kg + 20 µg/kg IM + 0.2 mg/kg SQ + 2 mg/kg IP | 13           | 2                      | Tachypnoea                                                                            | Rufiange et al. <sup>[21]</sup> |
| Robenacoxib                                                                                       | 1 mg/kg PO                                                               | 37           | 5                      | GI emesis (3); neurological (1); skin (1)                                             | Adrian et al. <sup>[41]</sup>   |
| Robenacoxib                                                                                       | 2 mg/kg SQ                                                               | 173          | 26                     | Incision site infection, dehiscence, bleeding; vomiting; decreased appetite; lethargy | King et al. <sup>[34]</sup>     |

**Table 7. Continue**

| Compound                                       | Dose & Route                             | Treated Cats | Cats with Side Effects | Side Effect                                                                  | Reference                          |
|------------------------------------------------|------------------------------------------|--------------|------------------------|------------------------------------------------------------------------------|------------------------------------|
| Robenacoxib                                    | 2 mg/kg SQ                               | 101          | 30                     | Diarrhoea, emesis                                                            | Speranza et al. <sup>[40]</sup>    |
| Meloxicam                                      | 0.3 mg SQ                                | 46           | 12                     | Diarrhoea, emesis                                                            | Speranza et al. <sup>[40]</sup>    |
| Frunevetmab (NGF)                              | 1.0-2.8 mg/kg SQ, IV                     | 64           | 5                      | Renal Insufficiency                                                          | Gruen et al. <sup>[42]</sup>       |
| Dipyrone                                       | 12.5 and 25 mg/kg PO                     | 20           | 20                     | Sialorrhea                                                                   | Pereira et al. <sup>[25]</sup>     |
| Hydromorphone + Buprenorphine or + Butorphanol | 0.1 mg/kg + 0.02 mg/kg or + 0.2 mg/kg IV | 6            | 6                      | Euphoria (rolling, kneading, vocalising, and purring)                        | Simon et al. <sup>[50]</sup>       |
| Hydromorphone                                  | 0.1 mg/kg IV                             | 7            | 7                      | Hyperthermia                                                                 | Wegner & Robertson <sup>[52]</sup> |
| Methadone                                      | 0.3 mg/kg IV or 0.6 mg/kg OTM            | 16           | 16                     | Mydriasis                                                                    | Ferreira et al. <sup>[51]</sup>    |
| Oxymorphone                                    | 0.1 mg/kg IV or 0.5 mg/kg buccal         | 12           | 6                      | Restlessness (4), vomiting (1), muscle rigidity (1)                          | Pypendop et al. <sup>[54]</sup>    |
| Tapentadol                                     | 25-50 mg/kg PO                           | 12           | 11                     | Salivation                                                                   | Doodnaught et al. <sup>[53]</sup>  |
| Butorphanol                                    | 0.4 mg IV                                | 70           | 2                      | Dysphoria                                                                    | Taylor et al. <sup>[37]</sup>      |
| Tramadol + Meloxicam                           | 3 mg/kg + 0.05 mg/kg OTM                 | 8            | 5                      | Mydriasis, decreased appetite, hypersalivation, vomiting                     | Monteiro et al. <sup>[43]</sup>    |
| Meloxicam                                      | 0.01 - 0.03 mg/kg drops in feed PO       | 46           | 4                      | Vomiting                                                                     | Gunew et al. <sup>[44]</sup>       |
| Morphine                                       | 1 drop (50 µL) Ocular                    | 17           | 3                      | Blepharospasm, hyperaemia, chemosis                                          | Thomson et al. <sup>[39]</sup>     |
| Oxymorphone                                    | 0.2 mg/kg                                | 8            | 8                      | Restlessness                                                                 | Briggs <sup>[48]</sup>             |
| Alfaxalone                                     | 5 mg/kg IV                               | 10           | 5                      | Opisthotonus, limb stiffness, shivering, excitation, myoclonus, vocalisation | Guerrero et al. <sup>[33]</sup>    |

Summary of the reported adverse effects of analgesic interventions reported in 38 studies. OTM: oral transmucosal; PO: per os, IV: intravenous, SQ: subcutaneous, IM: intramuscular

**Table 8. An Evidence-Based Tiered Framework for Feline Analgesia and Integrated Patient Care**

| Evidence-based Tier | Recommendation                 | Agent Example                                     | Rationale                                            | Availability                                                              |
|---------------------|--------------------------------|---------------------------------------------------|------------------------------------------------------|---------------------------------------------------------------------------|
| E1                  | Foundational analgesics        | Robenacoxib, Meloxicam, Bupivacaine, lidocaine    | Strong evidence, core of therapy                     | Widely available globally                                                 |
| E2                  | Potent analgesic add-ons       | Buprenorphine, Hydromorphone, Ketamine            | High efficacy; logistical constraints                | Variable (often controlled substances)                                    |
| E3                  | Adjunct for stress and anxiety | Synthetic facial pheromones (Feliway), Pregabalin | Reduces non-painful stress; supports welfare         | Feliway: globally available. Pregabalin: controlled in the USA and the UK |
| E4                  | Not recommended                | Dipyrone, Tramadol                                | significantly lower palatability and safety concerns | Banned or restricted in major jurisdictions (USA, Canada, Japan)          |

E1-E4 indicates the recommended order of intervention, from foundational (E1) to not recommended (E4)

were synthesised. The resulting Evidence-Based Tired Framework for feline pain management is presented in [Table 8](#), classifying interventions according to their recommended hierarchy and supporting evidence.

## DISCUSSION

This review concludes that available evidence strongly supports the use of multimodal analgesia, with drug

selection tailored according to the nature of individual cases. For severe acute pain cases, a combination of NSAIDs (meloxicam/robenacoxib), full opioid agonists (hydromorphone) and local anaesthesia is effective in ameliorating pain. For moderate pain such as routine ovariohysterectomy (OVH) procedures, a comprehensive multimodal approach encompassing opioid-sparing adjuvants and local analgesia is indicated.

Findings from the rescue analgesia analysis provide strong, empirical and evidence-based data with qualitative and severity-based insight. The need for rescue analgesia clearly indicates inadequate pain management, and the analyses assist in identifying effective strategies. The rescue analgesia analysis depicted a subtle image determined by the intensity of pain models and sample sizes. The low pooled rescue rate in individual therapy arms (5.3%) is diluted primarily by multiple large-scale trials ( $n > 100$ ) of effective single agents. Some of these agents include the use of robenacoxib in normal procedures like ovariohysterectomy. A single effective drug is often adequate in these controlled environments with a predictable pain burden, leading to few rescue events in a large population of animals. In contrast, the multimodal therapy arms were frequently used in more complicated cases or studies with a small sample size ( $n=10$  per arm). Under such circumstances, characterised by a more intense or unpredictable pain, the objective of a multimodal protocol is to offer a strong analgesic base. Although this approach results in an increased pooled rescue rate (16.6%) compared to idealised single-agent cases, it offers a significant improvement over the rescue rate recorded in the control group (41.6%).

Thus, the available data provides no evidence on the superiority of monotherapy. Instead, these findings support the selection of a multimodal therapy when dealing with complex or severe pain, given its ability to address the situation reliably [21,63,64]. On the other hand, monotherapy can be very useful in moderate pain models [21]. The substantial difference in the rescue rates between both treatment groups (multimodal and monotherapy) and the control group underscores the necessity of active analgesic intervention.

The drug-class analysis provided a well-defined hierarchy of the efficacy of monotherapy options. The very low rescue rate for opioids (3.11%) confirms their strong analgesic activity as a group of drugs [21,37]. The low rate in the miscellaneous category (4.9%), which incorporates local anaesthetics and other adjuncts, indicates the definitive effect of these interventions. While the average rate of NSAIDs (12.9%) confirms their effectiveness, it also depicts their limitations as a monotherapy approach, particularly in more painful procedures. The high rescue rate observed for the opioids and NMDA agonists groups (26.2%) does not indicate a regimen failure. Instead, it reflects this combination's use in models with extreme pain burden, and it still resulted in better outcomes [21].

Based on the rescue data from this review, the timing of analgesia administration is significant factors. The results strongly support the use of pre-emptive analgesia. The lowest rescue rate was, by far, for the pre-operative administration (4.6%). This reflects the effectiveness of administering

analgesia before surgical intervention and the onset of pain (central sensitisation). It is more effective to prevent pain in this way than attempting to ameliorate pain once it occurs [22,65,66]. The higher rescue rates in post-operative (19.6%) and pre- and intra-operative (19.2%) groups indicate that delaying intervention until the activation of pain results in poor control, despite subsequent usage of other effective drugs. This confirms that time of administering the drug is as critical as the drug itself.

This review also described the topography of pain assessment tools applied in feline research, due to their direct influence on intervention efficacy. The UNESP-Botucatu multidimensional composite pain scale and its subtypes [22-25,29,30,32,35,59,62] were the most frequently applied tools in surgical studies, especially for ovariohysterectomy and castration. These tools have been proven effective, demonstrating significant results compared to controls in various acute surgical conditions. These scales are designed to be comprehensive, incorporating behavioural observations, physiological parameters, and direct palpation to gauge discomfort. This tool assesses patient's behaviour both spontaneously and in response to interaction, incorporating features from established clinical pain measurement [67]. Its widespread use reflects a paradigm shift towards standardised, validated tools that assess multiple behavioural parameters, making them more sensitive and reliable than unidimensional scales.

The visual analogue scale (VAS) was also a frequently used [22,24-27,29,33], particularly in conjunction with other tools such as the UNESP-Botucatu, the Feline grimace scale, the Glasgow composite measure pain scale and physiological parameters. While its subjectivity is a limitation, its ongoing use highlights the continued need for tools that are practical for clinical application. However, the variability emphasises the significance of using VAS in conjunction with more objective measures or specific behavioural scales.

Physiological indicators like heart rate, blood pressure, respiratory rate [28,35,38], blood glucose [24] and cortisol level [27,40] are also used in assessing pain and stress in cats. Postoperative physiological parameters (HR, BP, and RR) and different biochemical markers (blood glucose and cortisol level) are inconsistent and unreliable for pain assessment, as evidenced in the reviewed studies [27,38,40]. These parameters and markers are often affected by different factors such as stress, fear, discomfort and anaesthesia recovery [27,35,38,40], thus contributing to their limitations in explaining significant differences between treatment groups as compared to validated pain scales. This implies that although physiological parameters are important in pain assessment, they are not adequate to be used as individual tools in explaining feline pain.

Mechanical Threshold (MT) <sup>[33,43,48,51,56]</sup> and Thermal Threshold (TT) <sup>[50,52-55,57]</sup> testing was generally used for quantitative, objective data. These tools were helpful in studies on healthy cats in establishing the pharmacodynamic profile of drugs, such as buprenorphine and hydromorphone <sup>[52,57]</sup>. They also yield a precise measure of efficacy that is less susceptible to observer bias. However, their use is limited to research settings because of the equipment and cooperation required from the patient. These instruments have their own advantages; however, drawbacks relating to habituation in cats following their repeated usage remain a big challenge. Despite their effectiveness in identifying nociceptive pain and hyperalgesia, their focus on sensory reaction may not fully reflect the animal's pain. They may overlook other aspects of the animals' experience, such as their behaviour and perception of pain. Overall, the common choice of tool was determined by the pain model. Activity monitors (AMs) and owner-completed questionnaires such as the Feline Musculoskeletal Pain Index (FMPI) were valuable and valid endpoints for chronic OA studies <sup>[41,42]</sup>. These tools provide a real-life measure of improvement that is unachievable by pure threshold testing. Overall, a universally optimal pain assessment technique for cats has not yet been established. Nevertheless, veterinary medicine is progressively embracing a flexible, multimodal methodology. Within this approach, validated composite scales such as the UNESP-Botucatu remain the most reliable in clinical settings.

Every analgesic protocol should be considered in view of its safety and tolerability profile. The analysis of adverse effects from this review identified significant trends to guide clinical decision-making. Opioids were commonly associated with dysphoric behaviours of euphoria (vocalising and rolling), and mydriasis <sup>[50,51]</sup>. While these effects are typically brief and rarely harmful, they can be unpleasant for owners and make post-operative monitoring more challenging. This emphasises the significance of patient monitoring and owner education when administering these potent medications. NSAIDs, especially robenacoxib and meloxicam, were linked to gastrointestinal effects such as vomiting and diarrhoea. However, the incidence was generally low and within acceptable limits for peri-operative use <sup>[40,41]</sup>. Dipyrone was consistently and significantly associated with ptyalism (excessive salivation) <sup>[25]</sup>. Despite not being life-threatening, dipyrone has significant detrimental effects on patient and owner comfort, thereby constituting a considerable limitation to its use.

The accumulated evidence from this review demonstrates that with multimodal therapy, effective analgesia could be achieved without a concomitant rise in severe adverse events. By leveraging synergistic pharmacological benefits,

lesser doses of each individual agent can be employed, potentially reducing the adverse effects associated with greater doses of individual treatments. The observed adverse effects were largely predictable and manageable. Opioid related dysphoria and GI effects from NSAIDs were the most common considerations. This information is crucial in making informed choices, preparing owners for what to expect and choosing the appropriate drug for a patient.

Based on the evidence on efficacy, safety, and assessment, the following levels of evidence-based tier (E1-E4) were suggested to range clinical practice despite the fluctuating international drug availability. This framework ranks interventions in order of strong evidence, high safety profiles, and wide regulatory acceptability to offer a flexible approach to international veterinarians (*Table 8*).

**Tier E1: Fundamentally Recommended and Accessible;** this tier includes agents having strong evidence for efficacy and a well-established safety profile in cats and having a widespread registration across major international regions (EU, North America, Australia). The cornerstone in managing the most painful conditions should be the pre-emptive administration of an NSAID (i.e., robenacoxib or meloxicam) combined with local anaesthetic techniques (i.e., lidocaine or bupivacaine nerve blocks or incisional infiltration). This multimodal approach produces effective, cost-effective analgesia with minimal dependence on controlled substances.

**Tier E2: Strong but Complex from a Logistical Perspective;** This tier contains drugs with strong evidence of efficacy, but the use is complicated by international controlled substance regulations, requirements for intensive monitoring or limited formulation availability. The combination of superior efficacy and a growing license for use in cats globally, buprenorphine, a partial opioid agonist, is the analgesic of choice in this tier. Where legal and under appropriate surveillance, full  $\mu$ -opioid agonists (hydromorphone, methadone) and low-dose ketamine infusions are pivotal in severe pain or as part of balanced treatment regimens. The application is often limited to environments that allow for addressing their side effects and regulatory requirements.

**Tier E3: Adjunct for stress and anxiety:** Tier E3 adds a very important aspect to the overall care of the patient, which is the management of non-painful stress and anxiety. It has been well established that stress may reduce pain thresholds and complicate recovery. Thus, the agents at this level are considered treatment adjuncts, which include synthetic feline facial pheromones (e.g. Feliway) and nutraceuticals. They produce a well-being by alleviating anxiety in hospitalised cats. This led to more accurate pain assessment, better adherence to treatment

and possibly even more effective core analgesics. Their presence raises the level of care beyond mere alleviation of a symptom of pain to a proactive support of the overall patient's well-being.

Tier E4: Not Recommended for Clinical Use; This tier contains drugs limited or inconsistent evidence regarding their efficacy. It also includes drugs with significant safety concerns that have led to restrictions or bans in key jurisdictions. Dipyrone (metamizole) is banned in the United States, Canada, and Japan. Tramadol has poor oral bioavailability and inconsistent analgesia in cats and is a controlled drug in many areas. These agents cannot be recommended as reliable analgesics.

This framework emphasises that excellent analgesia can be achieved even in resource-limited environments if the Tier E1 foundation of an NSAID and local anaesthetic block is used as a pre-emptive measure. The main challenge is in the Tier E2 group, where strong agents such as full  $\mu$ -opioid agonists are often limited by controlled substance legislation, which differs markedly between countries. This requires clinicians to be familiar with their local laws and use the best available agents, especially buprenorphine, which is becoming more widely available worldwide. This tiering provides a clear rationale for not using drugs such as dipyrone, moving clinical practice in line with available evidence and with international safety standards.

While this review has been focused on direct pharmacological control of pain, optimal patient care requires a holistic approach. Given the strength of the supporting evidence, we recommend formally integrating stress management as Tier E3 in our clinical framework. Although 10 studies on non-painful stress management<sup>[68-77]</sup> were not included in the main analysis, their results revealed an important adjacent field. These studies demonstrated that non-pharmacological treatments (e.g., synthetic pheromones; Feliway, nutraceuticals; alpha-casozepine, and environmental manipulation) and pharmacological agents like pregabalin can effectively reduce anxiety related to transportation, hospitalisation, and novel environments.

The relationship between stress and pain is well-documented<sup>[78,79]</sup>, stress can exacerbate pain perception and delay recovery<sup>[80]</sup>. Therefore, a key direction for future research is to investigate the synergy between evidence-based analgesic protocols and stress-reduction strategies. Although the tiered framework offers a definite route to pharmacological analgesia, its efficacy can be significantly enhanced by concurrently applying the Tier E3 stress reduction interventions. Uncontrollable pain is a profound stressor, and pain-related behaviour can be exacerbated by stress response. Consequently, agent such as synthetic feline facial pheromones (Feliway), alpha-

casozepine, and pregabalin should not be considered independent of pain management but rather as integral component of it. Implementing these modalities to create a less stressful environment helps achieve a calmer patient. This, in turn, facilitate a more successful pain assessment, easier administration of medications and reduced requirement for systemic analgesics. Future studies should quantitatively explore this multimodal synergism by determining whether the combination of stress-reduction interventions directly improves pain scores or less analgesic requirement in a clinical setting.

Looking beyond the existing evidence, several emerging methods have the potential to transform feline pain management. Targeted therapies such as the monoclonal antibodies like frunevetmab have a good safety profile and long duration of action, suggesting potential for widespread use. Cannabidiol (CBD), which represent a novel mechanism of action for conditions like feline chronic gingivitis (FCG), requires validation through more robust clinical trials. Future studies should investigate into long-acting formulations and localised delivery systems (e.g., extended-release local anaesthetics) to provide sustained analgesia from a single dose, thereby enhancing patient compliance and comfort. Lastly, the integration of precision medicine, such as genetic or biomarker testing to predict individual analgesic response, is a pertinent frontier of maximising efficacy and minimising adverse effects.

Furthermore, the application of objective neuro-physiological biomarkers should be considered in future work to overcome the subjectivity intrinsic to behavioural scales. Methods, including electroencephalography (EEG) with well-established potential in identifying neuro-physiological signals of pain and stress in other animals, are to be confirmed in cats. On the same note, the search for pain-specific plasma biomarkers is a crucial direction towards a blood-based objective test. Combining these objective measures with fine behavioural scales is pertinent for accurate and reliable pain assessment.

The relatively low number of studies (n=42) included in this systematic review reflecting the stringent inclusion and exclusion criteria required to address a specific research question. While this ensures the conclusions are based on high quality, relevant evidence, it also limits the generalisability of the findings and underscore the need for more primary research in this field. Furthermore, the review scope is constrained by the available research. The marked heterogeneity in study designs, pain models, and outcome measures precluded meta-analysis and prevented formal evaluation of publication bias. The accumulated evidence focused on acute peri-operative pain, with limited studies on chronic conditions. Finally, comparability between studies was influenced by the substantial inter-study differences in pain-assessment tools.

## CONCLUSION

This systematic review confirms that multimodal analgesia, founded on appropriate pre-emptive analgesia, is the cornerstone of effective feline pain management. The current evidence supports well-defined protocols based on NSAIDs and local anaesthetics, with addition of potent opioids for severe pain. A tiered system for global application is therefore suggested: a Tier E1 core of readily available NSAIDs and local anaesthetics, supported by Tier E2 opioids, such as buprenorphine, for more severe cases and Tier 3 stress-reducing agents as adjuncts. Drugs with limited efficacy or suboptimal safety profiles, such as dipyrone and tramadol, are not recommended. Future work should include further standardisation of outcomes, chronic pain management and investigation of synergies with stress reduction. This synthesis provides a concise, evidence-based roadmap to enhance feline analgesia, highlighting critical areas for translational research.

## DECLARATIONS

**Availability of Data and Materials:** All the generated data are included in the manuscript.

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## REVIEW ARTICLE

# Climate Change, Vector-Borne Animal Diseases: Impacts on Livestock Health: A Narrative Review

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## Abstract

Climate change is profoundly transforming animal health by intensifying vector-borne diseases (VBD) in livestock. Rising temperatures, shifting rainfall, and extreme weather events have expanded the geographic range and seasonality of vectors such as ticks, mosquitoes, and biting flies. This has led to increased incidence, emergence, and re-emergence of diseases like bluetongue, Rift Valley fever, trypanosomiasis, and tick-borne fevers. These infections reduce meat, milk, and reproductive performance, while also posing significant public health and socioeconomic threats, especially for pastoralists, smallholder farmers, and rural women. VBDs exacerbate poverty and gender inequities and heighten human exposure to zoonotic pathogens with epidemic potential. In response, climate-resilient agricultural practices, vector control, enhanced surveillance, and One Health-based strategies are being promoted to strengthen adaptive capacity. However, critical gaps persist, including weak integrated data systems, limited predictive modeling, unaffordable diagnostics and vaccines for neglected diseases, and poor understanding of community-level adaptive capacities. Addressing these challenges requires coordinated global action, investment in interdisciplinary research, and policies that enhance resilience and equity in animal health systems.

**Keywords:** Climate change, Disease modeling, Livestock, One health, Surveillance, Vector-borne diseases, Zoonoses

## INTRODUCTION

Climate change is increasingly recognized as one of the greatest global threats of the 21<sup>st</sup> century, with extensive implications for natural ecosystems, biodiversity, human health, and socioeconomic stability <sup>[1,2]</sup>. Driven primarily by human-induced greenhouse gas (GHG) emissions, most notably carbon dioxide (CO<sub>2</sub>), methane (CH<sub>4</sub>), and nitrous oxide (N<sub>2</sub>O), climate change is manifested through rising global temperatures, erratic precipitation, receding glaciers, sea-level rise, and an increased frequency of extreme weather events such as droughts, floods, and heatwaves <sup>[3,4]</sup>. These environmental shifts have severe and interrelated effects on ecosystems, including agricultural and livestock production systems. Crop and livestock agriculture, which is extremely sensitive to climatic variations, faces heightened threats to productivity, animal health, and welfare. Of particular concern is the increasing prevalence and spread of infectious livestock diseases, especially vector-borne diseases (VBDs) transmitted by ticks, mosquitoes, and flies <sup>[5]</sup>.

Vector-borne animal diseases are a significant threat to both animal and human health worldwide. Diseases

such as bluetongue, African swine fever, Rift Valley fever, tick-borne encephalitis, and trypanosomiasis cause major economic losses due to animal morbidity and mortality, decreased productivity, veterinary costs, and trade restrictions <sup>[6]</sup>. Their impact is particularly severe in low- and middle-income countries, where livestock are central to livelihoods. Many of these diseases are zoonotic, posing risks to both public and veterinary health <sup>[7]</sup>. The distribution and transmission of VBDs are strongly influenced by ecological and environmental factors such as temperature, humidity, host availability, and suitable vector habitats. Climate change alters these conditions, creating environments more favorable for the survival and dissemination of vectors and pathogens <sup>[8,9]</sup>.

The relationship between climate change and VBDs is complex and multifactorial. Rising temperatures accelerate pathogen development within insect vectors (extrinsic incubation), enhance vector survival, and expand their geographical range into previously unsuitable areas at higher latitudes and altitudes. Altered precipitation and humidity patterns can create new breeding grounds for vectors such as mosquitoes and midges, while droughts and heatwaves may force livestock and wildlife into closer



contact with vectors, increasing transmission risks <sup>[10]</sup>. These climatic shifts disrupt traditional epidemiological patterns and drive the emergence or re-emergence of infectious diseases. This review, therefore, highlights the critical interrelationship between climate change and animal vector-borne diseases, emphasizing their impacts on livestock production and rural livelihoods.

## CLIMATE CHANGE AND ITS IMPACTS ON LIVESTOCK SYSTEMS

Global temperatures have risen by about 1.2°C since pre-industrial times, and further warming is expected. Many livestock-rearing regions are projected to experience the highest temperature increases, exposing animals to greater thermal loads for longer periods. This will affect their performance, reproduction, and survival. Heat stress occurs when animals cannot dissipate body heat effectively. Species with dense coats or poor sweating ability, like cattle, are especially vulnerable <sup>[11]</sup>. Changes in precipitation and more frequent extreme weather events, such as storms, hurricanes, and wildfires, further strain livestock systems. These events can cause high animal losses, damage infrastructure and feed, and disrupt transport and veterinary services. For example, the 2019-2020 Australian bushfires killed or displaced around 100.000 livestock, causing major economic losses. The increasing severity and unpredictability of these events demand more resilient management strategies <sup>[12]</sup>.

Heat stress in cattle, sheep, and goats leads to lower feed intake and altered metabolism, reducing milk yield and weight gain. Dairy cows may lose up to 40% of milk production, with decreased fat and protein content. In poultry, high temperatures reduce egg production and quality and increase mortality, particularly in broilers <sup>[13,14]</sup>. Behaviorally, animals move less, seek shade, and drink more water, but reduced grazing or feeding time further lowers productivity. In extensive systems, concentrating in cooler areas can lead to overgrazing and pasture degradation <sup>[15]</sup>.

This literature review is novel in the sense that it brings an integrative and comprehensive outlook that was not available before. Several reviews have considered livestock diseases due to vectors or climate change effects on animals in general, but they did not include this global picture. The current review includes areas not often mentioned where the risk of diseases shifted by climate change is increasing. It is not limited to one continent or country but rather presents a compiled view that encompasses a number of different regions worldwide, including Africa, Asia, the Middle East, Europe, and Latin America. In addition, it integrates mechanistic knowledge on host immune response, vector competence, and pathogen dynamics

with quantitative modeling methods such as SIR, agent-based, and remote sensing-assisted models linking climate to key epidemiological metrics like  $R_0$ , degree-days, and entomological inoculation rates. The authors also mention emerging zoonotic and livestock pathogens, for instance, CCHF, leishmaniasis, and Japanese encephalitis, providing explicit practical recommendations for the risk communication and mitigation strategies. By merging mechanistic, quantitative, and applied perspectives into one framework, the review has addressed a critical gap between empirical observations, predictive modeling, and actionable guidance, and it provides a holistic and globally relevant synthesis that is beyond the scope of previous reviews.

## CHANGES IN FEED AND WATER AVAILABILITY

Feed and water are among the most critical inputs in animal production, both highly sensitive to climatic fluctuations. Temperature and rainfall affect the quantity and quality of forage and grains consumed by livestock. In grazing systems, pasture productivity and composition respond strongly to temperature and moisture. While moderate warming may initially boost plant growth in some temperate zones, excessive heat and water stress reduce biomass and nutritional quality <sup>[16,17]</sup>. Prolonged drought in arid and semi-arid areas lowers native grass and shrub canopies, forcing pastoralists to adopt costly supplementary feeding. Elevated CO<sub>2</sub> may stimulate plant growth but reduce protein content and digestibility, decreasing feed quality <sup>[18]</sup>. Animals on low-quality diets grow slower, produce less milk, and are more disease-prone <sup>[19]</sup>. Intensive farming systems dependent on maize and soybeans are also vulnerable to climate-induced fluctuations in crop yields and supply chains <sup>[20,21]</sup>.

Extreme weather, shifting agro-climatic zones, and competition between human food and animal feed crops can reduce feed affordability and availability, forcing producers to limit herd sizes or rations <sup>[22,23]</sup>. Water availability is similarly threatened. Climate change alters hydrological cycles, impacting both surface and groundwater <sup>[24]</sup>. Drought limits water for drinking, cooling, and feed production, while floods increase contamination risks. Inadequate or unsafe water leads to dehydration, reduced feed intake, impaired thermoregulation, and disease <sup>[25]</sup>. Competition for scarce water between agriculture, industry, and households often deprioritizes livestock, especially in developing countries with weak storage and distribution systems <sup>[26]</sup>. Climate-resilient water strategies, including rainwater harvesting, efficient irrigation, and improved watering systems, are essential <sup>[27]</sup>.

## GEOGRAPHICAL ALTERATIONS IN LIVESTOCK-FRIENDLY REGIONS

Climate change is expected to alter the geographical range of livestock production systems. Shifts in temperature and precipitation will determine which regions remain or become suitable for rearing specific animal species [28]. Warmer conditions in previously cold regions may extend grazing periods and reduce housing costs, making these areas more favorable for livestock. However, these benefits may be offset by emerging challenges such as pests, diseases, and unsuitable soils [29]. In contrast, excessive heat and water scarcity in tropical and subtropical zones may reduce system viability, prompting herd movement or replacement with more heat-tolerant breeds [29].

Pastoralist and nomadic communities are particularly vulnerable, as altered vegetation cycles, depleted water sources, and resource conflicts disrupt traditional migration routes. This can lead to grazing system collapse and higher livestock mortality [30]. Expanding vector ranges add further complexity; for example, bluetongue virus has moved into Northern Europe due to the northward spread of *Culicoides* midges, and ticks and tsetse flies are reaching higher elevations and temperate zones, increasing disease risk. Adapting to these shifts requires improved climate and disease forecasting, climate-resilient breeds, diversified systems, and strategic land-use planning [31]. Institutional support through mobility-enabling policies, secure land rights, and veterinary infrastructure is essential to help farmers adjust to new climatic realities [32].

## VECTOR-BORNE ANIMAL DISEASES

Vector-borne animal diseases (VBDs) are infections transmitted to livestock and sometimes humans by arthropod vectors, including insects (mosquitoes, flies, midges) and arachnids (ticks, mites). Their causative agents include viruses, bacteria, protozoa, and helminths. VBDs involve a tripartite system of host, pathogen, and vector, with many pathogens requiring development or multiplication within the vector before infecting the next host [33]. Examples include viral (bluetongue), bacterial (anaplasmosis), protozoan (trypanosomiasis), mosquito-borne (Rift Valley fever), tick-borne (babesiosis), fly-borne (trypanosomiasis), and midge-borne (bluetongue) diseases [34]. Some are strictly animal diseases, while others are zoonotic (e.g., Rift Valley fever) [35]. Understanding their classification and transmission dynamics is critical for surveillance and control, especially under a warming climate that enhances vector survival and spread [20].

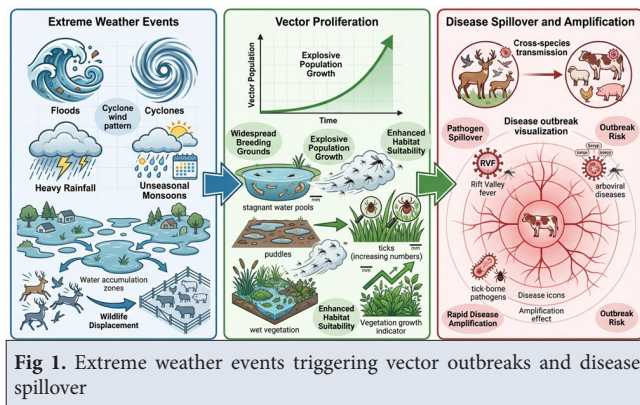
Bluetongue is a viral disease of ruminants, particularly sheep, caused by Bluetongue virus (BTV) of the *Orbivirus* genus (*Reoviridae*). It is transmitted by *Culicoides* midges,

notably *C. imicola* and *C. obsoletus*. Clinical signs include fever, mucosal inflammation, facial and tongue swelling, lameness, and sometimes death. Cattle often act as reservoirs, harboring subclinical infections that sustain viral circulation [36]. Formerly confined to sub-Saharan Africa and Asia, bluetongue has spread to Europe due to climate-driven vector expansion, with outbreaks in Germany, the Netherlands, and the UK [37]. The 2006–2008 Northern European outbreaks were linked to increased temperature and humidity. Economic impacts include trade restrictions, mortality, reduced productivity, and costs of vaccination and vector control [38].

The changes in temperature and precipitation across South and Southeast Asia have affected the outbreaks of diseases caused by mosquitoes and ticks that threaten livestock such as cattle, buffalo, goats, and sheep, and have even worsened them. Four major zoonotic diseases associated with livestock production, i.e., hemorrhagic septicemia, lumpy skin disease (LSD), Japanese encephalitis (JE), and anaplasmosis, have been detected with seasonal variability and extreme heat in India, Pakistan, Bangladesh, and Nepal. The *Aedes* and *Culex* mosquitoes have migrated north due to the warm winters, thus exposing livestock more to the JE virus and other arboviruses. Climate change is also the reason for the increased Harshamma ticks in Pakistan and Iran, for which animals' CCHF disease incidence is rising as well as the humans' [8].

In the Middle East, the sequence of droughts and then sudden heavy rains has become more common and creates the perfect environment for the vectors of the *Aedes*, *Culicoides*, and *Phlebotomus* types to breed. One of the consequences of this is the re-emergence of Rift Valley fever (RVF) outbreaks in Saudi Arabia and Yemen, where heavy rainfall, often linked to warm ocean currents and El Niño-like patterns, leads to the rise in mosquito numbers, causing large-scale infections in sheep, goats, and camels. The heat stress caused by these conditions also makes the livestock more susceptible to infection with *Theileria annulata*, which *Hyalomma* ticks transmit, and consequently, there is an increase in cases of tropical theileriosis, mainly in cattle [9].

Latin America provides proof of the similar climate-induced changes in vector ecology. Higher temperatures and deforestation-related microclimate changes have extended the range of sandfly species in Brazil, Colombia, and Venezuela, thereby facilitating the transmission of leishmaniasis in cattle and horses. In Brazil and Argentina, the occurrence of Bluetongue virus (BTV) outbreaks has been more and more connected to the changes in precipitation and humidity, which in turn, support *Culicoides* midge activity. Moreover, the climate fluctuations in Central America have led to the movement of *Rhipicephalus microplus* ticks to new regions, thereby



increasing the incidence of cattle diseases such as babesiosis and anaplasmosis. The warmer and moister conditions have also helped *Aedes* vectors to invade the mountainous regions of Mexico and Peru, which is, in turn, raising the alarm about the possible reappearance of arboviruses that would affect humans and livestock again in the future (Fig. 1) [30].

Rift Valley fever (RVF) is a viral zoonotic disease caused by the Rift Valley fever virus (RVFV), which belongs to the *Phlebovirus* genus of the *Bunyaviridae* family. It affects livestock such as sheep, goats, cattle, and camels and is primarily transmitted by mosquitoes, with *Aedes* and *Culex* species being the most common vectors [39]. Outbreaks of RVF are commonly associated with periods of intense rainfall and flooding, which create ideal breeding conditions for mosquitoes. The disease is characterized in animals by high rates of abortion and mortality, particularly in juveniles. In humans, RVF can cause influenza-like symptoms, hemorrhagic fever, encephalitis, or death.

The 2006-2007 East African RVF outbreak was triggered by El Niño-related flooding, demonstrating the role of climate anomalies in disease emergence. Infected livestock act as amplifying hosts, increasing spillover risk during outbreaks. Because of its significant public health implications and severe impact on livestock reproduction and survival, RVF is considered an important transboundary animal disease [40]. Control measures include vaccination (where available), mosquito eradication, and movement control [41].

Trypanosomiasis in livestock, also known as *Nagana*, is a protozoan disease caused by *Trypanosoma* species, particularly *T. congolense*, *T. vivax*, and *T. brucei*. Transmission occurs through tsetse flies (*Glossina* spp.), which are primarily distributed in sub-Saharan Africa. Clinical signs include fever, weakness, weight loss, anemia, abortion, and death. Chronic infections result in reduced productivity, infertility, and loss of draught power in cattle and other livestock [42]. The disease affects nearly 10 million km<sup>2</sup> of Africa and threatens more than 50 million cattle. Its economic impact exceeds US\$4.5

billion annually [43]. Climate change is expected to shift tsetse fly habitats, potentially expanding disease transmission to new areas. Rising temperatures and vegetation changes can modify fly density and infectivity, necessitating new surveillance and vector control strategies [20].

Ticks are among the most significant disease vectors affecting animals globally. Tick-borne diseases (TBDs) include several serious infections, such as Babesiosis, caused by *Babesia* spp., mainly *B. bigemina* and *B. bovis*, transmitted by *Rhipicephalus* ticks. It causes fever, anemia, hemoglobinuria ("redwater"), and high mortality in cattle [44,45]. Anaplasmosis, caused by *Anaplasma marginale*, is transmitted by ticks, leading to fever, jaundice, and emaciation in cattle. Theileriosis, caused by *Theileria* spp., e.g., *T. parva*, results in East Coast fever in cattle in eastern Africa.

Ticks thrive in warm, humid environments, and climate change is expanding their range and increasing their activity periods. Prolonged survival, increased reproduction, and longer seasonal activity raise tick burdens and disease risks. TBDs reduce productivity, milk yield, and fertility and cause major economic losses through animal deaths, reduced draught power, and treatment costs. Acaricide resistance further complicates control [46]. Ticks are arachnids with four life stages: egg, larva, nymph, and adult. Most hard ticks (*Ixodidae*) are three-host ticks, feeding on different hosts at each stage and molting in the environment between feedings. Pathogens are transmitted primarily through saliva during blood feeding, both transstadially (larva → nymph → adult) and transovarially (adult → egg), enabling long-term disease persistence [47]. Climate change extends tick seasons and permits their spread into cooler, higher-altitude regions. For example, *Ixodes Ricinus*, a vector of *Anaplasma* and *Borrelia*, has expanded northward and upward in Europe due to increased temperatures [20].

Tsetse flies are large, blood-feeding insects restricted to sub-Saharan Africa. They transmit *Trypanosoma* parasites to both animals and humans. Tsetse flies are viviparous, giving birth to live larvae that pupate in the soil. Transmission occurs during blood feeding, as the parasite undergoes development in the fly's midgut and salivary glands. The cycle takes approximately 20-30 days, and infection risk depends on temperature, humidity, and host availability [48]. Climate change alters tsetse habitats, especially in riverine and savannah ecosystems. Temperatures exceeding 32°C may reduce fly survival and fecundity, contracting their range, while cooler areas may become more suitable [49].

Mosquitoes, including *Aedes*, *Culex*, and *Anopheles* species, transmit numerous viral and parasitic diseases. Their life cycle consists of egg, larva, pupa, and adult stages,

| <b>Table 1. Factors about VBD and their prevention strategies</b> |                                                                               |                                                   |                   |
|-------------------------------------------------------------------|-------------------------------------------------------------------------------|---------------------------------------------------|-------------------|
| <b>Factor</b>                                                     | <b>Causes</b>                                                                 | <b>Mitigation Strategies</b>                      | <b>References</b> |
| Biodiversity Loss                                                 | Climate change, habitat destruction                                           | Ecosystem-based adaptation (EbA), protected areas | [1]               |
| GHG Emissions                                                     | CO <sub>2</sub> , CH <sub>4</sub> , N <sub>2</sub> O from livestock & farming | Renewable energy, emission regulation             | [3]               |
| Vector-Borne Diseases (VBDs)                                      | Climate shifts, globalization                                                 | One Health, vector surveillance                   | [5]               |
| Emerging VBDs in Europe                                           | Habitat shifts, vector importation                                            | Cross-border One Health strategies                | [7]               |
| Extreme Weather Events                                            | Increased frequency & severity                                                | Disaster preparedness                             | [11]              |
| Australia Risk Landscape                                          | Climate threats, zoonoses                                                     | Strategic preparedness                            | [12]              |
| Heat Stress in Cattle                                             | High temperature, humidity                                                    | Cooling, shade, hydration                         | [13]              |
| Tibetan Pasture Degradation                                       | Overgrazing, warming                                                          | Sustainable rangeland management                  | [15]              |
| Low Pasture Diversity                                             | Overgrazing, variable rainfall                                                | Diversified pasture species                       | [16]              |
| Methane from Ruminants                                            | Poor forage quality                                                           | Methane-mitigating forages                        | [18]              |
| Milk Quality Issues (Kenya)                                       | Poor hygiene, handling                                                        | Farmer training, hygiene improvements             | [19]              |
| Urban VBDs (Africa)                                               | Waterlogging, sanitation failure                                              | Vector control, sanitation upgrades               | [20]              |
| Poultry & Security                                                | Global price shocks                                                           | Poultry resilience strategies                     | [24]              |
| Rural Water Delivery                                              | Poor infrastructure                                                           | Community governance                              | [25]              |
| Water Allocation Imbalance                                        | Ecological undervaluation                                                     | Integrated valuation                              | [27]              |
| Global Livestock Trends                                           | Climate pressure, population growth                                           | Climate-smart livestock                           | [28]              |
| Silvopasture Integration                                          | Monoculture inefficiency                                                      | Agroforestry integration                          | [29]              |
| Environmental Impacts on Husbandry                                | Heat stress, pollution                                                        | Green, adaptive systems                           | [30]              |
| VBDs in Developing Countries                                      | Climate stress, weak infrastructure                                           | Disease mapping, education                        | [32]              |
| VBDs in Afghanistan                                               | Conflict, warming                                                             | Regional vector control                           | [32]              |
| Cattle as VBD Risk                                                | Vector proximity                                                              | Spatial management                                | [33]              |
| Asian Vector Ecology                                              | Regional vector diversity                                                     | Localized control plans                           | [34]              |
| Wildlife Trypanosomiasis                                          | Wildlife–livestock interface                                                  | Interface regulation                              | [34]              |
| Bluetongue Virus                                                  | Midge vectors, climate sensitivity                                            | Vaccination, vector control                       | [36]              |
| Climate & VBD Trends                                              | Vector behavior, pathogen cycles                                              | Regional vector plans                             | [37]              |
| VBDs in the UK                                                    | Changing vector habitats                                                      | Monitoring, early warning                         | [38]              |
| Rift Valley Fever (RVF)                                           | Mosquito vectors, flooding                                                    | Vaccines, vector control                          | [39]              |
| RVF Epidemiology                                                  | Climate, animal movement                                                      | Forecasting, vaccination                          | [41]              |
| Trypanosomosis (Africa)                                           | Tsetse exposure, grazing                                                      | Education, drug delivery                          | [42]              |
| Surveillance in Zambia                                            | Remote settings                                                               | Mobile vet units                                  | [43]              |
| Tick-Borne Diseases                                               | Climate effects on ticks                                                      | Repellents, resistant breeds                      | [44]              |
| Global Climate & Disease                                          | Vector range expansion                                                        | Climate-smart health                              | [46]              |
| Ticks in Pets                                                     | Pet mobility, tick spread                                                     | Owner education, tick control                     | [47]              |
| Mosquito Biology                                                  | Water habitats, breeding                                                      | Source elimination                                | [50]              |
| Livestock Diseases                                                | Poor vet capacity                                                             | Capacity building, One Health                     | [52]              |
| Climate-VBD-Conflict Link                                         | Fragile ecosystems, migration                                                 | Conflict-sensitive climate adaptation             | [53]              |
| Transboundary Diseases                                            | Trade, migration                                                              | Regional surveillance                             | [54]              |
| Zoonoses in SE Asia                                               | Deforestation, bushmeat                                                       | One Health coordination                           | [55]              |
| Animal Health Actors (Africa)                                     | Fragmented roles                                                              | Intervention mapping                              | [56]              |
| Veterinary Data Gaps                                              | Fragmented datasets                                                           | Integrated data systems                           | [57]              |
| RVF Modelling                                                     | Climate-based forecasting                                                     | Climate-sensitive models                          | [58]              |
| VBD Resilience in Europe                                          | Low preparedness                                                              | Knowledge sharing                                 | [59]              |

with larval and pupal development occurring in water. Pathogens typically require an extrinsic incubation period within the mosquito before becoming transmissible, a process accelerated by higher temperatures. Increased rainfall and flooding create more breeding sites, while warming extends mosquito activity and range. Outbreaks of RVF, for example, are linked to breeding of *Aedes* and *Culex* mosquitoes in rainwater pools [50].

Midges (*Culicoides* spp.) are small biting flies that transmit bluetongue virus and *Schmallenberg* virus in ruminants. Their life cycle (egg-larva-pupa-adult) depends on moist soil or dung for larval development. Adults are active at dawn and dusk and transmit pathogens biologically after viral multiplication in the insect. Higher temperatures accelerate viral replication and shorten incubation periods, increasing vector competence. Climate change has facilitated *Culicoides* survival in new areas, including temperate Europe. Warmer winters promote the overwintering of midges and viruses, allowing for year-round transmission cycles that were previously constrained [51] (Table 1).

## CLIMATE CHANGE-INDUCED ALTERATIONS IN DISEASE DYNAMICS

### Expansion of Vector Habitats and Breeding Grounds

One of the most important mechanisms through which climate change influences disease dynamics is the widening of ecological niches for vectors. Temperature and humidity affect the distribution, reproductive performance, and survival of vectors such as mosquitoes, ticks, midges, and flies. As these parameters shift, so do suitable habitats for vector development [20]. Warmer temperatures accelerate the development of ectothermic vectors, allowing them to reproduce faster and expand their geographic ranges. For example, populations of *Culicoides* midges that transmit bluetongue virus have become established in northern Europe, where they previously could not overwinter [36]. Similarly, *Aedes* mosquitoes, vectors of Rift Valley fever (RVF), are now able to breed in higher-altitude and latitude regions of East and North Africa [60].

Changes in precipitation patterns play a dual role in vector ecology. In some regions, excessive rainfall and flooding create stagnant water bodies ideal for mosquito breeding. In others, drought concentrates animals and vectors around limited water sources, increasing contact rates and transmission potential. *Glossina* spp. (tsetse flies), for instance, depend on humid environments for reproduction and survival; rising humidity in parts of southern and central Africa has increased their breeding areas [61].

Land cover and vegetation changes driven by both climate and human adaptation also affect vector ecology. Bush

encroachment linked to desertification may enhance tick survival, while deforestation can expose livestock to new vector populations and associated diseases. Such habitat expansions increase contact between vectors, livestock, and wildlife reservoirs, promoting cross-species transmission and raising the risk of zoonotic spillover events [62-64]

### Emerging and Re-emerging Diseases Under Climate Stress

Climate change has triggered the emergence of new vector-borne diseases and the re-emergence of previously controlled infections through ecological disruption, altered vector behavior, and increased contact among livestock, humans, and wildlife. Emerging diseases often arise when pathogens adapt to new vectors or hosts. For example, *Schmallenberg* virus, a novel *orthobunyavirus* detected in 2011, appeared in Northern Europe and was transmitted by *Culicoides* midges, the same vectors responsible for bluetongue virus. Although the direct role of climate change remains debated, rising temperatures likely enabled vector persistence and virus spread [36, 51]. Re-emergence occurs when climatic variability reignites transmission cycles of controlled diseases. Rift Valley fever, once localized in Eastern Africa, has resurfaced multiple times over recent decades, often following El Niño-driven flooding, causing major livestock losses, trade disruptions, and human infections [65].

Climate stress also weakens animal immunity, increasing susceptibility to co-infections. Heat-stressed cattle, for instance, are more vulnerable to tick-borne pathogens such as *Anaplasma* and *Babesia* in areas with prolonged tick activity [20]. Additionally, climate change interacts with globalization, urbanization, and deforestation to create ideal conditions for novel vector-pathogen-host dynamics. The expansion of livestock into wild habitats and climate-driven human migration further elevate outbreak risks. This underscores the urgent need for a One Health approach integrating human, animal, and environmental health perspectives to strengthen vector-borne disease surveillance, prevention, and control [66] (Fig. 2).

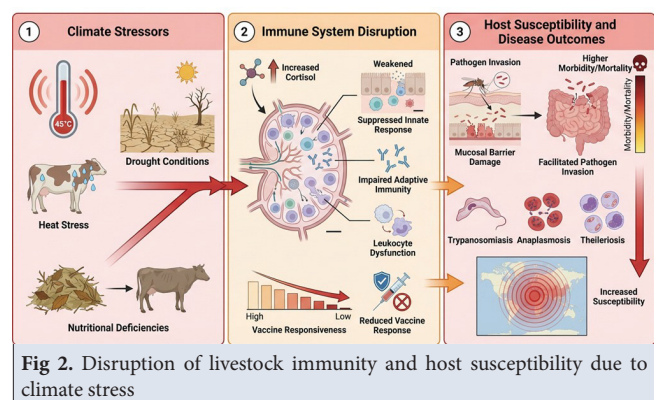


Fig 2. Disruption of livestock immunity and host susceptibility due to climate stress

## EFFECTS ON ANIMAL HEALTH AND PRODUCTION

### Higher Morbidity and Mortality Among Livestock

Climate change amplifies disease risks through increased heat stress and environmental hazards, leading to higher livestock morbidity and mortality. Prolonged exposure to high ambient temperatures and humidity elevates respiration rate, body temperature, and reduces feed intake, compromising immune function and increasing vulnerability to infections<sup>[67]</sup>. Major vector-borne diseases such as trypanosomiasis, anaplasmosis, babesiosis, and Rift Valley fever (RVF) remain significant causes of livestock deaths. African trypanosomiasis alone kills about 3 million cattle annually. Mortality is highest in young or naïve herds and is exacerbated by concurrent climate events like drought or flooding, which limit access to feed and water. These shocks reduce herd size, alter genetic selection, and undermine long-term productivity and resilience<sup>[68]</sup>.

### Decreased Milk, Meat, and Reproductive Performance

Thermal stress depresses milk yield by lowering feed intake, impairing metabolism, and damaging mammary function, with reductions of up to 40% reported during prolonged heat waves<sup>[69]</sup>. Vector-borne infections compound this by causing fever, anemia, and systemic inflammation, leading to sharp declines in both milk quantity and quality<sup>[70]</sup>. Meat-producing animals such as beef cattle, sheep, goats, and poultry experience reduced growth rates and carcass weights due to combined heat stress and disease pressures<sup>[71,72]</sup>.

Climate stressors also impair reproductive efficiency. In females, heat and disease disrupt estrous cycles, ovarian activity, and pregnancy maintenance, causing abortions, stillbirths, and low offspring survival. In males, semen quality and libido decline at high temperatures. RVF outbreaks, for example, are linked to mass abortions in ruminants, while trypanosomiasis delays puberty, lowers fertility, and compromises fetal development. These reproductive disruptions prolong calving intervals, reduce herd expansion, and threaten profitability and sustainability<sup>[73]</sup>.

### Costs of Disease Control and Veterinary Services

Disease prevention and control impose substantial financial burdens on livestock producers and governments. Preventive measures, including vaccination, use of antiparasitic drugs, insecticides, acaricides, and housing improvements, require significant investment. For example, large-scale bluetongue vaccination programs in Europe cost tens of millions of euros annually per country. Similarly, trypanosomiasis control in Africa relies on

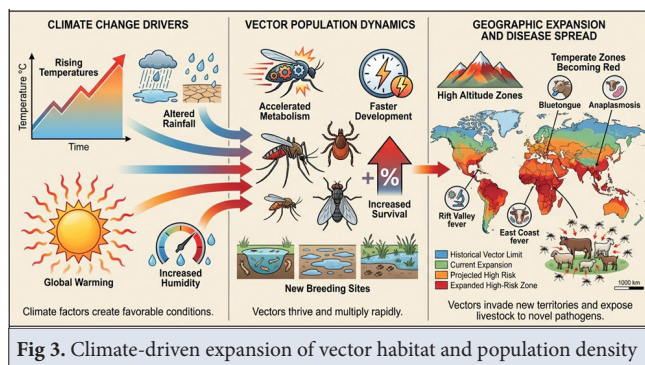
trypanocidal drugs, which are expensive and increasingly threatened by drug resistance due to overuse and misuse<sup>[36,74]</sup>. Tick control necessitates regular acaricide applications; however, rising product costs, frequent treatments, and resistance development make long-term control financially challenging.

Once the disease occurs, veterinary consultations, diagnosis, and treatment further escalate costs. In rural and remote regions, limited access to veterinary services often leads to delayed or inappropriate treatment. In tick-endemic areas, drugs such as buparvaquone for theileriosis are essential but expensive and frequently in short supply<sup>[75]</sup>. Outbreak control requires government interventions, including movement restrictions, quarantines, and mass vaccination campaigns, which add logistical and financial burdens to both producers and public health systems<sup>[40]</sup>. The economic impact is compounded when outbreaks disrupt domestic and international livestock trade. For instance, detection of Rift Valley fever can halt the export of meat, milk, and live animals, affect national economies, and undermine the livelihoods of smallholder farmers<sup>[76]</sup>.

### Economic and Livelihood Impacts

Smallholder farmers are highly vulnerable to climate-driven vector-borne disease (VBD) shocks due to limited access to adaptive technologies, weak veterinary infrastructure, and dependence on a few animals for subsistence. The loss of even one cow or ox can push a household into poverty by eliminating its primary source of milk, draught power, or income<sup>[77]</sup>. Repeated disease outbreaks and climatic stressors reduce herd sizes, force distress livestock sales, and diminish household access to animal-source foods, undermining both income and nutrition security. Women, who play key roles in small-scale livestock production in many developing countries, are disproportionately affected. Livestock loss limits their economic empowerment, reduces their ability to invest in household welfare, and increases their labor burden in sourcing feed and water<sup>[78]</sup>.

Commercial farms, though better resourced, also face significant risks. VBD outbreaks can cause large-scale production losses, reputational damage, and increased spending on biosecurity, veterinary care, and insurance. Intensive, high-density farming systems facilitate rapid disease spread, while heat stress requires costly cooling and ventilation. Extreme disease or heat events can wipe out major investments in infrastructure, feed, and genetics. Livestock insurance programs, often accessible only to large farms, remain out of reach for most smallholders. Climate-resilient risk management measures such as index-based insurance, microcredit, and public-private partnerships in animal health are crucial to protect both



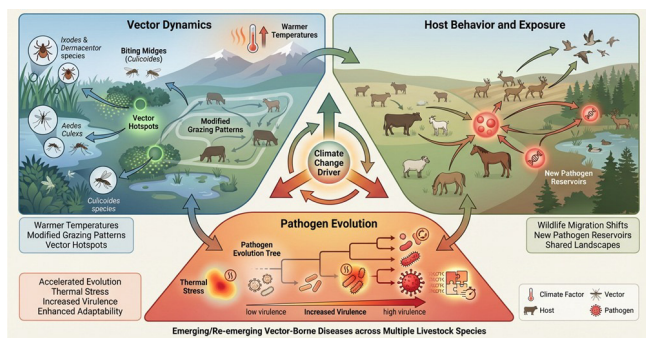
small and commercial producers from climate-related shocks <sup>[79]</sup> (Fig. 3).

## ZOONOTIC POTENTIAL OF VECTOR-BORNE DISEASES

Many vector-borne animal diseases carry zoonotic potential, i.e., the potential to be transmitted from animals to humans with grave consequences to human health. Climate change enhances the risk of such transmissions through the modification of the vectors' distribution and the bringing together of populations of humans, livestock, and wildlife <sup>[33]</sup>. Rift Valley fever (RVF), which is among the most recognized zoonotic VBDs, infects both humans and animals and causes flu-like symptoms that can progress into hemorrhagic fever, encephalitis, or blindness <sup>[81]</sup>. Abnormal rain and flooding, which aid in the breeding of mosquitoes and amplification of the virus among livestock, trigger outbreaks of RVF. Infections in humans usually arise from direct contact with infected blood, milk, or meat, especially among farmers, butchers, and veterinarians <sup>[82]</sup>.

In East Africa, the 2006-2007 RVF outbreak led to more than 300 human fatalities and huge losses of livestock, illustrating the lethal nexus between animal and human health under climate stress <sup>[83]</sup>. Likewise, West Nile virus, another temperature-sensitive arbovirus, is transmitted by mosquitoes and has had rising cases in Europe and North America with warming temperatures extending vector ranges <sup>[33]</sup>. Trypanosomiasis, while mainly its consequence for livestock (as Nagana), also manifests in human form as human African trypanosomiasis (HAT or sleeping sickness). Climate-induced shifts in tsetse fly distribution may potentially raise the risk of HAT in hitherto non-endemic areas <sup>[42]</sup>.

Zoonotic VBD outbreaks impose significant burdens on frequently underfunded public health infrastructures, particularly in the developing world. Zoonotic disease outbreaks often overwhelm hospital and clinic resources with patients presenting with a broad array of clinical signs and symptoms, frequently without clear diagnostic



tests or treatments <sup>[40]</sup>. Integrated surveillance systems that monitor diseases across the animal-human interface also do not exist. Veterinary and human health services in most countries are running independently, downplaying the capacity to detect outbreaks early and respond promptly <sup>[84]</sup>. It hinders containment, as this does not enable diseases to be contained geographically and between species <sup>[85]</sup> (Fig. 4).

## SURVEILLANCE, ADAPTATION, AND MITIGATION MEASURES

Early surveillance and detection are the pillars of successful VBD control. In a time when the environment is under threat and disease dynamics are becoming increasingly erratic, active disease surveillance systems offer the opportunity for real-time intervention, reduce the severity of outbreaks, and safeguard both animal and human health <sup>[33]</sup>. Integrated surveillance entails frequent gathering, analysis, and interpretation of data relating to the occurrence of disease in vectors and animals. Successful surveillance systems track clinical expression in animals along with environmental factors like temperature, vegetation, and rainfall, factors that under usual circumstances would mirror vector multiplication. These are to be supplemented with field-level data collection by veterinary officers, extension workers, and community-based animal health workers for responding and accuracy <sup>[86]</sup>. Early warning systems (EWS) use climate models and epidemiological data to forecast the occurrence of disease weeks or months in advance. For example, El Niño events have been associated with elevated risk for RVF in East Africa based on improved breeding conditions for mosquitoes. Rainfall anomaly prediction and vegetation index models have aided nations in pre-emptive vaccination and vector control measures. Challenges remain, however, in translating forecasts into action, particularly where there is poor governance or poor communication infrastructure. Getting more linkage between prediction and policy response is required for effective use of early warning <sup>[87]</sup>.

When climate change makes vectors expand their range and season of activity, maintaining their populations under control and at bay from disease transmission by vaccination is a high priority. Vector control is a chemical and biological intervention for reducing the vector population or preventing vector–host contact. The primary methods are treated livestock and shelters: Spraying acaricides or insecticides on animal hide, housing, and beddings reduces the infestation of ticks, flies, and mosquitoes [88]. Environmental management: Water drainage, changing landscape vegetation, or animal housing design can discourage vector breeding [89]. Biological control: Introducing natural enemies (e.g., larvivorous fish) or pathogens (e.g., Wolbachia-infected mosquitoes) provides a cost-effective and environmentally friendly alternative to chemicals [90]. On the other hand, extensive use of chemicals can result in resistance, environmental contamination, and health hazards. Integrated Vector Management (IVM) places strong emphasis on the coordinated use of several methods adapted to local ecological and socioeconomic situations [91].

Vaccination is an inexpensive way of preventing outbreaks of diseases, especially in endemic areas. Successful experiences include the Development of RVF vaccine campaigns in Kenya and Tanzania, which have cut outbreak severity by half. Bluetongue virus (BTV) vaccination in Europe, where large-scale immunization has contained the spread through affected areas [36]. Trypanosomiasis control, which is frustrated by the absence of a commercial vaccine but is instead addressed via chemoprophylaxis and insect control. The principal hindrances to successful vaccination are cold-chain supply chains, lack of funding, and scarcity of vaccine doses for some diseases. There is also an urgent need to investigate new candidate vaccines, especially for vector-borne diseases with narrow therapeutic windows [92].

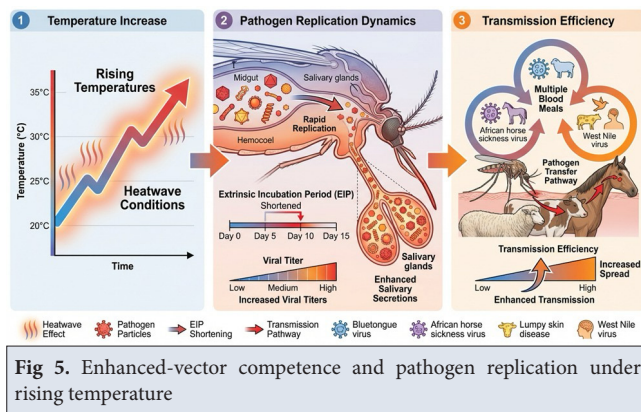
### Immunopathogenesis and Host Susceptibility Under Heat Stress

Heat stress is a significant factor affecting the immunopathogenesis of vector-borne diseases through the weakening of host defense mechanisms and the creation of physiological conditions favorable for the pathogen's establishment and growth. The high temperatures trigger the hypothalamic-pituitary-adrenal axis and lead to continuous cortisol secretion, which in turn suppresses both innate and adaptive immunity by reducing macrophage activity, interfering with T-cell proliferation, and blocking antigen presentation. Water loss due to heat affects the epithelial and mucosal barriers, thus increasing the skin and oral-nasal tissues' permeability and making it easier for the pathogen to get in after a vector bite. The oxidative stress that is produced during heat exposure at the cellular level interferes with the neutrophils, dendritic

cells, and natural killer cells' functions, thus delaying the process of recognizing and clearing the pathogen [38]. Nutritional stress linked to lower forage intake during hot weather makes immune dysfunction worse, mainly through the lack of zinc, selenium, and antioxidant vitamins, which are crucial for interferon signaling, antibody production, and leukocyte activation. These immune system weaknesses give a chance to intracellular pathogens like *Theileria*, *Babesia*, and *Anaplasma* to grow more rapidly and to arboviruses such as the Bluetongue virus or the lumpy skin disease virus to reach higher levels in the bloodstream. Consequently, stressed animals not only show more severe symptoms but also stay infectious for a longer period, which in turn makes them more open to secondary infections and helps the spread of the pathogen within the herd. In general, heat stress changes the host from a strong immunological barrier to a tolerant reservoir, thereby increasing the vector-borne disease spread in the warmer climate [12].

### Modeling Temperature Effects on Vector-Borne Disease Transmission

The effect of climate change on the spread of vector-borne diseases in livestock has been a subject of varied modeling techniques, one of which is SIR (Susceptible–Infected–Recovered) compartmental models, along with agent-based models (ABMs), and remote sensing-assisted predictive frameworks. SIR models are capable of estimating the time-dependent prevalence and incidence of the disease, as they provide a mechanistic framework for doing so, and allow the use of temperature-sensitive parameters like vector biting rate, pathogen extrinsic incubation period, host recovery rate, etc. Agent-based models give the advantage of better resolution in the simulations as they create individual vectors, hosts, and their interactions, and hence more accurately reflecting the dynamics of the disease caused by different patterns of movement, contact, and microclimate exposure. Remote sensing-assisted models, through the access to environmental data like land surface temperature, rainfall, and vegetation indices, enable the identification of vector habitats, the prediction of spatiotemporal risk patterns, and this is especially relevant in areas with limited epidemiological surveillance. In a numerical way, temperature has a direct effect on the basic reproduction number ( $R_0$ ), which increases with vector survival, biting frequency, and pathogen replication rates in a nonlinear manner. High temperatures lead to a faster transmission of pathogens and consequently, there is an increase in the number of degree-days, the total of thermal exposure required for the pathogen development, so  $R_0$  and EIR (entomological inoculation rates) are increased proportionately [90]. As an example, one can take the case of the *Culicoides*-transmitted Bluetongue virus, which is



said that the transmission intensity can be increased by 15-30% if the virus is allowed to mature in the vector through every 1-2°C warming. The models, once put together, clearly show that temperature is a quantitative factor that links the microclimate changes to the vector physiology, pathogen development, and finally, the livestock infection risk through the provision of a predictive surveillance and targeted mitigation framework under future climate scenarios <sup>[80]</sup> (Fig. 5).

### Vector-Borne Disease Transmission and Risk Communication under Climate Change

Climate variability plays a major role in the understanding the transmission dynamics of vector-borne diseases that affect humans and animals, such as Crimean–Congo hemorrhagic fever (CCHF), leishmaniasis, and Japanese encephalitis (JE). Increased temperatures, changes in precipitation, and natural disasters, which are all consequences of climate change, are also factors that expand the habitats of the vectors, speed up the replication of the pathogen, and increase the contact rates of the host with the vector. The Middle East and South Asia regions are noted for being affected the CCHF virus through the heat generated from the proliferation of *Hyalomma* ticks <sup>[38]</sup>. Besides, the reduction of water sources due to the drought causes the animals to congregate, which results in a higher incidence of tick bites. In the case of Latin America and South Asia, the fluctuations in climate are directly related to the changes in sandfly populations that are already assisting in the leishmaniasis transmission among the cattle, goats, and wildlife reservoirs. On the other hand, warmer winters and monsoon-associated waterlogging in the South and Southeast Asian regions are the primary factors that increase *Culex* mosquito abundance, thus exacerbating JE outbreaks in pigs, cattle, and humans <sup>[77]</sup>. Quantitative modeling suggests that warming up the ante in terms of the duration of pathogen extrinsic incubation periods (EIP), the accumulation of degree-days, and the rate of infectiousness via vector (EIR), thus, in unison pushing the basic reproduction number ( $R_0$ ) and the probability of an epidemic outbreak up. To

cope with these hazards, risk communication plans must be multi-pronged, region-specific, and aimed at different stakeholders: Besides learning about preventive measures against vectors, proper husbandry, and signs of disease early, the livestock owners and farmers should be informed; timely alerts using weather and vector surveillance should be issued by public health officials; and participation of the community should include mapping of vector hot spots using participatory approach and vaccination campaigns where necessary. The community messaging must couple climate predictions with local epidemiological data to lead preventive actions and stress the steps that are already in place such as insecticide application, controlled grazing, and installing protective barriers during the peak of vector activity. Climate-vector-pathogen interaction has been mechanistically understood and is now coupled with evidence-based communication, which means these strategies can raise the alarm, decrease livestock death rates, and cut down on the transmission of zoonotic diseases to humans <sup>[65]</sup>.

### Tick-Borne *Babesia* Infections in Livestock

Ticks are key mediators of transmission for a wide range of protozoan pathogens, namely, *Babesia ovis* and *Babesia* species in small ruminants and equids. Their pathogenicity has been a matter of great concern from the epidemiological and economic standpoint in the Mediterranean Basin, the Middle East, and in Türkiye, where the farming of sheep, goats, and equids is prevalent. The main symptoms of *B. ovis* infection in sheep and goats are hemolytic anemia, weight loss, and lower production of wool or milk, while, on the other hand, *B. caballi* and *B. equi* cause equine babesiosis, which is manifested by fever, anemia, lethargy, and sometimes, death. Consequently, the affected animals perform poorly, and their trade is restricted. Climatic conditions are critical in determining disease transmission, as a warmer climate with changes in rainfall leads to increased tick survival, faster *Babesia* cycle, and longer active period for the vectors, resulting in more infections. Furthermore, it was observed that seasonal shifts and prolonged warm periods in Türkiye and the Eastern Mediterranean have led to earlier and longer transmission seasons of the diseases, thereby increasing both disease burden and economic losses. The review gets its strength and acceptance from the regional and global aspects by including these species as a part of it, which eventually gives a better picture of the climate change impact on the protozoal diseases of ruminants and equids <sup>[38]</sup>.

## RESEARCH GAPS AND FUTURE DIRECTIONS

Climate variability, vector dynamics, and livestock disease are closely linked, yet data on climate, animal health,

and vector ecology remain fragmented across sectors. Separate meteorological, veterinary, and environmental data systems hinder early detection and coordinated responses [57]. For example, RVF outbreaks are often preceded by heavy rainfall and mosquito proliferation, but disconnected data streams reduce the ability to act in time [58]. Establishing interoperable, real-time data platforms is crucial for linking climate, vector, and disease information, tracking population trends, and detecting hotspots [66]. Many high-risk regions, especially in sub-Saharan Africa, Southeast Asia, and Latin America, lack baseline surveillance. Strengthening local infrastructure, such as weather stations, diagnostic labs, and electronic records can close these gaps [59].

Predictive models can guide early warning, vaccination, and vector control strategies. Current models often rely on historical data, ignoring evolving vector behavior, pathogen adaptation, and host immunity [93]. Coarse-scale forecasts also limit local decision-making. High-resolution, context-specific models that incorporate real-time weather, land use, livestock density, and socioeconomic factors are needed [94]. Neglected tropical diseases (NTDs) such as East Coast fever and anaplasmosis remain under-modeled due to poor surveillance and limited investment [20]. AI and ML offer opportunities to improve predictions using satellite, drone, and health record data, but transparency and accessibility are essential to build trust [95].

Underdiagnosis is common due to limited, slow, or outdated diagnostic tools [96]. In many rural areas, poor laboratory capacity delays detection and response [97]. Effective vaccines are unavailable for several major VBDs, and existing ones often face issues like cold chain dependence, genetic variability, and high costs [98]. Investment in field-adapted rapid diagnostics and thermostable vaccines is essential. Vulnerability is amplified by poverty, marginalization, and poor infrastructure [99]. Women play central roles in livestock care but face gender-specific barriers to veterinary services and resources [100]. Integrating behavioral insights and community engagement into control strategies can enhance adoption and sustainability [101].

## CONCLUSION

Climate change is increasing the transmission and impacts of vector-borne animal diseases, threatening livestock health, security, and rural livelihoods. Rising temperatures and ecosystem shifts are creating new habitats for vectors, driving more outbreaks that reduce animal productivity and disrupt supply chains. Smallholder farmers, women, and vulnerable communities are most affected, both economically and nutritionally. Effective strategies such as disease surveillance, vector control, vaccination, and climate-resilient livestock production are essential for

adaptation. These must be supported by fair policies, strong infrastructure, and education, and anchored in the One Health approach that integrates animal, human, and environmental health. To build resilience, research must address data integration gaps, predictive modeling, improved diagnostics, vaccine development, and social vulnerability. A unified international effort is needed to protect livestock systems and promote sustainable and equitable livelihoods in a changing climate.

## DECLARATION

**Availability of Data and Materials:** All the generated data are included in the manuscript.

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**Conflict of Interest:** The author declares that there is no conflict of interest.

**Generative Artificial Intelligence:** No Generative Artificial Intelligence was used in this research

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## REVIEW ARTICLE

# Research Progress of Bovine Interferon Alpha (BoIFN- $\alpha$ ) in Veterinary Science: A Review

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## Abstract

Interferons (IFNs) are a class of cytokines with antiviral, antitumor, and immunoregulatory functions, and they have great potential for use in veterinary science. Bovine interferon alpha (BoIFN- $\alpha$ ), as a crucial member of the type I IFN family, is essential for keeping Bovine population healthy and preventing diseases. In recent years, research on BoIFN- $\alpha$  has been increasing, covering aspects such as its structural characteristics, biological functions, and mechanisms of action. But in practical real-world application in clinical settings, BoIFN- $\alpha$  still has a variety of significant challenges, like keeping its clinical effectiveness stable and managing production costs. This review gives a thorough overview of the research on BoIFN- $\alpha$ , particularly its applications in antiviral, immune regulation, and disease treatment, and also introduces its clinical applications in the prevention and control of common viral, bacterial, and parasitic diseases in Bovine population. Additionally, the review discusses the challenges BoIFN- $\alpha$  faces in veterinary practice and future development directions, with the goal of giving solid theoretical support and practical advice for its in-depth research and widespread application in veterinary science.

**Keywords:** Antiviral, Bovine interferon alpha (BoIFN- $\alpha$ ), Clinical application, Disease treatment, Immune regulation, Veterinary science

## INTRODUCTION

The bovine industry is crucial for global farming, impacting meat and dairy quality, but diseases threaten herds, causing production losses and economic damage <sup>[1]</sup>. Traditional disease control methods like vaccination and antibiotics have helped, but pathogen mutations and drug resistance necessitate new prevention strategies.

Interferons (IFNs) are crucial for the immune defense, recognized since 1957 for their antiviral, immunoregulatory, and antitumor effects <sup>[2,3]</sup>. Based on their structure, receptor specificity, and functional characteristics, IFNs can be classified into type I, II, and III. Among them, type I IFNs are the most diverse, including several subtypes such as IFN- $\alpha$ , IFN- $\beta$ , and IFN- $\omega$  <sup>[3]</sup>. Bovine interferon alpha (BoIFN- $\alpha$ ) rapidly triggers the immune response after pathogen infection,

crucial for antiviral and immune regulation, protecting bovine health <sup>[4]</sup>.

Significant progress in veterinary science on BoIFN- $\alpha$  includes its gene structure, protein characteristics, mechanisms, and clinical applications (*Fig. 1*), showing its role in bovine antiviral immunity and potential in regulating immune responses and reducing diseases like respiratory issues and dermatitis <sup>[5]</sup>.

A deeper understanding of BoIFN- $\alpha$ 's biological characteristics and mechanisms is crucial for advancing bovine health and industry sustainability, with molecular biology research providing new evidence for improving bovine health and informing related fields <sup>[6]</sup>.

This review systematically summarizes BoIFN- $\alpha$  research in veterinary medicine, covering its classification, structure,



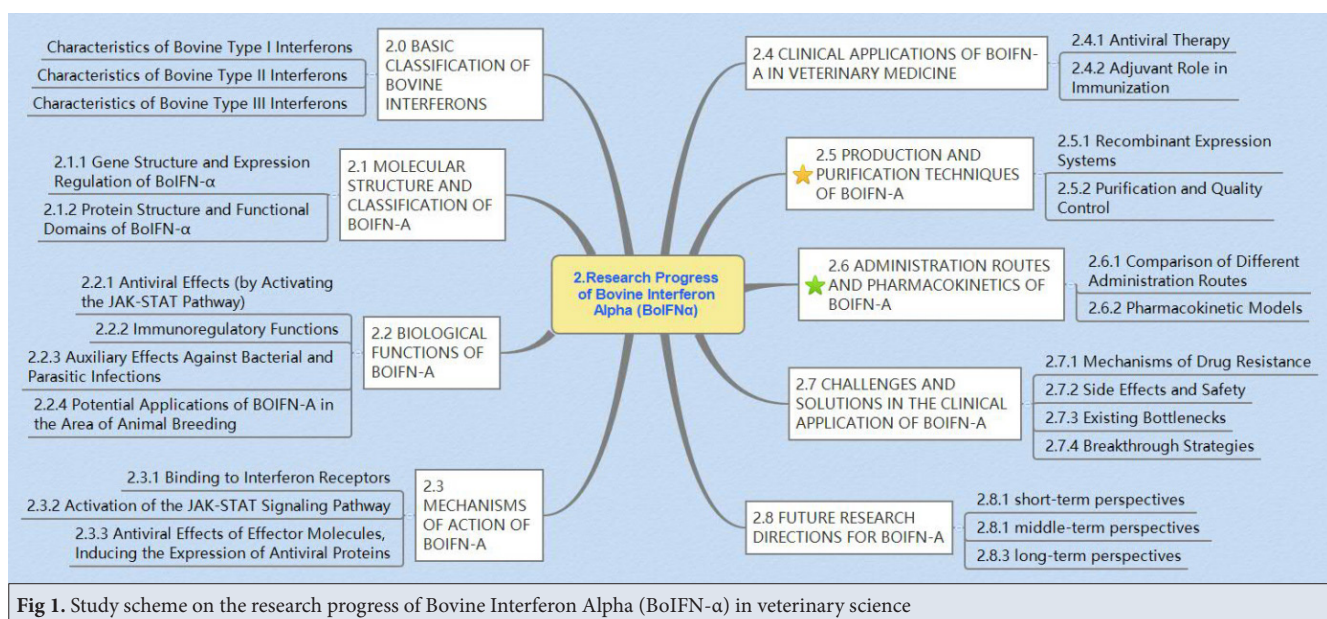


Fig 1. Study scheme on the research progress of Bovine Interferon Alpha (BoIFN- $\alpha$ ) in veterinary science

functions, mechanisms, applications, production, challenges, and future directions to enhance its use in bovine herd health and livestock sustainability.

## BASIC CLASSIFICATION OF BOVINE INTERFERONS

Bovine interferons (BoIFNs) are crucial for bovine immunity, classified into type I, II, and III, each with distinct gene families and functions. Type I (IFN- $\alpha$ , IFN- $\beta$ , IFN- $\omega$ , IFN- $\tau$ , IFN- $\chi$ , IFN- $\epsilon$ , IFN- $\kappa$ ) mediates antiviral responses, while type II (IFN- $\gamma$ ) and type III (IFN- $\lambda$ ) are involved in immune response networks, aiding in disease prevention and control.

### Characteristics of Bovine Type I IFNs

Type I IFNs, particularly IFN- $\alpha$  and IFN- $\beta$ , vary in tissue expression and are regulated by viral infections, influencing their antiviral activity [7,8]. Bovine interferon-omega (BoIFN- $\omega$ ), a type I IFN, has broad antiviral functions, inducing ISGs via the JAK (Janus kinase)-STAT (signal transducer and activator of transcription) pathway and showing potential as a therapeutic agent due to its efficacy in other species [9,10]. Bovine interferon-tau (BoIFN- $\tau$ ) is vital for early pregnancy by promoting maternal recognition and endometrial receptivity through Interferon regulatory factor 1 (IRF1) and Leukemia inhibitory factor receptor (LIFR) expression, enhancing implantation, and regulating prostaglandin synthesis for pregnancy maintenance [11,12]. Bovine interferon-chi (BoIFN- $\chi$ ) is a type I IFN with immune functions, enhancing innate immunity and promoting IFN production during viral infections via the JAK-STAT pathway [13]. Bovine interferon-epsilon (BoIFN- $\epsilon$ ), cloned from bovine liver, has a specific gene structure, shows

lower antiviral activity than BoIFN- $\alpha$ 1, and is expressed in various organs, indicating its typical type I IFN properties and therapeutic potential [14]. Bovine interferon-kappa (BoIFN- $\kappa$ ) is a 215-amino acid protein with 63% similarity to human IFN- $\kappa$ , showing type I IFN traits-like antiviral activity. It binds to the bovine type I IFN receptor, inducing Mx1, ISG15, and ISG56 transcription, and operates mainly via the JAK-STAT pathway [15]. The summary of Bovine interferon subtypes are listed in Table 1. Based on the retrieved PubMed literature, it is important to note that for Bovine Type I IFNs, all entries remain at the laboratory stage, and no specific trade names or commercially approved products are mentioned in the literature (Table 2). Therefore, we clarified the regulatory status of all products are “experimental”. This statement highlights the gap between Bovine Type I IFNs research and practical application, emphasizing challenges like production, stability, delivery, safety, and regulation.

### Characteristics of Bovine Type II IFNs

Bovine Type II IFN, mainly IFN- $\gamma$ , is produced by T cells and NK cells, enhancing immune responses through macrophage activation and antigen presentation via the STAT1 pathway, playing a crucial role in antiviral and immune regulation [19]. This literature highlights IFN- $\gamma$ 's key role in immune responses and its application in disease control, but does not address the STAT1 pathway.

### Characteristics of Bovine Type III IFNs

Bovine Type III IFNs (IFN- $\lambda$ ) resemble type I IFNs but are limited to epithelial cells, aiding mucosal immunity. Research shows IFN- $\lambda$ 's antiviral activity against bovine viral diarrhea virus (BVDV), reducing viral load and symptoms, and it primarily induces local antiviral states, minimizing systemic inflammation, highlighting its

| Table 1. Summary of bovine interferon subtypes |                                                                                                                                                                                                                   |                                                                                                                  |                                                                                                                                                                                   |                                                                                                                                                                                             |                                                                                                                                                               |                                                                                                                                                                                             |                                                                                                                                                                                                                            |                                                                                                                  |                                                                                                                                                              |
|------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Parameter                                      | BoIFN- $\alpha$ (Bovine IFN- $\alpha$ )                                                                                                                                                                           | BoIFN- $\beta$ (Bovine IFN- $\beta$ )                                                                            | BoIFN- $\omega$ (Bovine IFN- $\omega$ )                                                                                                                                           | BoIFN- $\tau$ (Bovine IFN- $\tau$ )                                                                                                                                                         | BoIFN- $\chi$ (Bovine IFN- $\chi$ )                                                                                                                           | BoIFN- $\epsilon$ (Bovine IFN- $\epsilon$ )                                                                                                                                                 | BoIFN- $\kappa$ (Bovine IFN- $\kappa$ )                                                                                                                                                                                    | BoIFN- $\gamma$ (Bovine IFN- $\gamma$ )                                                                          | BoIFN- $\lambda$ (Bovine IFN- $\lambda$ )                                                                                                                    |
| Subtype                                        | Multigene family; multiple subtypes [16]                                                                                                                                                                          | 3 subtypes [17]                                                                                                  | Different subtypes exist in species like cats and pigs [10,18]                                                                                                                    | At least 18 different BoIFN- $\tau$ cDNA variants                                                                                                                                           | 4 subtypes                                                                                                                                                    | Not specified                                                                                                                                                                               | Likely 1 major subtype                                                                                                                                                                                                     | Single gene                                                                                                      | Multiple IFN- $\lambda$ genes (e.g., $\lambda 1$ , $\lambda 2$ , $\lambda 3$ )                                                                               |
| Source Tissue/Cell                             | Peripheral blood mononuclear cells (PBMC), lymphoblast cell lines                                                                                                                                                 | Bovine kidney cells (e.g., BK, CRIB cells)                                                                       | Inferred from studies in pigs and cats, likely various cells                                                                                                                      | Trophoblast cells                                                                                                                                                                           | Can be expressed in various cells (e.g., virus-infected cells)                                                                                                | Cloned from bovine liver genomic DNA                                                                                                                                                        | Gene amplified from bovine tissues, specific source not specified                                                                                                                                                          | Mainly produced by activated T cells and natural killer (NK) cells                                               | Mucosal epithelial tissues, source include epithelial cells and dendritic cells                                                                              |
| Amino Acid Number                              | Varies by subtype                                                                                                                                                                                                 | Varies by subtype                                                                                                | 195 amino acid residues (including 23-residue N-terminal signal peptide)                                                                                                          | Varies by subtype, protein molecular weight approximately 23 kDa                                                                                                                            | Varies by subtype                                                                                                                                             | 193 amino acids (including 21-amino acid signal peptide) [14]                                                                                                                               | 215 amino acids [15]                                                                                                                                                                                                       | 143 amino acids                                                                                                  | 200 amino acids. Includes 19-23-residue N-terminal signal peptide                                                                                            |
| Signaling Pathway                              | Activates JAK-STAT pathway via IFNAR receptor complex, induces ISG expression                                                                                                                                     | Activates JAK-STAT pathway via common type I interferon receptor                                                 | Induces ISG expression via JAK-STAT pathway [10]                                                                                                                                  | (Based on type I IFN characteristics) speculated to possibly act through JAK-STAT pathway                                                                                                   | JAK-STAT pathway                                                                                                                                              | Inferred as type I IFN, likely through JAK-STAT pathway                                                                                                                                     | JAK-STAT signaling pathway                                                                                                                                                                                                 | Activates JAK-STAT pathway via IFNGR receptor complex, promotes Th1 immune response [19]                         | Speculated BoIFN- $\lambda$ function via JAK-STAT signaling pathway, its specificity determined by the IFNLR1 receptor.                                      |
| Characteristics                                | 1. Subtype expression differences; 2. Rigid expression patterns; 3. Activity differences among subtypes; 4. Potent, broad-spectrum antiviral activity, induce systemic responses; 5. Side effects like fever [19] | 1. Virus-induced specificity; 2. Promoter differences among subtypes; 3. Viral infection leads to immune evasion | 1. Induces ISG expression; 2. Cross-species activity; 3. Affects miRNA composition in blood cell-derived extracellular vesicles, possesses novel immunomodulatory mechanisms [20] | 1. Induces IRF1 and LIFR expression; 2. Reduces E-cadherin expression; 3. Enhances endometrial receptivity; 4. Regulates prostaglandin synthesis [11,12]                                    | 1. Highly sensitive to trypsin, but stable under pH and temperature changes; 2. Multigene family, possesses antiviral and antiproliferative activities        | 1. Antiviral activity lower than BoIFN- $\alpha$ ; 2. Highly transcribed in EBK cells; 3. Constitutively expressed in liver, thymus, kidney, small intestine, testis, but not in heart [14] | 1. Sensitive to trypsin; 2. Stable under pH and temperature changes; 3. Possesses antiviral and antiproliferative activities [15]                                                                                          | 1. Core pro-inflammatory cytokine; 2. Primarily activates macrophages; 3. Involved in cellular immune responses. | 1. Possesses antiviral activity; 2. Good safety profile; 3. Its receptor is mainly expressed on epithelial cells; 4. Involved in mucosal immune defense [21] |
| Function                                       | 1. Establishes systemic antiviral state; 2. Activates innate immune cells; 3. Key cytokine in defense against viral infections [7]                                                                                | Mediates antiviral responses and plays roles in immune regulation [22]                                           | 1. Broad-spectrum antiviral; 2. Participates in immune regulation; 3. Has therapeutic potential [9,10]                                                                            | Related to reproduction, including: 1. Maternal recognition; 2. Enhancing endometrial receptivity and embryo implantation; 3. Regulating prostaglandin synthesis to maintain pregnancy [23] | 1. Antiviral and antiproliferative activities; 2. Enhances IFN production via positive feedback regulation (e.g., activates IFN-gulating prostaglandins [13]) | 1. Antiviral activity; 2. Potential therapeutic agent                                                                                                                                       | 1. Antiviral activity: binding to bovine type I IFN receptor, can be blocked by specific antibodies; 2. Induces ISG gene transcription; 3. Activates promoters including NF- $\kappa$ B, ISRE, and BoIFN- $\beta$ promoter | 1. Regulates adaptive immunity; 2. Activates macrophages; 3. Enhances antigen presentation [19]                  | 1. Anti-infection; 2. Induces immune responses, promotes early production of bovine viral diarrhea virus (BVDV) neutralizing antibodies post-infection       |

**Table 2.** Summary of commercial and experimental use of bovine type I IFNs

| Trade Name     | Active Ingredient | Target Species    | Indication                                      | Formulation and Route of Administration | Common Dosage/Regimen          | Regulatory Status | Key References |
|----------------|-------------------|-------------------|-------------------------------------------------|-----------------------------------------|--------------------------------|-------------------|----------------|
| No information | BoIFN-alpha 1-1   | Swine population  | Antiviral (transmissible gastroenteritis virus) | Recombinant protein, oral               | 1mg orally every 12 hours      | Experimental      | [24]           |
| No information | rIFN-alpha        | Bovine population | Antiviral diseases (e.g., viral infections)     | Recombinant protein, expression system  | No specific dosage information | Experimental      | [25]           |
| No information | BoIFN-omega       | Bovine population | Antiviral (e.g., VSV, BEV)                      | Recombinant protein, yeast expression   | No specific dosage information | Experimental      | [26]           |
| No information | BoIFN-epsilon     | Bovine population | Antiviral                                       | Recombinant protein, E. coli expression | No specific dosage information | Experimental      | [14]           |
| No information | BoIFN-kappa       | Bovine population | Antiviral                                       | Recombinant protein                     | No specific dosage information | Experimental      | [15]           |

Based on the retrieved PubMed literature, the above table summarizes the commercial and experimental applications of Bovine Type I IFNs. It is important to note that all entries are derived from research contexts, and no specific trade names or commercially approved products are mentioned in the literature

importance in mucosal immunity [21]. The study had a small sample size (n=4 treatment, n=2 control), limiting its statistical power and generalizability, and focused on BVDV type-2, suggesting varying effects of IFN- $\lambda$  with other strains.

## MOLECULAR STRUCTURE AND CLASSIFICATION OF BOIFN- $\alpha$

### Gene Structure and Expression Regulation of BoIFN- $\alpha$

BoIFN- $\alpha$  is a key antiviral cytokine in cattle, with its gene on chromosome 1 consisting of 5 exons and 4 introns, coding for about 567 bp. There are at least 10 subtypes, showing 80%-95% sequence homology, possibly linked to bovine adaptation to pathogens [27,28].

Research indicates that BoIFN- $\alpha$  subtypes regulate immune responses via different pathways, offering insights into bovine immune systems [29]. The subtypes differ in antiviral, antibacterial, and immune regulation, impacting bovine herd health. BoIFN- $\alpha$  levels are affected by factors like viral infections, which significantly upregulate its expression, highlighting its role in antiviral responses [30].

Further research shows BoIFN- $\alpha$  levels are linked to the JAK-STAT pathway; using the BacMam system allows effective expression of BoIFN- $\alpha$  in cells to observe its viral replication inhibition [31]. The key to this process is that BoIFN- $\alpha$  induces ISG transcription, enhancing antiviral capacity and supporting its veterinary use.

### Protein Structure and Functional Domains of BoIFN- $\alpha$

The BoIFN- $\alpha$  precursor protein has 189-192 amino acids, a 23-amino acid signal peptide, and a mature form of 166-169 amino acids, weighing about 19 kDa with a pI of 5.0-

6.5, featuring a structure of 5 anti-parallel  $\alpha$ -helices (A-E) crucial for receptor binding [32].

Compared to IFN- $\alpha$  from other species, BoIFN- $\alpha$  has the highest amino acid homology with sheep IFN- $\alpha$  (about 85%), about 60% with human IFN- $\alpha$ , and about 70% with pig IFN- $\alpha$ . This species difference causes some specificity in BoIFN- $\alpha$  activity, yet there is cross-reactivity with related species [33].

BoIFN- $\alpha$ , a secreted glycoprotein, has a central  $\alpha$ -helical structure and  $\beta$ -sheet segments, allowing it to bind to IFN receptors and trigger signal transduction pathways for its biological functions. In the structure of BoIFN- $\alpha$ , the receptor-binding domain is one of its important functional regions [34]. The domain composition affects BoIFN- $\alpha$ 's receptor binding affinity and selectivity, influencing its biological effects through stable interactions and activation of signaling pathways that enhance antiviral and immune responses.

Additionally, the signaling domain of BoIFN- $\alpha$  is also crucial [35]. The signaling domain transduces external signals into cells, activating the JAK-STAT pathway after BoIFN- $\alpha$  binds to its receptor, which regulates cell proliferation, differentiation, and immune responses by recruiting and activating JAK enzymes firstly and then followed by activating STAT transcription factors that express specific genes involved in antiviral, antitumor, and immune regulation.

It is noteworthy that the glycosylation modifications of BoIFN- $\alpha$  also have a significant impact on its function [36-38]. Glycosylation not only affects its stability and biological activity but may also influence its binding ability to receptors [39,40]. By regulating the glycosylation pattern, the biological activity of BoIFN- $\alpha$  can be enhanced or reduced,

resulting in different effects in clinical applications <sup>[41-43]</sup>. Therefore, in-depth studies of the protein structure and functional domains of BoIFN- $\alpha$  are of great significance for understanding its role in veterinary science and developing new therapeutic approaches.

## BIOLOGICAL FUNCTIONS OF BOIFN-A

### Antiviral Effects (by Activating the JAK-STAT Pathway)

BoIFN- $\alpha$  is a crucial cytokine that plays a key role in antiviral immunity. Studies have shown that BoIFN- $\alpha$  significantly inhibits the replication of viruses by activating the JAK-STAT signaling pathway <sup>[44,45]</sup> (Fig. 2). The JAK-STAT pathway is crucial for cytokine signaling, regulating immune responses; BoIFN- $\alpha$  activates it, through boosting ISG expression and enhancing antiviral capacity.

The specific process is as follows: after IFN- $\alpha$  binds to the IFN- $\alpha$ -receptor on the cell surface, it subsequently activates intracellular Janus kinases (Janus kinases 1 (JAK1) and tyrosine-protein kinase 2 (TYK2)). The phosphorylation of JAK kinases leads to the activation of STAT proteins, causing STAT1 and STAT2 to be phosphorylated and form heterodimers, which bind to IRF9 to form “interferon-stimulated gene factor 3 (ISGF3).” ISGF3 enters the cell nucleus and binds to the “interferon-stimulated response

element (ISRE)” in the promoter region of target genes, initiating downstream gene transcription. Ultimately, it regulates the expression of ISGs, thereby enhancing the cell's resistance to viruses.

### Antiviral Effects of Effector Molecules, Inducing the Expression of Antiviral Proteins

BoIFN- $\alpha$  exerts significant antiviral effects in veterinary science by inducing the expression of antiviral proteins and regulating the immune responses of host cells <sup>[46]</sup> (Fig. 3). Key effector molecules induced by BoIFN- $\alpha$  include: (1) 2',5'-oligoadenylate synthetase (OAS): catalyzes the production of 2',5'-oligoadenylate from ATP, activating RNase L to degrade viral RNA; (2) Protein kinase R (PKR): phosphorylates the eukaryotic translation initiation factor eIF2 $\alpha$ , inhibiting viral protein synthesis; (3) Mx protein: prevents the release and replication of viral nucleic acids by binding to viral nucleocapsid proteins (e.g., inhibition of BVDV); (4) Interferon regulatory factors (IRF): further amplify immune signals and enhance antiviral responses.

Research indicates that BoIFN- $\alpha$  activates ISGs like MxA, OAS, and PKR, crucial for antiviral immunity; rBoIFN- $\alpha$  transduced via BacMam significantly inhibited virus replication and induced ISG transcription <sup>[31]</sup>. It was proved that BoIFN- $\alpha$  enhances antiviral response via

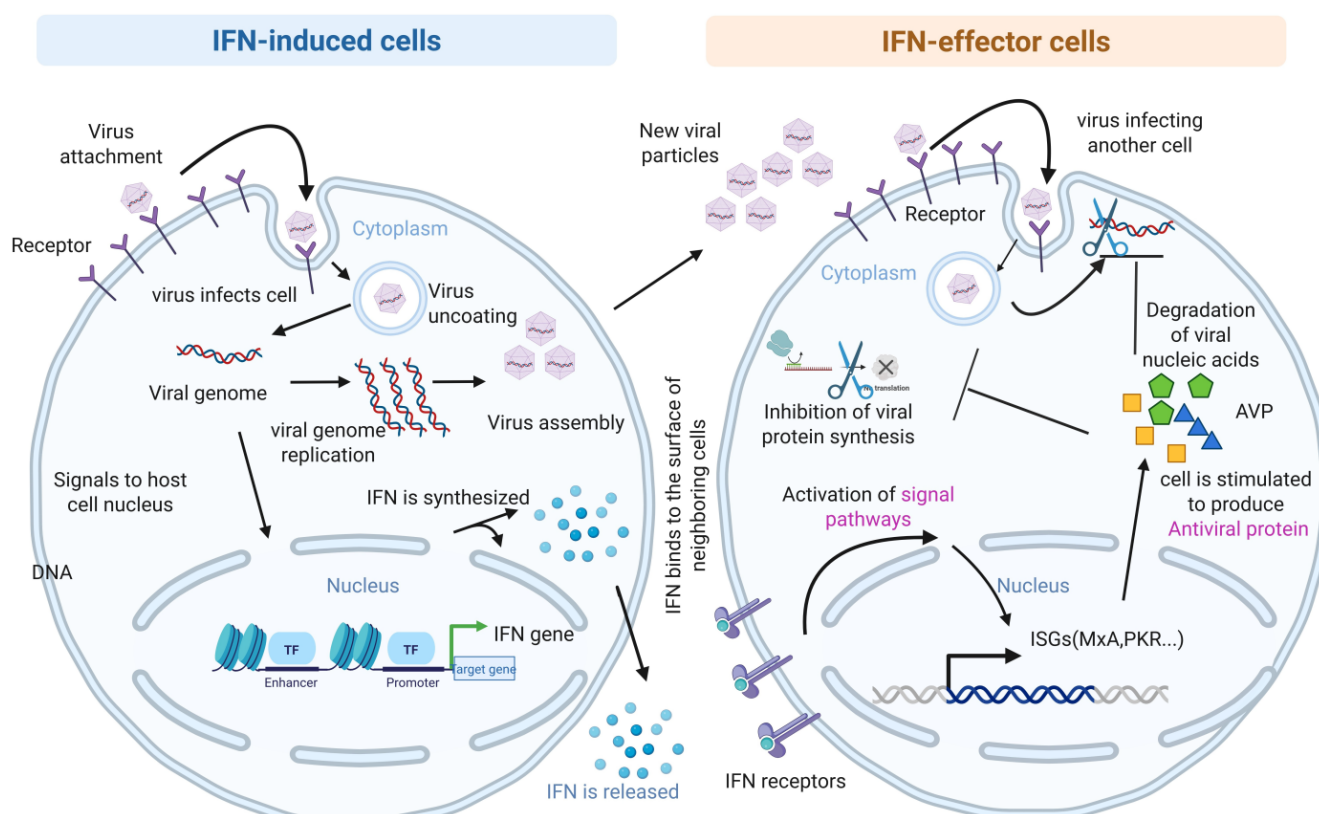


Fig 2. Presentation of the induction and effectiveness of interferon (IFN)

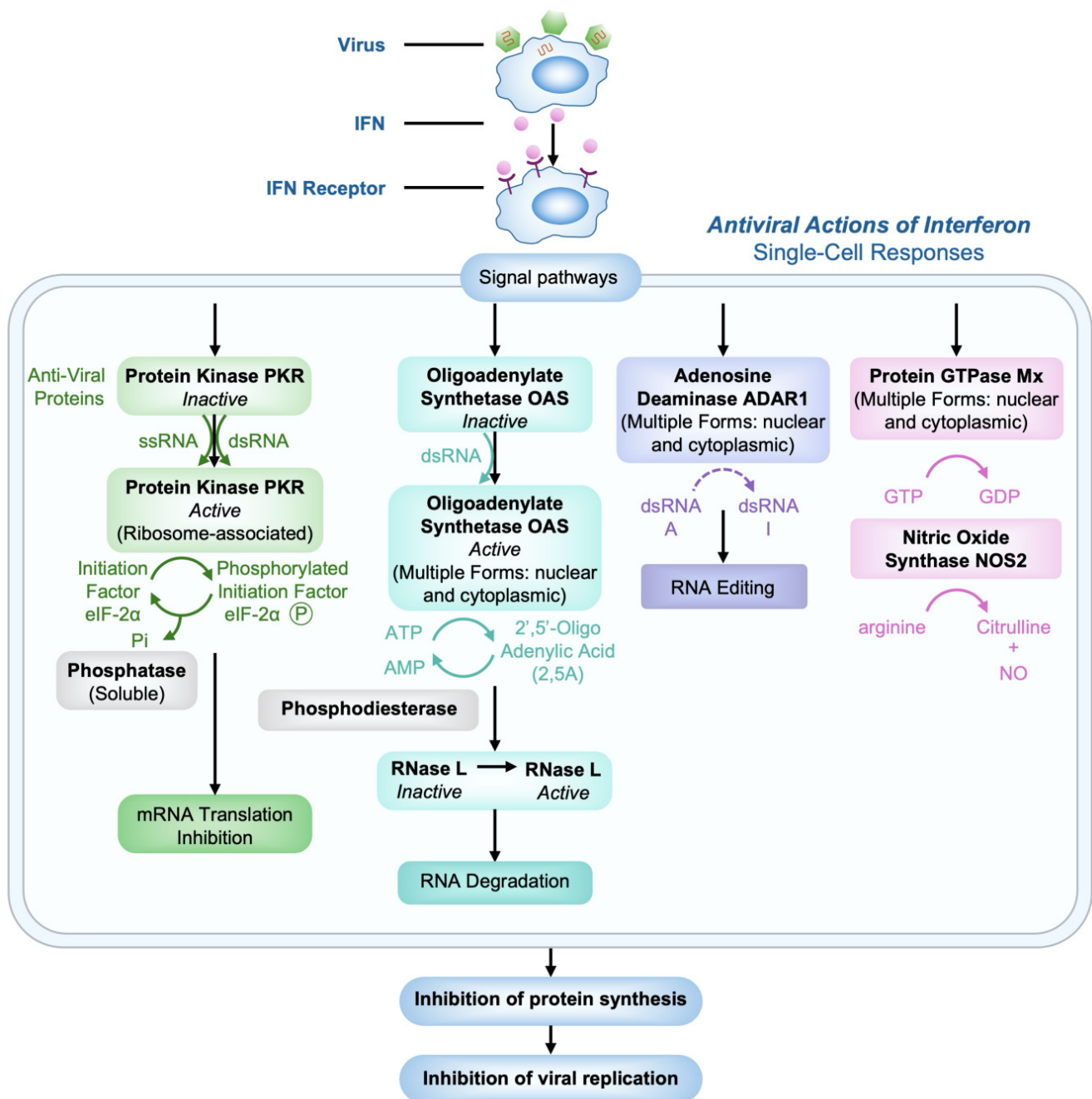


**Fig 3.** Functions of selected IFN-inducible proteins are critical. Among the IFN-induced proteins that affect virus multiplication within single cells are PKR kinase, the OAS synthetase family, the Mx protein GTPase family, and ADAR. These proteins appear to target viral nucleocapsids and inhibit RNA synthesis, thereby preventing viral replication

JAK-STAT, but the study lacks focus on long-term effects like viral adaptation or immune tolerance.

Additionally, IFNs have antiviral effects through various mechanisms; herbal extracts like matrine and icariin upregulate IFN- $\alpha$  and IFN- $\beta$ , enhancing antiviral protein production and inhibiting BVDV replication *in vitro*, but more research is needed for “synergistic effect” conclusions<sup>[47]</sup>.

BoIFN- $\alpha$ 's antiviral mechanisms also include regulating

host cell metabolism, with IFNs inhibiting viral infections by altering metabolic states. For example, SAMHD1 limits some RNA virus (such as Hepatitis C Virus and yellow fever virus) replication by regulating lipid synthesis, highlighting IFNs' antiviral effects<sup>[48]</sup>. However, the studied viruses are human viruses, while our BoIFN- $\alpha$  is bovine-derived, indicating potential functional differences in IFN and SAMHD1 across species.

Moreover, BoIFN- $\alpha$  is linked to interactions with other

cytokines. For example, ZAP protein, as an ISG, exhibits broad antiviral activity, and in cells lacking a robust type I IFN system, ZAP can also inhibit the replication of certain viruses [49]. However, Further studies are warranted to elucidate this IFN-alpha and -beta independent anti-Zika virus activity and involvement of ZAP.

### Immunoregulatory Functions

BoIFN- $\alpha$  is important for immune regulation, enhancing NK cells and macrophages' activity, and boosting NK cells' ability to kill tumors and viruses-infected cells [50]. Besides, BoIFN- $\alpha$  boosts macrophage phagocytosis and bactericidal functions, crucial for innate immunity, and enhances cytokine secretion like tumor necrosis factor alpha (TNF- $\alpha$ ) and IFN beta (IFN- $\beta$ ) in bovine infection models [51].

In adaptive immunity, BoIFN- $\alpha$  promotes Th1 responses and inhibits Th2, regulating immune balance by enhancing Th1 cell differentiation and cytokine production, activating T and B cells [52]. This regulatory effect is significant for fighting viral infections and tumors, as Th1 responses eliminate infected cells and tumors while suppressing excessive humoral responses, reducing allergy and autoimmune risks. Moreover, BoIFN- $\alpha$  has notable antitumor effects, such as inhibiting cell proliferation, suppressing angiogenesis, and boosting immune cell activity. One study showed it significantly inhibits tumor growth by upregulating tumor suppressor genes and downregulating oncogenes [53]. At the same time, BoIFN- $\alpha$  can induce apoptosis in tumor cells [54], and enhance the immune surveillance capacity of macrophages and NK cells against tumor cells, providing new ideas and potential applications for cancer immunotherapy.

### Auxiliary Effects Against Bacterial and Parasitic Infections

The use of BoIFN- $\alpha$  in veterinary science is gaining attention for aiding in bacterial and parasitic infections by enhancing immune functions, crucial for combating diseases that affect bovine health and farming economics. In terms of combating bacterial infections, BoIFN- $\alpha$  has been found to significantly enhance the phagocytic ability of bovine macrophages against *Brucella* [55]. *Brucella* causes brucellosis in cattle, impacting reproduction and milk yield. BoIFN- $\alpha$  activates macrophages, boosting their ability to kill *Brucella* by enhancing activation, cytokine secretion, and antibacterial proteins. It also helps against calf diarrhea from *E. coli* [56]. *Escherichia coli* diarrhea in calves can cause high mortality, but BoIFN- $\alpha$  enhances local immunity and intestinal barrier functions, aiding infection control and informing prevention strategies. BoIFN- $\alpha$  shows promise against parasitic infections like bovine babesiosis and theileriosis, which cause anemia and reduced production performance, by boosting the

host's immune response [57]. BoIFN- $\alpha$  enhances T cell activation and cytokine secretion, such as IFN- $\gamma$  and TNF- $\alpha$ , improving bovine resistance to parasites.

### Potential Applications of BOIFN-A in the Area of Animal Breeding

BoIFN- $\alpha$  improves animal reproduction by increasing pregnancy rates and regulating immunity, with sheep studies showing rates rising from 58% to 79% without teratogenic effects [58]. Additionally, BoIFN- $\alpha$  is linked to embryo signaling molecules like Bovine trophoblast protein-1 (bTP-1), which aids in maternal immune regulation to prevent embryo rejection [59]. Furthermore, BoIFN- $\alpha$  inhibits lymphocyte migration from lymph nodes but does not affect their entry into lymph nodes, supporting embryo implantation and pregnancy maintenance, suggesting its immunity may influence reproduction [60]. Overall, BoIFN- $\alpha$  improves pregnancy rates and immune responses in animals, but further research is needed for safety in species like cattle, requiring more clinical trials for livestock management.

## MECHANISMS OF ACTION OF BOIFN-A

BoIFN- $\alpha$  functions through the "receptor-signaling pathway-effector molecules" axis, primarily activating the JAK-STAT pathway to express antiviral proteins [46].

### Binding to IFN Receptors

BoIFN- $\alpha$  binds to type I IFN  $\alpha$  receptors (IFNAR) on target cells, with IFNAR2 binding BoIFN- $\alpha$  and IFNAR1 involved in signaling; this high-affinity binding (Kd  $\sim 10^{-10}$  M) relies on BoIFN- $\alpha$ 's  $\alpha$ -helical structure, activating antiviral gene expression and highlighting its role in immunity through bioassays in MDBK cells that stimulate Toll-like receptors TLRs (e.g. TLR3, TLR7, and TLR8) and RIG-I-like receptors (RIG-I and MDA5) [61].

Moreover, the antiviral mechanism of BoIFN- $\alpha$  includes regulating host immune responses, not just direct effects [62]. BoIFN- $\alpha$  significantly increases during BVDV infection, enhancing antiviral factors and improving bovine resistance by upregulating cytokines like IFN- $\beta$ , interleukin-1(IL-1), and TNF- $\alpha$  [47].

After binding to the IFN receptor, the activated IFN receptor signaling pathways induce ISG expression, crucial for antiviral, antitumor, and immunoregulatory responses, with BoIFN- $\alpha$  enhancing antiviral gene expression like Mx1, OAS1, and IFIT in host cells [63].

It is noteworthy that the effects of BoIFN- $\alpha$  on IFN receptors depend on factors like the host's genetics and the virus type, indicating the need to consider immune status and viral circumstances for effective treatment

strategies. Future research should investigate BoIFN- $\alpha$ 's efficacy against various pathogens for improved veterinary options.

## CLINICAL APPLICATIONS OF BOIFN-A IN VETERINARY MEDICINE

### Antiviral Therapy

**Treatment of Bovine Viral Diarrhea Virus (BVDV) Infection:** BVDV is a harmful virus in cattle, causing symptoms like calf diarrhea, respiratory issues, and abortions, leading to economic losses <sup>[64]</sup>.

Research shows that BoIFN- $\alpha$  can significantly inhibit the replication of different biotypes of BVDV (cytopathic and non-cytopathic) <sup>[47]</sup>, and its antiviral effect is time-dependent and dose-dependent. In the early stages of infection (0-20 h), BoIFN- $\alpha$  maximally inhibits BVDV RNA synthesis in the early stages of infection (0-20 h), reducing viral load significantly without relying on the PKR pathway, indicating alternative antiviral mechanisms <sup>[65]</sup>. Experimental results show rBoIFN- $\alpha$  significantly inhibits BVDV replication in Madin Darby Bovine Kidney (MDBK) cells, indicating its antiviral potential <sup>[4]</sup>. Additionally, rBoIFN- $\alpha$  boosts antiviral capacity by activating immune cells and inducing interferon-stimulated genes (ISGs) like ISG15 and OAS1, lowering viral load and disease severity <sup>[4]</sup>.

In scientific experiments, BoIFN- $\alpha$  significantly relieved BVDV symptoms in cattle, reducing viral load and improving CD<sub>4</sub><sup>+</sup> and CD<sub>8</sub><sup>+</sup> T cell function <sup>[66]</sup>.

**Prevention and Treatment of Infectious Bovine Rhinotracheitis Virus (IBRV):** Infectious Bovine Rhinotracheitis Virus (IBRV) is a common virus in the bovine respiratory tract, which early infection damages the respiratory mucosal barrier, leading to decreased immune defense and susceptibility to secondary bacterial infections <sup>[67]</sup>. The early use of BoIFN- $\alpha$  in IBR infection boosts antiviral responses in respiratory cells and immune cells, strengthening local immune barriers and lowering viral replication. Studies indicate BoIFN- $\alpha$  activates ISGs in respiratory mucosa, boosting NK cells and macrophages to enhance immune clearance of IBR virus <sup>[68]</sup>. Meanwhile, The combination of BoIFN- $\alpha$  and the IBR vaccine enhances immune protection and lowers viral transmission risk <sup>[69]</sup>.

**Prevention of Bovine Respiratory Disease (BRD):** Akiyama et al. <sup>[6]</sup> described a clinical trial testing the effect of recombinant bovine interferon alpha 1 (rBoIFN- $\alpha$ 1) on the incidence of bovine respiratory disease (BRD). The experiment with 60 bulls showed that rBoIFN- $\alpha$ 1 injections significantly reduced respiratory disease symptoms, with lower incidence, recurrence rate, and average days of illness, and slightly higher weight gain,

leading to the conclusion that it may prevent BRD.

**Treatment of Foot-and-Mouth Disease Virus (FMDV), Bovine Rotavirus (BRV), Coronaviruses (CoV), and Influenza Virus (IFV) Infections:** Regarding Foot-and-Mouth Disease Virus (FMDV), BoIFN- $\alpha$  has inhibits effects by regulating immune responses and promoting antibody production <sup>[70]</sup>. BoIFN- $\alpha$  activates the IFN pathway, inhibiting the virus and boosting immune response for better resistance.

Regarding Bovine Rotavirus (BRV), BoIFN- $\alpha$  treatment positively affects calf diarrhea, alleviating the condition and promoting recovery <sup>[71,72]</sup>.

Regarding Coronaviruses (CoV), Recombinant bovine interferon-alpha I1 (rBoIFN- $\alpha$ ) is a sustained release treatment for bovine respiratory disease, indicating broad antiviral potential, but its effect on coronaviruses is not mentioned in the literature <sup>[69]</sup>.

Regarding the Influenza Virus (IFV), BoIFN- $\alpha$  inhibits viral replication by impairing early protein synthesis in bovine cells, unlike BoIFN- $\gamma$  <sup>[44]</sup>. The antiviral effect relates to two Mx-related proteins from BoIFN- $\alpha$ , akin to the resistance against influenza in mice <sup>[44]</sup>.

**Adjunctive Therapy for Bovine Leukemia Virus (BLV) Infection:** BLV infection is a chronic disease causing lymphocyte proliferation and immune dysfunction, potentially leading to leukemia or lymphoma <sup>[73]</sup>. Currently, there is no cure, so management focuses on controlling the virus and regulating immunity.

BoIFN- $\alpha$  enhances T cell and NK cell recovery <sup>[66]</sup>, boosts cytokine production, reduces viral load, inhibits replication, and improves immune function against BLV.

BoIFN- $\alpha$  can enhance conventional vaccines in managing BLV-infected cattle, alleviating symptoms, improving health indicators, and extending lifespan while strengthening immune responses.

### Adjuvant Role in Immunization

BoIFN- $\alpha$  is a key immunoregulatory factor studied in veterinary science, particularly as a vaccine adjuvant, enhancing immunogenicity by promoting cytokine secretion, boosting antibody production, and regulating T cell activity, thus improving vaccine efficacy. Traditional adjuvants like aluminum salts stimulate immune responses but have limitations and side effects, making the search for new adjuvants crucial. BoIFN- $\alpha$  enhances vaccine immunogenicity by inducing strong cell-mediated and humoral immune responses, as shown in a study where it increased antibody levels and T cell responses with the foot-and-mouth disease vaccine <sup>[74]</sup>.

More importantly, Vaccines with BoIFN- $\alpha$  enhance

specific T cell responses, increasing IFN- $\gamma$ -positive CD<sub>8</sub><sup>+</sup> lymphocytes, offering new insights for foot-and-mouth disease vaccine research and application. Optimizing adjuvants may improve vaccine efficacy and safety for sustainable farming.

## PRODUCTION AND PURIFICATION TECHNIQUES OF BOIFN- $\alpha$

### Recombinant Expression Systems

The choice of expression system is vital for producing recombinant BoIFN- $\alpha$ , with options like *Escherichia coli*, yeast, and mammalian cells, each having pros and cons. *Escherichia coli* is fast and cost-effective for large-scale production but lacks post-translational modifications<sup>[32]</sup>, potentially impairing protein folding and antiviral activity<sup>[31]</sup>. Yeast, a eukaryotic system, allows some modifications and is suitable for medium-scale production, but may still struggle with glycosylation and stability issues. Mammalian cell systems like HEK293T better mimic *in vivo* conditions, enabling crucial post-translational modifications for BoIFN- $\alpha$  activity. Baculovirus-mediated gene transfer (BacMam) effectively expresses BoIFN- $\alpha$  in these cells, yielding a biologically active product with antiviral properties, showing promise in veterinary science<sup>[31]</sup>. Researchers optimize BoIFN- $\alpha$  yield and activity through strategies like media optimization, culture condition adjustments, and genetic engineering, enhancing protein effectiveness and safety for clinical use.

### Purification and Quality Control

Purification and quality control of BoIFN- $\alpha$  are vital for its biological activity and safety, with methods like affinity and ion exchange chromatography influencing the final product's characteristics. Affinity chromatography uses specific binding to enhance purification<sup>[32]</sup>, often employing antibodies against IFN- $\alpha$  as ligands<sup>[75]</sup>. Ion exchange chromatography separates proteins based on charge at varying pH levels, using appropriate resins for effective purification.

Activity testing uses bioactivity assays like MTT, cell proliferation, or ELISA to assess BoIFN- $\alpha$ 's effects on cells, showing its role in immune responses against BVDV by upregulating gene expression<sup>[75]</sup>. Stability assessments ensure product effectiveness during storage, with research indicating BoIFN- $\alpha$ 's long stability under proper conditions, supporting its clinical potential.

## ADMINISTRATION ROUTES AND PHARMACOKINETICS OF BOIFN- $\alpha$

### Comparison of Different Administration Routes

The choice of administration routes for BoIFN- $\alpha$  in

veterinary science is vital for treatment efficacy. Subcutaneous injections provide slower release and sustained immune activation, while intramuscular injections offer quicker delivery and higher bioavailability<sup>[76]</sup>, though they may cause more local reactions. Nebulized inhalation delivers BoIFN- $\alpha$  directly to the respiratory tract, minimizing systemic side effects and enhancing local immunity, with good tolerance and bioavailability in small animals, but requires further validation for large animals<sup>[77]</sup>. The half-life varies by administration route, with nebulized inhalation achieving higher initial concentrations but shorter half-life, impacting its use in chronic diseases<sup>[78]</sup>. Thus, careful selection of administration routes and frequencies is crucial for maximizing therapeutic effects.

### Pharmacokinetic Models

BoIFN- $\alpha$  is a key immune regulator in bovine population with significant pharmacokinetic traits essential for clinical use. It quickly enters the bloodstream post-injection, maintaining high concentrations influenced by administration route, dosage, and bovine physiological state<sup>[79]</sup>. It is widely distributed, mainly in immune organs like the liver, spleen, and lungs, which relates to its immune regulation function<sup>[76]</sup>. Metabolized by the liver and excreted by the kidneys, BoIFN- $\alpha$  has a short biological half-life, limiting long-term efficacy<sup>[79]</sup>. Thus, dosing regimens must account for its rapid clearance, possibly requiring frequent doses or sustained-release formulations<sup>[78]</sup>, while individual bovine differences may necessitate tailored clinical applications.

The dose-response relationship of BoIFN- $\alpha$  is vital in drug research and requires systematic clinical trials, as it significantly influences cattle's immune response; low doses enhance antiviral capacity while high doses may cause immunosuppression<sup>[80]</sup>. Establishing this relationship involves controlled experiments to monitor immune indicators like cytokines and clinical symptoms, aiding in determining optimal dosages and providing evidence for clinical applications<sup>[81]</sup>.

## CHALLENGES AND SOLUTIONS IN THE CLINICAL APPLICATION OF BOIFN- $\alpha$

### Mechanisms of Drug Resistance

In the context of viral infections, working procedures of drug resistance mainly manifest in two aspects: the emergence of viral escape mutants and adaptive changes in host immune responses.

Firstly, the emergence of viral escape mutants involves viruses altering their antigenic epitopes through mutations to evade the host immune system. Research shows BoIFN- $\alpha$  is vital in regulating immune responses,

particularly for CD $_8^+$  T cell responses against viral infections <sup>[50]</sup>. Besides, studies on CpHV-1 (Caprine herpesvirus type 1) infection have found that this virus can regulate immune genes within host cells, thereby affecting the host's immune response <sup>[82]</sup>. This phenomenon is crucial in viral transmission and persistent infections, showcasing virus-host interactions.

Secondly, adaptive changes in host immune responses involve adjustments to the immune system due to viral infections, with research indicating significant changes in immune gene expression, including ISG upregulation crucial for antiviral immunity <sup>[82]</sup>. Studies show that heat-inactivated *Mycobacterium* (HIMB) and P22PI protein complexes stimulate the innate immune response, increasing cytokine expression and enhancing pathogen defense <sup>[83]</sup>. This adaptive change enhances the host's defense against infections and may create lasting "trained immunity".

### Side Effects and Safety

BoIFN- $\alpha$  has therapeutic effects in veterinary science but also presents side effects and safety concerns, necessitating careful monitoring. Common side effects include fever and leukopenia, which can affect bovine health and performance. Long-term use is generally safe at proper doses, with studies showing good tolerance in cattle when monitored effectively.

However, the use of BoIFN- $\alpha$  must adhere to dosage guidelines to prevent misuse and side effects, while veterinarians should tailor treatment plans based on the health and disease of the bovine population, adjusting them through regular monitoring.

### Existing Bottlenecks

BoIFN- $\alpha$  faces significant clinical application bottlenecks, primarily due to its short half-life, which complicates maintaining effective body concentrations and reduces therapeutic efficacy <sup>[61]</sup>, necessitating frequent dosing that may lower compliance from patients and veterinarians.

BoIFN- $\alpha$  production costs are much higher than other drugs, hindering its use, especially in economically disadvantaged areas where affordability is a concern <sup>[84]</sup>.

The administration of BoIFN- $\alpha$  via injection faces challenges like animal cooperation and stress, complicating large-scale operations <sup>[50]</sup>. Oral administration research is still exploratory and lacks a mature regimen.

BoIFN- $\alpha$  can trigger immunogenic reactions, posing a significant risk; exogenous proteins may cause antibody production, reducing IFN efficacy and leading to immune-related diseases, complicating clinical management <sup>[85]</sup>.

### Breakthrough Strategies

The use of gene editing (such as CRISPR/Cas9) in BoIFN- $\alpha$

research enhances its activity and production, optimizing expression systems for higher yields and aiding vaccine and drug development <sup>[86]</sup>.

Multi-omics research is crucial for understanding BoIFN- $\alpha$  by integrating genomic, transcriptomic, proteomic, and metabolomic data, revealing its effects on viral infections and host responses, and aiding in new therapeutic strategies and personalized medicine <sup>[87]</sup>.

The development of new formulations is key for improving BoIFN- $\alpha$ 's clinical use, with researchers investigating drug delivery systems like nanoparticles, liposomes, and hydrogels to enhance IFNs' stability and bioavailability, protect against degradation, and enable targeted release, thus improving therapeutic effects and sustaining antiviral responses, particularly in veterinary medicine <sup>[86]</sup>.

Combination therapy with BoIFN- $\alpha$  and other antivirals or immune modulators shows promise in enhancing efficacy, improving host resistance, reducing viral replication, and minimizing drug resistance, requiring personalization for different pathogens <sup>[87]</sup>.

Personalized dosing regimens for BoIFN- $\alpha$  enhance treatment efficacy by adjusting dosage and frequency based on individual immune responses, pathogen types, and resistance characteristics, relying on detailed patient assessments <sup>[88]</sup>.

## FUTURE RESEARCH DIRECTIONS FOR BOIFN-A

### Short-term Perspectives

The development of BoIFN- $\alpha$  derivatives advances veterinary science by enhancing activity and stability through genetic engineering, with BacMam systems effectively expressing recombinant BoIFN- $\alpha$  in mammalian cells against viruses like bovine parainfluenza virus 3 (BPIV3) and BVDV <sup>[31]</sup>. This process boosts BoIFN- $\alpha$  expression and antiviral effects both *in vitro* and *in vivo*, showing genetic engineering's effectiveness.

### Middle-term Perspectives

PEGylation attaches PEG chains to biomolecules, improving pharmacokinetics and biocompatibility; in BoIFN- $\alpha$  development, it enhances stability, extends half-life, reduces immune clearance, and boosts therapeutic effects while minimizing immunogenicity and allergic reactions, crucial for veterinary medicine safety <sup>[89]</sup>.

### Long-term Perspectives

BoIFN- $\alpha$  shows potential in veterinary science, particularly in its synergistic effects when combined with antiviral drugs or vaccines. Research indicates that IFNs play an important role in enhancing the immune response, improving the

efficacy of antiviral drugs, and enhancing vaccine immune responses. This combination therapy strategy enhances the overall therapeutic effects by boosting the body's antiviral response, making the synergistic effects of IFNs with antiviral drugs or vaccines more pronounced.

In co-infections, IFNs offer unique benefits by enhancing immune responses against multiple pathogens, as shown in studies with pigs infected by porcine epidemic diarrhea virus (PEDV) and porcine delta coronavirus (PDCoV), where IFN- $\alpha$  improved disease severity and immune response<sup>[90]</sup>.

Moreover, research on canines has shown that in the treatment of canine vascular sarcoma, the combined use of IFNs with chemotherapy drugs showed better efficacy, enhancing the immune system's ability to recognize and eliminate tumors<sup>[91]</sup>. This combination therapy strategy not only reduces recurrence rates but also provides new treatment options for clinical practice.

## CONCLUSION

The prospects of BoIFN- $\alpha$  in veterinary science are promising, particularly in antiviral and immune regulation, showing great potential in tackling various viral infections and offering novel ideas for antiviral treatments. However, research on BoIFN- $\alpha$ 's antiviral effects and applications varies, with some studies showing effective virus inhibition while others highlight species and method differences, necessitating consideration of experimental conditions.

Future research should focus on optimizing BoIFN- $\alpha$  production and administration methods for better clinical use and personalized plans to enhance effectiveness and reduce side effects. Systematic clinical trials will evaluate its safety and efficacy for veterinary applications.

At the same time, exploring BoIFN- $\alpha$ 's potential for new treatments is vital, as combining it with vaccines and antiviral drugs may enhance clinical outcomes and improve animal immunity against diseases.

In summary, BoIFN- $\alpha$ , as a biologic with significant therapeutic potential, has promising development prospects in veterinary science. By examining molecular, clinical, and epidemiological research perspectives, we can gain a deeper understanding of BoIFN- $\alpha$ 's role in antiviral activity and immune regulation. Future research should focus on enhancing BoIFN- $\alpha$ 's clinical applications. This will facilitate its broader adoption in the veterinary field, helping to address the continuously evolving challenges posed by infectious and immune-related diseases. We can only unlock BoIFN- $\alpha$ 's true potential through ongoing exploration and validation, which will provide more effective solutions for animal health and welfare.

## HIGHLIGHT KEYPOINTS

1. BoIFN- $\alpha$ , as a key cytokine, can effectively inhibit virus replication, activate host immune responses, and protect the health of bovine population.
2. The gene structure, protein characteristics, signaling pathways, antiviral activities, and immunoregulatory functions of BoIFN- $\alpha$  were analyzed. It shows significant effects in antiviral, immune regulation, antitumor, and assisting in anti-bacterial and anti-parasitic infections.
3. The effects of BoIFN- $\alpha$  in clinical treatment, vaccine adjuvant applications, and production purification technologies were evaluated. The clinical application potential of BoIFN- $\alpha$  is enormous, but it needs to overcome limitations such as stability, production costs, and drug delivery methods.
4. The pharmacokinetic characteristics and safety issues of BoIFN- $\alpha$  were discussed.
5. Future research for BoIFN- $\alpha$  should focus on optimizing production technology, developing new drug delivery methods, and creating personalized therapy plans to promote the widespread application of BoIFN- $\alpha$  in veterinary medicine and the sustainable development of the bovine industry.

## DECLARATIONS

**Availability of Data and Materials:** Data availability is not applicable to this article as no new data were created in this study.

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## RESEARCH ARTICLE

# Enhancing Dairy Safety: A User-Friendly Rapid Kit for Sensitive Detection of Antibiotic Residues in Milk

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## Abstract

Milk is a nutrient-dense food that provides essential nutrients to support growth, development, and overall health. Currently, over 80% of food animals receive antibiotics as therapeutics, feed additives, and growth promoters. Significant amounts of antibiotics and their metabolites in milk pose public health risks. Antibiotic residues can be detected using different techniques. Detection of antibiotic residues is limited by complex pretreatment, high cost, time-consuming procedures, and required expertise. To overcome these limitations, the current research was conducted to establish a sensitive and time-saving detection kit containing *Bacillus subtilis* as an indicator to detect antibiotic residues in milk. The kit was validated using 14 antibiotic-spiked samples by measuring the limit of detection, specificity, and sensitivity. The kit successfully detected the antibiotic residues of ampicillin, oxytetracycline, streptomycin, spectinomycin, sulphadimidine, sulphadiazine, tylosin, lincomycin, enrofloxacin, ciprofloxacin, trimethoprim, spiramycin, chloramphenicol, and florfenicol. The limit of detection of the kit was lower than the maximum residue limit (MRL) for various antibiotics, including oxytetracycline, sulphadiazine, tylosin, enrofloxacin, ciprofloxacin, and trimethoprim. High specificity and sensitivity of 98.2% and 99.2%, respectively, were obtained. The kit showed accurate results for most antibiotics over 4 months, indicating good shelf life and suitability for regular use. The developed kit offers a rapid, cost-effective, and reliable method for detecting antibiotic residues in milk, ensuring routine monitoring and safeguarding public health.

**Keywords:** Antibiotic residues, *Bacillus subtilis*, public health, food safety, milk

## INTRODUCTION

Antimicrobials are widely used in dairy animals as a primary means of controlling infectious diseases. They play a crucial role in treating and preventing mastitis, respiratory tract infections, and reproductive tract infections, which are common health challenges in dairy herds [1-3]. The use of antimicrobials helps in maintaining animal health, improve milk production, and reduce economic losses [4-6]. Improper or prolonged antibiotic use leaves residues in milk and dairy products, posing public health challenges, including allergies, disturbance of gut flora, and promotion of antibiotic resistance [7,8]. Furthermore, antibiotic residues also cause significant financial losses in the dairy industry by disrupting fermentation processes [9]. The presence of bacterial pathogens and antimicrobial residue in milk, poultry or other animal products poses a serious concern for public health, especially with reference to emergence of antimicrobial resistance [10-12]. The regulatory organizations such as Codex Alimentarius

and European Union have set specific MRLs for each antibiotic in milk, to mitigate the public health concerns [13]. Veterinary drug approval requires the establishment of withdrawal periods (WPs) based on residue data. WPs define the minimum time after the last dose during which animals cannot be slaughtered, and milk or eggs cannot be consumed, ensuring that drug residues fall below MRLs and remain safe for human health [14].

Advanced physicochemical methods like mass spectrometry (MS) and high-performance liquid chromatography (HPLC) can detect antibiotic residues, but these methods are expensive, require expertise, and involve complex preparations [15]. Immunological methods such as ELISA allow rapid and specific detection but are limited to certain antibiotic classes [16]. Microbiological inhibitory approaches, conversely, provide a low-cost, broad-spectrum, and user-friendly solutions for identifying antibiotic residues in milk, but these methods face challenges such as milk matrix effects causing false positive results and labor-intensive procedures [17].



To address these limitations, a cost-effective and stable kit was developed using a 96-well microtiter plate containing minimal agar medium and a highly sensitive *Bacillus subtilis* spore suspension. This approach reduces the need for fresh bacterial cultures, as spores remain dormant under harsh conditions. It also permits processing of multiple samples, with results obtained within 6-7 h, and the kit remains stable for several months.

## MATERIAL AND METHODS

### Bioethical Statement

This research was conducted following approval from the Institutional Biosafety/Bioethics Committee (IBC) of the University of Agriculture, Faisalabad, Pakistan (D. No. 3275/ORIC, Dated: 24/05/2024).

### Isolation and Identification of *B. subtilis*

A total of five soil samples were collected from agricultural fields of Faisalabad to isolate *B. subtilis*. Twenty-gram soil samples were collected 5 cm from the surface and placed in a sterile plastic container and sealed. The soil samples were cultured on LB agar using ten-fold serial dilution. Based on colony morphology, distinct bacterial colonies were isolated and sub-cultured repeatedly on nutrient agar to obtain pure cultures<sup>[18]</sup>. The purified colonies were identified using Gram staining, spore staining<sup>[19]</sup>, and several biochemical tests according to Bergey's Manual and confirmed with molecular characterization by PCR and sequencing<sup>[20]</sup>.

### Molecular Characterization of *B. subtilis*

**DNA Extraction and Polymerase Chain Reaction:** The GeneJET PCR Purification Kit® (Thermo Scientific) was used for DNA extraction of pure culture. To differentiate *B. subtilis* from other *Bacillus* species, species-specific oligonucleotide forward and reverse primers for the pyruvate carboxylase (*pyrA*) and shikimate dehydrogenase (*aroE*) genes were used for PCR DNA amplification<sup>[21]</sup>. After PCR amplification, PCR products were subjected to gel electrophoresis on a 1.5% agarose gel<sup>[21]</sup>. Detection of the *aroE* (278bp) gene and *pycA* (233bp) confirmed the isolation of *B. subtilis*.

**Sequence Analysis and Phylogenetic Relationship:** The Gene JET Gel Extraction Kit® (Thermo Scientific) was used for the excision of DNA from agarose gels. The retrieved DNA was sequenced by the ABI PRISM® 3100 Genetic Analyzer at Macrogen Sequencing Facilities (Macrogen, Korea). The nucleotide sequences of isolates were submitted to GenBank NCBI for accession numbers. Phylogenetic tree analysis was conducted for sequenced *aroE* and *pycA* genes using MEGA 11<sup>[22]</sup>.

**Antibiotic Susceptibility Testing:** The disc diffusion

method was employed using Mueller-Hinton agar (MHA) plates and bacterial suspensions adjusted to 0.5 McFarland turbidity. Bacterial culture was swabbed onto MHA plates, followed by placement of antimicrobial discs. The plates were incubated at 37°C for 24 h<sup>[23]</sup>. The results of AST were based on literature confirmation and microbiology manuals. The bacterial isolates were classified as sensitive, intermediate, or resistant based on zone of inhibition (*Table 1*), following CLSI guidelines<sup>[24]</sup>.

**Spore Suspension Preparation:** Of the two isolated strains, the more sensitive *B. subtilis* strain was swabbed onto nutrient agar and incubated at 30°C for 10 days to induce spore formation. Spore production was confirmed by spore staining<sup>[19,25]</sup>. The spore suspension was subjected to viable count using serial dilution, and spore concentration was adjusted to  $4.096 \times 10^9$  CFU/mL<sup>[26]</sup>.

**Preparation of the Kit Medium:** The kit medium was prepared by dissolving 28 g of nutrient agar, 0.25 g K<sub>2</sub>HPO<sub>4</sub>, 5.25 g glucose, 0.006 g MgSO<sub>4</sub>, 7 g carboxymethylcellulose sodium, 0.003 g MnSO<sub>4</sub>·H<sub>2</sub>O, 0.5 g NaCl, and 0.004 g CaCl<sub>2</sub> in 1 L dH<sub>2</sub>O<sup>[27,28]</sup>. After autoclaving, the pH was adjusted to 6.6-7.0, and bromocresol purple (0.1 mg/L), along with *B. subtilis* spore suspension, was added. The mixture was vortexed, and 150 µL was dispensed into each well of microtiter plate, which was then sealed with a foil-wrapped lid and stored at 4°C until use<sup>[27]</sup>.

**Preparation of the Stock Solution and the Working Solution of Antibiotics:** Fourteen pure anti-biotics were used in powdered form: ampicillin, oxytetracycline, streptomycin, spectinomycin, sulphadimidine, sulphadiazine, tylosin, lincomycin, enrofloxacin, ciprofloxacin, trimethoprim, spiramycin, chloramphenicol, and florfenicol. Stock solutions (1 mg/mL) and working solutions (1 µg/mL) were prepared and stored at -20°C until use.

**Blank and Spiked Milk Samples:** Cow milk samples were collected from animals that had not received antibiotics during the previous 30 days. Working solutions of individual antibiotics were added to antibiotic-free milk to prepare spiked milk samples<sup>[29]</sup>.

**Microbiological Inhibition Test:** Milk samples (blank and spiked) were first incubated at 80°C for 10 min in water bath. The treated milk samples were then added to the microtitration plate containing prepared culture media. Results were interpreted based on color change (purple or yellow). The experiment was repeated four times with each concentration tested in triplicate to ensure accuracy and consistency<sup>[27]</sup>.

### Validation Protocols

**Limit of Detection:** The ISO13969: 2003 criteria were followed in the establishment of the dose-response curves for these drugs (ISO, 2003). Eight distinct concentrations

of each drug were tested over different days, with 20 replicates of each concentration being examined. The lowest concentration that yielded 95% positive results was determined as the limit of detection (LOD) [30].

**Specificity and Sensitivity:** A total of 168 blank milk samples were tested with the kit to determine the false-positive rate. To assess the false-negative rate, 100 blank samples spiked at the LOD and MRL for each antibiotic were analyzed, as a method is considered reliable when false negatives remain  $\leq 5\%$  at both levels [31]. The specificity, sensitivity, positive predictive value and negative predictive value were calculated using standard formulae [32,33].

**Stability:** The stability of the kit was evaluated over four months (0, 30, 60, 90, and 120 days) at 4°C by monitoring appearance, odor, performance, incubation time, and detection capacity. Each month, the kit was tested with antibiotics at, above, and below their detection concentrations [29].

### Statistical Analysis

Logistic regression was applied to assess the impact of antibiotic concentration on detection likelihood, with model fit evaluated by Hosmer-Lemeshow, Pearson, and Deviance tests. ANOVA was conducted using SPSS version 26 (Armonk, USA), and graphs were generated with SigmaPlot version 14.0 [34].

## RESULTS

### Identification, Molecular Characterization and Phylogenetic Analysis

Initial soil samples yielded seven distinct bacterial colonies: five were Gram-positive rods and two were cocci. Spore staining revealed that all bacilli were spore-formers. Colony characteristics included circular, rough, opaque, fuzzy, white, or slightly yellow colored with jagged edges. After biochemical confirmation, PCR was performed on five bacterial cultures, of which four showed positive amplification with distinct bands at 233 bp (*pycA*) and 278 bp (*aroE*) to a 100 bp DNA ladder (Fig. 1). Two strains (NF-S1, NF-S2) were further tested by AST, and

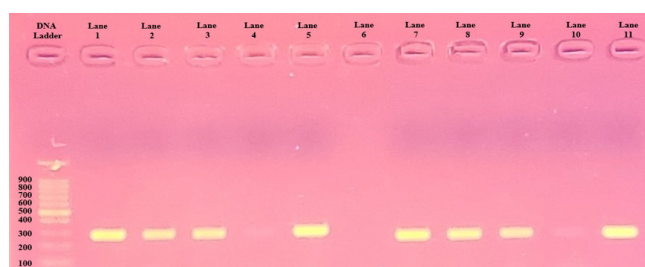


**Fig 1.** Species-specific PCR genes *aroE* and *pycA* of *B. subtilis*. Lane 0 size marker (100 bp DNA ladder); lanes 1-5 *aroE* (278bp) gene fragments for *B. subtilis*, Lane 6 negative control, lanes 7-11 *pycA* (233bp) gene fragments

the more sensitive strain (NF-S2) was sequenced. The verified sequences were submitted to GenBank (accession Nos. PP820929 and PP898198), and phylogenetic analysis confirmed the isolates as *B. subtilis* (Fig. 2).

### Antibiotic Susceptibility Testing

The susceptibility of two bacterial strains to 48 antibiotics revealed that all antibiotics posed a significant response against both strains ( $P < 0.05$ ). NF-S1 strain showed resistance to 24, intermediate resistance to 6, and sensitivity to 18 antibiotics. In contrast, the NF-S2 strain was resistant to only 14 antibiotics, with intermediate resistance to 3 and sensitivity to 31 (Table 1; Fig. 3). This indicated that the NF-S2 strain was more sensitive than the NF-S1 strain. Therefore, NF-S2 strain was selected for sequencing and kit preparation. The number of sensitive antibiotics against the two strains was compared using Chi-square, suggesting that there was a non-significant relationship between the two strains ( $P > 0.05$ ). Therefore, there was a significant difference between the strains, the NF-S2 strain presented more sensitivity than the NF-S1 strain (Table 1).

### Microbiological Inhibition Test

Color changes in the kit were observed till 7 h: yellow indicated negative results (no antibiotic residues), while the purple indicated positive results (antibiotic residues present) (Fig. 4). Table 2 shows the mean limit of detection for the kit at different concentrations of each antibiotic using microbiological inhibition tests conducted four times. Fig. 5 represents the mean LOD of all batches compared the MRL for each antibiotic. The concentrations determined in batches 3 and 4 were considered the true limit of detection of the kit, at which the results were stable and consistent (Fig. 6). The results revealed that kit was highly sensitive for oxytetracycline (25 µg/L), sulphadiazine (50 µg/L), tylosin (30 µg/L), enrofloxacin

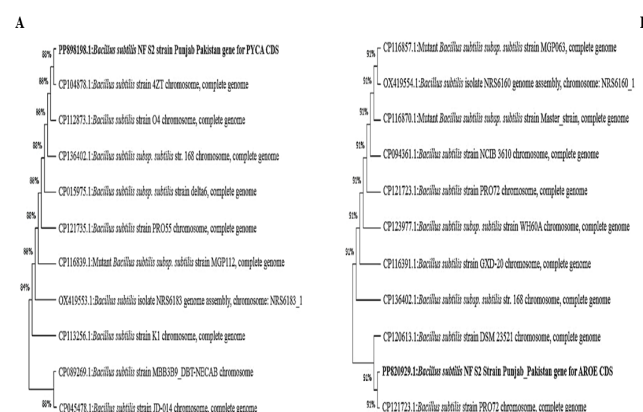


**Fig 2.** Phylogenetic tree of *Bacillus subtilis*. A- Phylogenetic tree of the *Bacillus subtilis* targeting species-specific gene *pycA*, pyruvate carboxylase, with 1000 bootstraps. B- Phylogenetic tree of the *Bacillus subtilis* targeting species-specific gene *aroE* shikimate dehydrogenase, with 1000 bootstraps

**Table 1.** Mean zones of inhibition of antibiotics against NF-S1 and NF-S2 *B. subtilis* isolates

| Sr. No. | Antibiotic Discs        | Zone of Inhibition (mm) |         |       |          |
|---------|-------------------------|-------------------------|---------|-------|----------|
|         |                         | NF-S1                   | S, I, R | NF-S2 | S, I, R, |
| 1       | Penicillin G            | 0                       | R       | 0     | R        |
| 2       | Ampicillin              | 8                       | R       | 29    | S        |
| 3       | Amoxicillin             | 12                      | R       | 25    | S        |
| 4       | Carbenicillin           | 0                       | R       | 13    | R        |
| 5       | Piperacillin            | 15                      | I       | 28    | S        |
| 6       | Piperacillin Tazobactam | 14                      | R       | 14    | R        |
| 7       | Methicillin             | 0                       | R       | 0     | R        |
| 8       | Oxacillin               | 0                       | R       | 11    | I        |
| 9       | Tetracycline            | 15                      | I       | 22    | S        |
| 10      | Oxytetracycline         | 13                      | R       | 27    | S        |
| 11      | Tigecycline             | 9                       | R       | 19    | S        |
| 12      | Doxycycline             | 12                      | R       | 19    | S        |
| 13      | Gentamycin              | 18                      | S       | 19    | S        |
| 14      | Streptomycin            | 16                      | S       | 25    | S        |
| 15      | Lincomycin              | 25                      | S       | 18    | S        |
| 16      | Enrofloxacin            | 26                      | S       | 46    | S        |
| 17      | Amikacin                | 16                      | I       | 20    | S        |
| 18      | Norfloxacin             | 18                      | S       | 28    | S        |
| 19      | Bacitracin              | 9                       | I       | 0     | R        |
| 20      | Ciprofloxacin           | 24                      | S       | 40    | S        |
| 21      | Erythromycin            | 22                      | S       | 40    | S        |
| 22      | Vancomycin              | 10                      | I       | 15    | S        |
| 23      | Tobramycin              | 8                       | R       | 11    | R        |
| 24      | Azithromycin            | 30                      | S       | 34    | S        |
| 25      | Rifampicin              | 20                      | R       | 23    | I        |
| 26      | Clindamycin             | 28                      | S       | 22    | S        |
| 27      | Tylosin                 | 32                      | S       | 38    | S        |
| 28      | Flumequin               | 20                      | I       | 38    | S        |
| 29      | Ceftriaxone             | 0                       | R       | 0     | R        |
| 30      | Meropenem               | 0                       | R       | 0     | R        |
| 31      | Imipenem                | 33                      | S       | 42    | S        |
| 32      | Entrapenem              | 8                       | R       | 0     | R        |
| 33      | Ceftazidime             | 0                       | R       | 0     | R        |
| 34      | Cephalexin              | 9                       | R       | 21    | S        |
| 35      | Trimethoprim            | 0                       | R       | 40    | S        |
| 36      | Sulphamethoxazole       | 30                      | S       | 32    | S        |
| 37      | Polymyxin B             | 8                       | R       | 8     | R        |
| 38      | Cefoxitin               | 0                       | R       | 0     | R        |
| 39      | Nitrofuratoin           | 20                      | S       | 30    | S        |
| 40      | Linezolid               | 26                      | S       | 40    | S        |
| 41      | Cefepime                | 0                       | R       | 0     | R        |

**Table 1.** Continue

| Sr. No. | Antibiotic Discs | Zone of Inhibition (mm) |         |       |          |
|---------|------------------|-------------------------|---------|-------|----------|
|         |                  | NF-S1                   | S, I, R | NF-S2 | S, I, R, |
| 42      | Ceftiofur        | 0                       | R       | 0     | R        |
| 43      | Chloramphenicol  | 22                      | S       | 25    | S        |
| 44      | Florfenicol      | 34                      | S       | 38    | S        |
| 45      | Spectinomycin    | 18                      | S       | 40    | S        |
| 46      | Spiramycin       | 33                      | S       | 44    | S        |
| 47      | Sulphadiazine    | 12                      | R       | 22    | S        |
| 48      | Sulphadimidine   | 12                      | R       | 13    | I        |

I = Intermediate, R = Resistant, S = Sensitive, 0 = No Zone of Inhibition

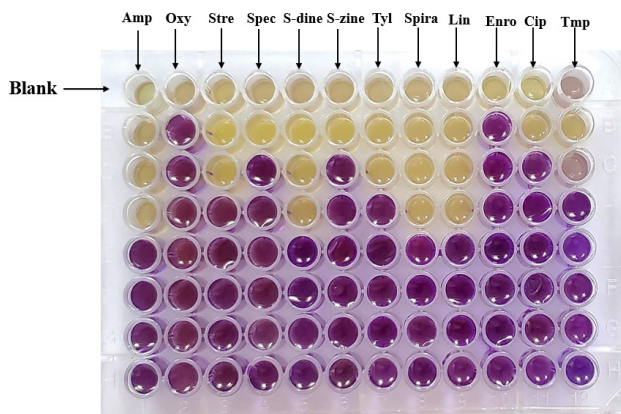
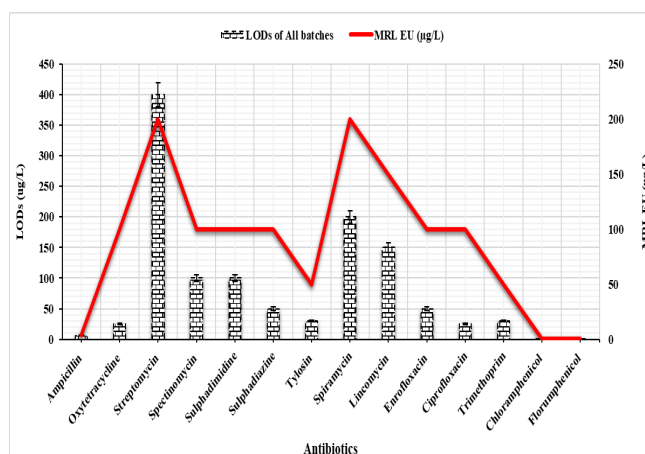
**Fig 3.** Antibiotic sensitivity of NF-S2 strains against various antibiotics. 1. enrofloxacin, 2. erythromycin, 3. linezolid, 4. azithromycin, 5. flumequine, 6. chloramphenicol, and 7. Imipenem**Fig 4.** Results of batch 4 LOD concentrations to detect antibiotic residues. The first row A shows the blank samples, all the column shows the concentrations at which antibiotic residues were detected ampicillin (4 µg/L), oxytetracycline (25 µg/L), streptomycin (300 µg/L), spectinomycin (100 µg/L), sulphadimidine (100 µg/L), sulphadiazine (50 µg/L), tylosin (30 µg/L), spiramycin (250 µg/L), lincomycin (150 µg/L) enrofloxacin (50 µg/L), ciprofloxacin (25 µg/L), trimethoprim (30 µg/L)

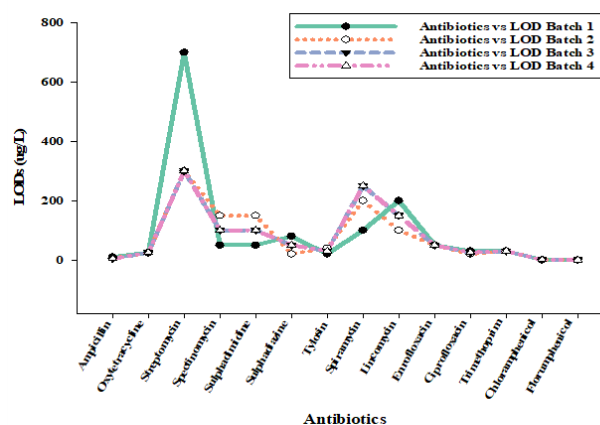
**Table 2.** Estimation of the limit of Detection of the kit

| Antibiotics     | MRL EU (µg/L) | Mean of LOD of Batch 1 (µg/L) | Mean of LOD of Batch 2 (µg/L) | Mean of LOD of Batch 3 (µg/L) | Mean of LOD of Batch 4 (µg/L) |
|-----------------|---------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| Ampicillin      | 4             | 10                            | 6                             | 4                             | 4                             |
| Oxytetracycline | 100           | 25                            | 25                            | 25                            | 25                            |
| Streptomycin    | 200           | 700                           | 300                           | 300                           | 300                           |
| Spectinomycin   | 100           | 50                            | 150                           | 100                           | 100                           |
| Sulphadimidine  | 100           | 100                           | 200                           | 100                           | 100                           |
| Sulphadiazine   | 100           | 80                            | 20                            | 50                            | 50                            |
| Tylosin         | 50            | 20                            | 40                            | 30                            | 30                            |
| Spiramycin      | 200           | 100                           | 200                           | 250                           | 250                           |
| Lincomycin      | 150           | 200                           | 100                           | 150                           | 150                           |
| Enrofloxacin    | 100           | 50                            | 50                            | 50                            | 50                            |
| Ciprofloxacin   | 100           | 30                            | 20                            | 25                            | 25                            |
| Trimethoprim    | 50            | 30                            | 30                            | 30                            | 30                            |
| Chloramphenicol | N.A.          | 0.6                           | 0.6                           | 0.6                           | 0.6                           |
| Florfenicol     | N.A.          | 0.5                           | 0.5                           | 0.5                           | 0.5                           |

N.A. = Not Available

**Fig 5.** The overall mean of the limit of detection of 4 batches for antibiotic residues. The red line indicates the maximum residue limit of the European Union (EU) for the respective antibiotic residue's mean concentrations

(50 µg/L), ciprofloxacin (25 µg/L), and trimethoprim (30 µg/L). Except for streptomycin (300 µg/L), spiramycin (250 µg/L), chloramphenicol (0.6 µg/L) and florfenicol (0.5 µg/L), which showed that concentrations must be present at least 1-3 times higher than MRLs to detect the positive results through this bioassay. Furthermore, ampicillin (4 µg/L), spectinomycin (100 µg/L), sulphadimidine (100 µg/L) and lincomycin (150 µg/L) were detected at a level equivalent to MRL. Logistic regression revealed significant positive associations between antibiotic concentration and probability of detection, having odds ratios that range

**Fig 6.** Four experiments were conducted to detect the LOD of the respective antibiotics mentioned. The trend showed that batches 3 and 4 reveal the final LOD of antibiotics

from 1.0098 to 1.35E+06. Detection of quantities below allowable limits and goodness-of-fit tests demonstrated a good-to-acceptable model fit.

### Validation Protocols

**Limit of Detection:** The final LOD values for the kit are summarized in [Table 3](#), and the dose-response curve range for 14 antibiotics was generated. The limit of detection of the kit for oxytetracycline, sulphadiazine, tylosin, enrofloxacin, ciprofloxacin, and trimethoprim was lower than the MRL. On the other hand, streptomycin, spiramycin, chloramphenicol and florfenicol showed higher LOD than the MRL of the EU. Furthermore, ampicillin, spectinomycin, sulphadimidine and lincomycin had LOD equivalent to MRL ([Fig. 7](#)).

**Table 3.** Dose-response range and the LOD of the antibiotics at different concentrations according to the EU

| Sr. No. | Antibiotics     | LOD | MRL EU (µg/L) | Range of Dose-response Curve |
|---------|-----------------|-----|---------------|------------------------------|
| 1       | Ampicillin      | 4   | 4             | 3-5                          |
| 2       | Oxytetracycline | 25  | 100           | 25-50                        |
| 3       | Streptomycin    | 300 | 200           | 200-300                      |
| 4       | Spectinomycin   | 100 | 100           | 100-150                      |
| 5       | Sulphadimidine  | 100 | 100           | 80-100                       |
| 6       | Sulphadiazine   | 50  | 100           | 50-80                        |
| 7       | Tylosin         | 30  | 50            | 20-30                        |
| 8       | Spiramycin      | 250 | 200           | 250-300                      |
| 9       | Lincomycin      | 150 | 150           | 100-200                      |
| 10      | Enrofloxacin    | 50  | 100           | 50-100                       |
| 11      | Ciprofloxacin   | 25  | 100           | 25-50                        |
| 12      | Trimethoprim    | 30  | 50            | 20-40                        |
| 13      | Chloramphenicol | 0.6 | N.A.          | 0.5-0.7                      |
| 14      | Florfenicol     | 0.5 | N.A.          | 0.4-0.6                      |

N.A. = Not Available

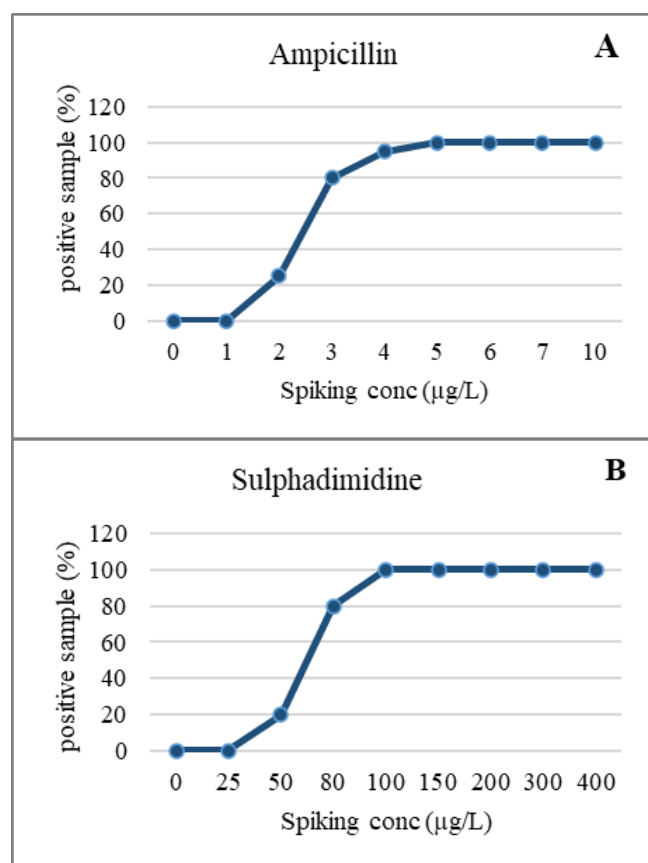


Fig 7. Dose response curve of ampicillin and sulphadimidine. A- Dose response curve of ampicillin, B- Dose response curve of sulphadimidine. The curves are generated by percentage of positive samples (y-axis) against spiking concentration (µg/L) (x-axis)

Table 4. Results of the False-Negative Rate of the kit against 100 samples tested for each drug

| Sr. No. | Antibiotics     | Total Samples | Positive (%) | Negative (%) | LOD | MRL  | False Negative Rate |
|---------|-----------------|---------------|--------------|--------------|-----|------|---------------------|
| 1       | Ampicillin      | 100           | 98           | 2            | 4   | 4    | 2                   |
| 2       | Oxytetracycline | 100           | 100          | 0            | 25  | 100  | 0                   |
| 3       | Streptomycin    | 100           | 97           | 3            | 300 | 200  | 3                   |
| 4       | Spectinomycin   | 100           | 100          | 0            | 100 | 100  | 0                   |
| 5       | Sulphadimidine  | 100           | 99           | 1            | 100 | 100  | 1                   |
| 6       | Sulphadiazine   | 100           | 100          | 0            | 50  | 100  | 0                   |
| 7       | Tylosin         | 100           | 100          | 0            | 30  | 50   | 0                   |
| 8       | Spiramycin      | 100           | 98           | 2            | 250 | 200  | 2                   |
| 9       | Lincomycin      | 100           | 100          | 0            | 150 | 150  | 0                   |
| 10      | Enrofloxacin    | 100           | 100          | 0            | 50  | 100  | 0                   |
| 11      | Ciprofloxacin   | 100           | 100          | 0            | 25  | 100  | 0                   |
| 12      | Trimethoprim    | 100           | 100          | 0            | 30  | 50   | 0                   |
| 13      | Chloramphenicol | 100           | 99           | 1            | 0.6 | N.A. | 1                   |
| 14      | Florfenicol     | 100           | 98           | 2            | 0.5 | N.A. | 2                   |

**Specificity and Sensitivity:** A total of 168 blank samples were analyzed using the kit: 165 were negative and 3 were positive. Therefore, the false positive rate of the kit was 1.78%. A total of 1400 samples were analyzed through the kit; 11 samples were negative and 1389 were positive. The results of the false negative rates of each of the 14 antibiotics were less than 5% at the MRL and limit of detection. Consequently, the kit's false-negative rates met ISO13969: 2003 requirements [30]. False-negative results are mentioned in Table 4. High specificity (98.2%) and sensitivity (99.2%) were obtained during kit evaluation.

**Predictive Values:** The positive and negative predictive values were evaluated using the specificity and sensitivity values of the experiment and concluded that the actual proportion of the positive rate was 99.8% while the negative rate was 93.9%.

**Stability:** The parameters (odor, performance, appearance, incubation time and detection capacity) of the kit did not change over 2 months (Fig. 8). Odor and incubation time remained stable over 4 months, whereas the detection capacity for ampicillin, streptomycin, spectinomycin, sulphadimidine and lincomycin changed during the 3<sup>rd</sup> and 4<sup>th</sup> months, as shown in Table 5. Different correlations between concentration and detection were seen in the logistic

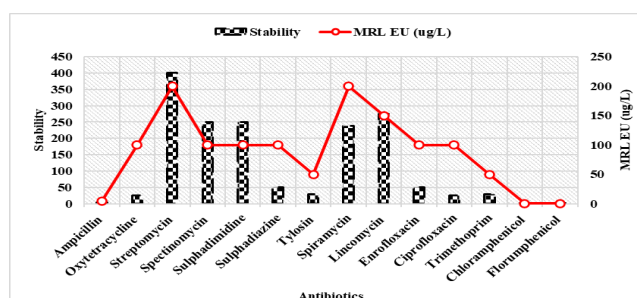


Fig 8. Four-month trend of stability study. The maximum residue limit and the mean results of the stability of the kit for four months of each antibiotic are shown. The red line indicates the maximum residue limit

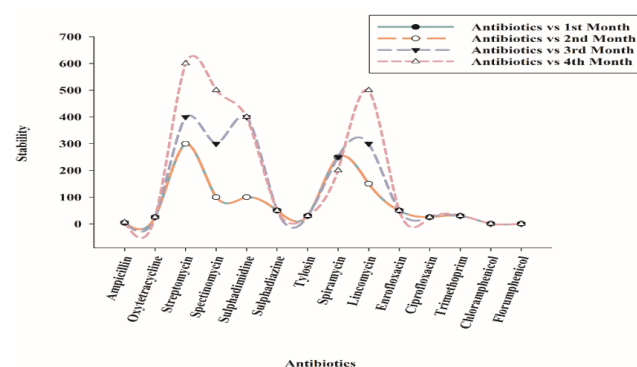


Fig 9. Month-wise antibiotic residues detected on the kit and their limit of detection. The trend showed that in the 1<sup>st</sup> and 2<sup>nd</sup> months, the antibiotic residue detection was highly stable, while in the 3<sup>rd</sup> and 4<sup>th</sup> months, changes in detection were observed

**Table 5.** Limit of detection of the four-month stability study

| Antibiotics     | MRL EU (µg/L) | Results (µg/L)             |                            |                            |                            |
|-----------------|---------------|----------------------------|----------------------------|----------------------------|----------------------------|
|                 |               | 1 <sup>st</sup> -month LOD | 2 <sup>nd</sup> -month LOD | 3 <sup>rd</sup> -month LOD | 4 <sup>th</sup> -month LOD |
| Ampicillin      | 4             | 4                          | 4                          | 5                          | 6                          |
| Oxytetracycline | 100           | 25                         | 25                         | 25                         | 25                         |
| Streptomycin    | 200           | 300                        | 300                        | 400                        | 600                        |
| Spectinomycin   | 100           | 100                        | 100                        | 300                        | 500                        |
| Sulphadimidine  | 100           | 100                        | 100                        | 400                        | 400                        |
| Sulphadiazine   | 100           | 50                         | 50                         | 50                         | 50                         |
| Tylosin         | 50            | 30                         | 30                         | 30                         | 30                         |
| Spiramycin      | 200           | 250                        | 250                        | 250                        | 200                        |
| Lincomycin      | 150           | 150                        | 150                        | 300                        | 500                        |
| Enrofloxacin    | 100           | 50                         | 50                         | 50                         | 50                         |
| Ciprofloxacin   | 100           | 25                         | 25                         | 25                         | 25                         |
| Trimethoprim    | 50            | 30                         | 30                         | 30                         | 30                         |
| Chloramphenicol | N.A.          | 0.6                        | 0.6                        | 0.6                        | 0.6                        |
| Florfenicol     | N.A.          | 0.5                        | 0.5                        | 0.5                        | 0.5                        |

regression analysis for 14 antibiotics. These odds range from modest (1.0089) to substantially high (1.24865E+06), accounting for 17.73% to 57.62% of detection variation. Tests for goodness-of-fit tend to validate practical applicability, even in the event of test inconsistencies. Effective detection at low concentrations (e.g. 25 µg/L) retains model utility, for antibiotics with non-significant associations ( $P=0.002$  to 0.609), showing reliability in field applications for determining whether milk samples contain antibiotic residues. Fig. 9 represents the mean LOD of four months of each antibiotic with their respective MRL.

## DISCUSSION

Various studies have highlighted the alarming extent of antibiotic resistance, often caused by antibiotic residues present in animals, food, and the environment [35-40]. Antibiotic residues in milk pose a significant public health risk because they contribute to antibiotic resistance and cause severe health issues. These residues occur when antibiotics administered to cattle are excreted in milk without proper metabolism or clearance [41]. These residues can cause allergic reactions in sensitive people and disturb gut microflora, leading to serious health issues. The detection of antibiotic residues is essential for ensuring food safety and safeguarding public health. It is crucial to monitor as well as control antibiotic residues in milk to preserve its quality and safety [42].

A kit was developed using an indigenously isolated *B. subtilis* strain. The isolated strain was confirmed using PCR amplification of two species-specific genes

and sequenced for phylogenetic analysis. To ensure validity and consistency of results, strains used in assay development must be well-characterized, traceable, and maintained under controlled conditions [43,44]. Two isolated strains depicted sensitivity to several antibiotics. The NF-S2 strain was suitable for detecting various antibiotic residues. Its high sensitivity to various antibiotics and extensive use in microbiological assays demonstrates its efficacy and reliability. *B. subtilis* is the most suitable species for the development of this detection assay due to its robust genetic profile and established precedent. There are many studies conducted to detect antibiotic residues using *B. subtilis* [25,45-47] their presence in foodstuff derived from animals is a potential public health hazard. They pose a serious threat as they are implicated in direct toxicity; allergic reactions; disturbance of the normal gut microbiota, carcinogenesis, and emergence of antibiotic-resistant bacteria.

The bacterium in this study demonstrates distinct inhibitory zones, confirming its strong susceptibility to various antibiotics. Ji et al. [48] study from 2022 employed antibiotics that closely matched the antibiotics used in this research and produced similar findings, with the exception that ampicillin (20 mm) and piperacillin (20 mm) exhibited moderate sensitivity, but high sensitivity was observed in the current study. The sensitivity of the bioassay must also be validated by AST to identify low antibiotic residue concentrations and confirm the *B. subtilis* as a bioindicator for monitoring milk safety.

After confirming the sensitivity of the bacteria, the antibiotic residue detection kit was developed using various stabilizers, buffering agents, nutrients and color indicators. Every component has a vital function in maintaining pH stability, promoting bacterial growth, preserving spore integrity, and facilitating enzymatic reactions [46]. The remarkable sensitivity of *B. subtilis* spores to antibiotics enables accurate residue detection. The kit changed color from purple to yellow (Fig. 4) in 6-7 h, indicating negative results and vice versa. This rapid, easy-to-use test will enable dairy producers and regulators to monitor antibiotic residues in milk efficiently [49]. Comparison of the results from the present detection kit with those from studies by Nagel et al. [26] and Wu et al. [50] revealed that this kit demonstrated greater sensitivity in detecting antibiotic residues in milk. The assay for antibiotic residue detection concentrations in the Wu et al. [50] study kit showed oxytetracycline (200 µg/L), sulphadiazine (75 µg/L), sulphadimidine (150 µg/L), lincomycin (300 µg/L), streptomycin (500 µg/L) and tylosin (50 µg/L). Similarly, when comparing the results of Nagel et al. [26] for ciprofloxacin (150 µg/L), enrofloxacin (160 µg/L), and spiramycin (340 µg/L), the present kit performed better. Due to the absence of several stabilizers, buffering agents,

and specific additives in the Nagel et al.<sup>[26]</sup> Kit, there was a notable disparity in detection concentrations between present kit and their study.

The media composition for the kit was developed from Wu et al.<sup>[27]</sup> and Posada Uribe et al.<sup>[28]</sup> studies, with minor modifications to support the growth and sporulation of *B. subtilis* as well as the functionality and stability of the kit. A combination of additives, such as carboxymethylcellulose sodium, prevented milk from interfering with the nutritional aspects of the medium and ensured even distribution of spores, thereby improving detection assay clarity<sup>[51]</sup>. To enhance detection capabilities, components including  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ ,  $\text{MgSO}_4$ , and glucose were added to stimulate bacterial growth, propagation, and acid production. As enzyme cofactors, manganese and magnesium ions enhanced enzyme activity, improving the kit's acid-producing capacity and sensitivity; however, lower bacterial concentrations increased detection time<sup>[52]</sup>. Nevertheless, the presence of nutrients such as glucose improved the detection process and time by enhancing bacterial proliferation and functionality. Comparing this detection kit to previous research shows its greater sensitivity in detecting lower antibiotic residual amounts in milk.

The validation of the detection kit was an essential aspect of the study, guaranteeing its dependability and precision for practical use. To determine the limit of detection (LOD), milk samples spiked with varying concentrations of antibiotics were analyzed, as performed by Yazdanpanah et al.<sup>[53]</sup>. The present kit identified traces of antibiotics at concentrations as low as below MRL (Table 3), thereby confirming its high sensitivity. The sensitivity testing assessed the kit's capacity to accurately detect positive samples, resulting in a high sensitivity rate of 99.2%. This high sensitivity ensures that present kit can effectively detect antibiotic residues, thus reducing false-negative results. To ensure that the kit could correctly identify samples without antibiotics, specificity testing was conducted. The kit's specificity rate was 98.25%, indicating that it consistently discriminates negative samples, thus lowering the probability of false-positive results. These findings align with the study by Wu et al.<sup>[54]</sup>.

There was no sample preprocessing except for heating the milk in a water bath for 10 minutes at 80°C before adding it to the detection system. A study by Andrew<sup>[52]</sup> used a similar approach for sample pretreatment. During the incubation process, antibiotics found in milk might contaminate the detection medium. Additionally, the detection medium can be contaminated by natural bacteriostatic compounds found in milk, including, fat, protein, somatic cells, bacteria, lysozyme, lactoferrin, and the lactoperoxidase system<sup>[52,55]</sup>. Inhibition of *B. subtilis* growth in the detection system by these natural

bacteriostatic compounds might lead to false-positive results because they impede acid production and the color change of the pH indicator. Moreover, antibiotics may lose part of their efficacy if they encounter certain milk components. When testing for tetracyclines, false-negative results are common because calcium ions in milk may chelate with them<sup>[56]</sup>. Furthermore, during incubation, a significant amount of milk protein diffuses throughout the media, and white lactoprotein may mask the pH indicator's color change, complicating visual detection. In this study, milk samples were incubated in a water bath at 80°C for 10 min before being added to the kit's medium to inactivate natural bacteriostatic compounds and eliminate false positive results, as also demonstrated by Houali et al.<sup>[57]</sup>. A low false-positive results (1.78%) indicated the importance of this kit. The kit's carboxymethylcellulose sodium can also bind with milk's calcium ions, blocking their interference and ensuring that the test will not provide a false-negative result for tetracyclines. Furthermore, the complex network formed by carboxymethylcellulose sodium prevents macromolecules such as fat and protein from entering detection system and prevents white lactoprotein from obstructing the pH indicator's color change<sup>[58]</sup>. In conclusion, the sample pretreatment technique used in this study was simple to use, cost-effective, time-saving, and applicable in any setting.

Kit stability was assessed for four months, and consistent results were obtained for most antibiotics (Fig. 9). However, for some antibiotics (streptomycin, ampicillin, spectinomycin, lincomycin, and sulphadimidine), LOD mildly increased after two months, which was possibly due to the lack of sensitizers in the kit. Therefore, to improve stability for more than six months, antibiotic sensitizers must be included for these antibiotics to increase bacterial susceptibility. Additionally, for the remaining antibiotics tested, the kit continued to function without changes the parameters such as color, odor, or detection time, indicating that the core medium, spores, and stabilizing additives/buffering remained functional, demonstrating its dependability for routine checking of antibiotic residues in milk. The study by Wu et al.<sup>[54]</sup> demonstrated that their kit remained stable for up to six months; however, their kit contained an antibiotic sensitizer. It is suggested that adding chloramphenicol and trimethoprim increases the sensitivity of the microbiological inhibition assay toward sulfonamide, tetracycline, and other antibiotics, including cephalosporin and penicillin. Another study by Nagel et al.<sup>[26]</sup> used trimethoprim as a sensitizer to increase the sensitivity and stability of the bioassay to detect antibiotic residues. The detection medium of this kit, along with the supplementary chemicals, microplates, aluminized film cover, and other materials was aseptically

treated. Moreover, sodium carboxymethylcellulose forms hydrogen bonds with water, further inhibiting water evaporation<sup>[54]</sup>. The kit's *B. subtilis* spores maintained their acid-producing abilities and antibacterial sensitivity for four months. In conclusion, the kit developed in this study demonstrated stability, with a shelf life of four months.

Growing concerns about antibiotic residues in milk and their connection to resistance and hazards to public health emphasizes the urgency of effective detection techniques. The detection kit utilized *B. subtilis* spore suspension to determine presence of antibiotic residues in milk. Altogether, this research established a practical, rapid microbiological screening method using *Bacillus subtilis* spores that enables dairy producers and regulators to detect multiple antibiotic residues in milk within 6-7 h with high accuracy, addressing critical food safety concerns while remaining accessible for routine field use.

## DECLARATIONS

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**Declaration of Generative Artificial Intelligence (AI):** The article and tables and figures were not written/created by AI and AI assisted technologies.

**Authors Contributions:** NF, Concept Design, Experimental work, Data Collection and Analysis, Writing-original draft, MSM, Supervision, Concept Design, Review of the manuscript, MKK, Supervision, Critical review of the manuscript, SA, Supervision, Analysis and Interpretations, Writing, review and Editing, Correspondence. All authors approved the final version of the manuscript and accept responsibility for the scientific integrity of the work in accordance with the journal's Ethical Principles and Authorship Policy.

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RESEARCH ARTICLE

# First Record of *Musca crassirostris* Stein, 1903 (Diptera: Muscidae) in Algeria, Confirmed from the Sétif Region, with Notes on Hematophagous Brachycera Diversity

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## Abstract

From December 2022 to November 2023, blood-feeding Diptera were studied in the Sétif region (Algeria) using Nzi and Vavoua traps installed at four sites: Beni Fouda, Ain Roua, Ain Azel and Ain Arnat. A total of 4.917 specimens were collected, including *Stomoxys calcitrans* (Linnaeus, 1758), *Haematobia irritans* (Linnaeus, 1758), *Tabanidae* spp. (Latreille, 1802) and *Musca crassirostris* (Stein, 1903). The latter species was identified and confirmed for the first time in Algeria using a dual morphological and molecular approach based on the characterisation of the mitochondrial COI gene, whose sequences have been deposited in GenBank (PV012500-PV012513). Abundance varied significantly according to season and altitude, with higher densities in summer and autumn, particularly in mountainous. The effectiveness of the traps was found to depend on the species: Nzi traps were more effective for *M. crassirostris*, while Vavoua traps captured more *S. calcitrans*. The detection of *M. crassirostris* extends its known range in North Africa and provides the first integrative, morphological and molecular reference for its presence in Algeria. This study provides essential baseline data for entomological vector surveillance and contributes to a better understanding of the diversity of blood-feeding and their potential role in the transmission of pathogens in the Sétif region.

**Keywords:** Blood-feeding flies, Molecular confirmation, Nzi trap, Vavoua trap, Seasonal dynamics

## INTRODUCTION

Blood-feeding flies form a diverse and ecologically important group within the order Diptera, including taxa such as horseflies (Tabanidae), stable flies (*Stomoxys* spp.), and related species that are found across a wide range of ecosystems worldwide <sup>[1]</sup>. Due to their hematophagous feeding habits and frequent interactions with vertebrate hosts, these insects play a significant role in the spread of pathogens and impact ecosystem dynamics <sup>[2]</sup>. Their populations often reach high densities in areas with intensive livestock farming, particularly in agricultural and rural settings, where they act as vectors for bacterial,

viral, and protozoan pathogens of considerable veterinary significance <sup>[3]</sup>. For example, *Musca* spp. and *Haematobia irritans* have been linked to the dissemination of *Escherichia coli* and *Moraxella bovis*, agents responsible for mastitis in dairy cattle and infectious bovine keratoconjunctivitis (pinkeye) in beef cattle, respectively <sup>[4]</sup>. Infectious diseases pose significant threats to animal health, productivity, and welfare, thereby underscoring the imperative for rigorous monitoring and effective control strategies.

In the Maghreb region (comprising Algeria, Morocco, Tunisia, Libya, and Mauritania), blood-feeding flies pose persistent challenges to cattle producers and veterinary services <sup>[5]</sup>. *M. crassirostris* Stein, 1903, commonly known



as the African housefly or cattle fly, is widely distributed and has been extensively studied due to its significant impact on livestock health. Like other hematophagous dipterans, *M. crassirostris* is capable of transmitting a diverse array of pathogenic microorganisms, including viruses such as equine infectious anaemia virus and protozoan parasites such as *Trypanosoma* sp. [6]. These infections are associated with substantial economic losses stemming from decreased productivity, increased veterinary expenditures, and, in severe cases, livestock mortality [7]. The species exhibits a high capacity for adaptation to a wide range of environmental conditions, accompanied by an extensive geographic distribution. This adaptability significantly increases its potential to disrupt local ecosystems and adversely affect agricultural economies [6].

Algeria's heterogeneous bioclimatic zones, particularly in the Sétif region of eastern Algeria, provide ideal conditions for diverse hematophagous fly populations. Characterised by semi-arid climates, fertile plains, and mountainous terrain, Sétif represents an understudied yet epidemiologically significant area for vector-borne disease dynamics. Despite the recognised threat of dipteran vectors to livestock health, entomological surveys in this region remain sparse, limiting the development of targeted control strategies. A comprehensive assessment of the hematophagous dipteran fauna in Sétif is therefore essential to enhance our understanding of their ecology, vectorial capacity, and role in disease transmission. This study aims to document the species composition, diversity, and relative abundance of blood-feeding flies across selected sites in the Sétif region, thereby generating baseline data to support future risk assessments and inform targeted vector control strategies.

## MATERIAL AND METHODS

### Sampling Sites and Insect Identification

Sampling was conducted monthly from December 2022 to November 2023 at four sites: Beni Fouda and Ain Roua (mountainous areas), Ain Azel (low-lying plains), and Ain Arnat (upper plains). The region covers 6,549 km<sup>2</sup> with a semi-arid climate characterised by cold winters (−9.5°C) and hot summers (40°C), as well as an annual rainfall of 200–500 mm, concentrated in winter and spring. Nzi and Vavoua traps were used. Insects were preserved in 70% ethanol and identified at the Higher National Veterinary School of Algiers (ENSV), Laboratory of Parasitology, Rabie-Bouchama, Issad Abbes, Oued Smar, Algiers, Algeria, using Afrotropical dipteran keys. Diagnostic characters (wing venation, coloration, and proboscis morphology) were photographed using a LEICA EZ4W stereomicroscope equipped with a digital camera, connected to a computer, and image acquisition and processing were performed using Leica Application Suite

LAS EZ software (version 3.3.0). Specimens were stored at −20°C before undergoing molecular analysis.

### Molecular Characterisation

Each sample was placed in a tube containing liquid nitrogen and crushed. DNA was isolated using a mixture of phenol, chloroform, and isoamyl alcohol [8]. After centrifugation, the DNA in the upper aqueous phase was collected and precipitated with ethanol in the presence of sodium acetate, following the protocol of the same authors. Confirmation of the species was performed by amplifying and sequencing the mitochondrial COI gene [9], using the primers LCO-1490-sense (GGTC AACAAATCATAAAGATATTGG) and HCO-2198-antisense (TAAACTTCAGGGTGACCAAAAA ATCA). Amplification products were visualised on 2% agarose gels with MIDORI intercalating agent, then sequenced. The amplicons were purified with Exo-SAP-IT (USB Corporation, Cleveland, Ohio, USA) and sequenced in both directions using the same PCR primers at a concentration of 3.2 µM, employing BigDye™ chemistry in a PCR analyser. ABI 3500 sequences (Applied Biosystems, California, USA). Sequence chromatograms of each strand were examined with 4Peaks software and compared with published sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST; [www.ncbi.nlm.nih.gov/blast](http://www.ncbi.nlm.nih.gov/blast))

### Statistical Analysis

Variations in the number of flies caught were assessed according to season (winter, spring, summer, autumn), study site, and type of trap used. Descriptive statistics (mean, standard deviation, median, minimum, and maximum values) were calculated for each factor. Statistical analyses were performed using R software (RStudio, version 2026.01.0+392). Group comparisons were conducted using one-way analysis of variance (ANOVA), applied separately for each factor (season, study site, and trapping method). When significant differences were detected ( $P < 0.05$ ), post-hoc pairwise comparisons were performed using the Wilcoxon-Mann-Whitney test to identify differences between groups.

## RESULTS

In the Sétif region, a total of 4,917 flies were collected. Our analysis showed significant differences in the number of flies captured ( $P = 0.001$ ) across different species. We identified several bloodsucking fly species, including *Stomoxys calcitrans*, Tabanidae sp, and *Hematobia irritans*. A notable finding was the presence of *M. crassirostris* among these species.

### Species Identification

The captured hematophagous Diptera specimens were

identified to species level through morphological examination under 10-40x magnification, following established taxonomic protocols for Afrotropical Diptera. *M. crassirostris* features four dark longitudinal stripes on the thorax. Its sturdy, bulbous proboscis includes a shiny black chitinous structure called the mentum or haustellum, bearing 6 to 8 long setae and 20 to 28 short setae. The M vein (M1+2) forms a sharp angle, bending nearly at a right angle to reach the costa before the wing apex, close to the third longitudinal vein (R4+5). The forehead is generally broader in females and narrower in males. In females, the ovipositor is slender and terminates in two dark cerci, and the legs exhibit a characteristic seta located at approximately two-thirds of the distal tibia (Fig. 2-A1,2,3,4,5,6). *S. calcitrans* (Fig. 1-B1,2,3) was identified by its stiff, forward-extending proboscis and a checkerboard pattern on the abdomen. The abdomen is wider than it is long, featuring a median brown spot and two lateral spots on the second and third segments, along with an angled wing vein (M1+2). *Haematobia irritans* (Fig. 1-C1,2,3) specimens (body length 2.5 to 3.5 mm) exhibited a grey colouration with dark thoracic stripes and a gently curved wing vein (M1+2), unlike related species, which display a more steeply angled curvature. Specimens of Tabanidae (Fig. 1-D1,2,3) have been classified at the genus level due to their partially globular heads and prominent compound eyes that occupy most of the head capsule. Their sizes vary from 5 mm to 30 mm, depending on the species and developmental conditions. Bicoloured wing venation patterns are observable.

### Molecular Confirmation

The sequences obtained were submitted to the GenBank database under accession numbers PV012500-PV012513, corresponding to *M. crassirostris* Stein, 1903. This constitutes the first molecular reference for this fly in Algeria and will support future entomological, taxonomic,

or epidemiological studies on this species in the region.

### Trap Performance and Species Specificity

**Overall Catches and Trap Efficacy:** A total of 4,917 flies were captured across all sites, seasons, and trap types. Nzi traps accounted for 51.7% (2,542/4,917) of the total catches, while Vavoua traps captured 48.3% (2,375/4,917). No significant difference was detected between the two trapping methods in terms of overall abundance (one-way ANOVA,  $P > 0.05$ ). However, clear differences in species composition were observed between trap types. Nzi traps captured a higher number of *M. crassirostris* individuals (1,786 specimens; 70.3% of all Nzi catches), whereas Vavoua traps showed higher captures of *S. calcitrans* (1,328 specimens; 55.9% of all Vavoua catches). Although these patterns suggest species-specific preferences, the differences were not statistically significant when analyzed separately for each insect group (one-way ANOVA,  $P > 0.05$ ). Both trap types were less effective for Tabanidae spp. and *H. irritans*, which together represented only a small proportion of the total catches (Fig. 3-A,B).

**Spatial and Seasonal Variation:** Our study revealed significant disparities in hematophagous fly populations between regions, particularly when comparing mountainous areas (Beni Fouda and Ain Roua) with plain areas (Ain Azel and Ain Arnat) (Fig. 4-A,B). Statistical analysis confirmed a highly significant difference in insect abundance between these two geographical settings ( $P = 0.00065$ ). Seasonal dynamics also showed a marked influence on fly captures ( $P = 0.01$ ), with higher abundances recorded during summer and autumn compared to winter and spring (Fig. 5-A,B). However, no significant difference was detected between summer and autumn captures ( $P = 0.1784$ ). These results emphasise the importance of including topographical, environmental, and climatic factors in entomological surveillance and

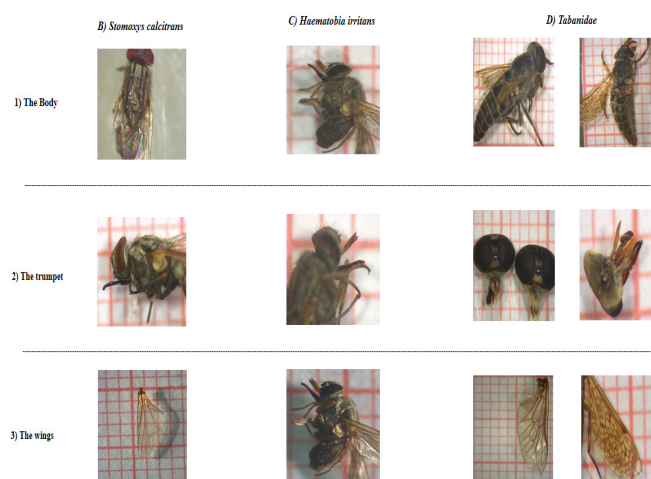


**Fig 1.** Morphological description of hematophagous flies: *Stomoxys calcitrans*, *Haematobia irritans*, and Tabanidae

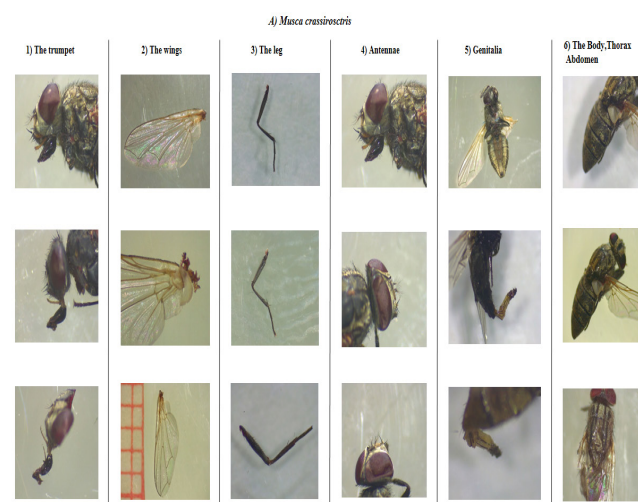


**Fig 2.** Morphological description of *Musca crassirostris* Stein, 1903

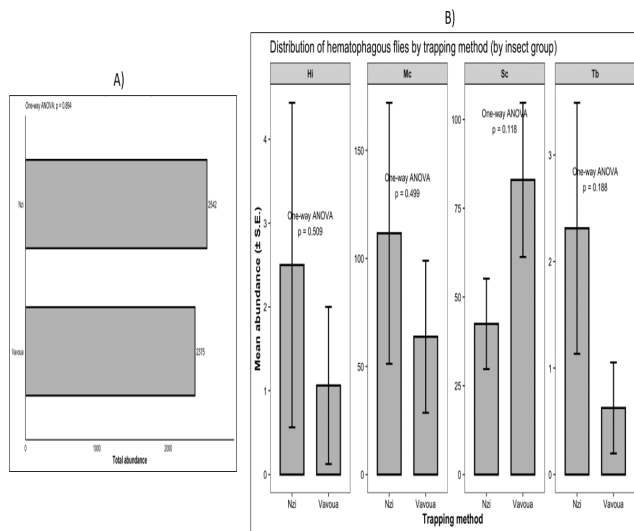


**Fig 3.** Distribution of hematophagous flies by trapping method (HI: *Haematobia irritans*; Mc: *Musca crassirostris*; Sc: *Stomoxys calcitrans*; Tb: *Tabanidae*)

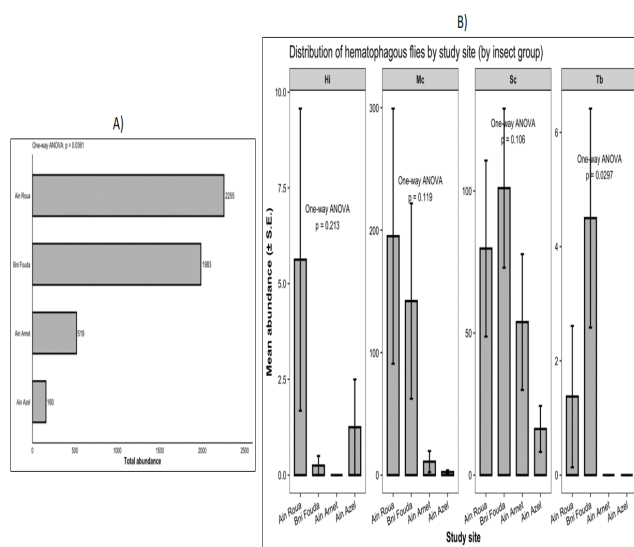


**Fig 4.** Distribution of hematophagous flies by study site (HI: *Haematobia irritans*; Mc: *Musca crassirostris*; Sc: *Stomoxys calcitrans*; Tb: *Tabanidae*)

vector control strategies, as they greatly affect fluctuations in fly populations throughout the year.

## DISCUSSION

This study provides the first integrated survey of hematophagous Diptera in Sétif and confirms the presence of *M. crassirostris* in Algeria. The results highlight significant differences in species composition, seasonal dynamics, and site-specific abundance, which can be attributed to both ecological conditions and methodological factors.

Horseflies (Tabanidae) were rare in our captures. They were completely absent in the spring, appeared only

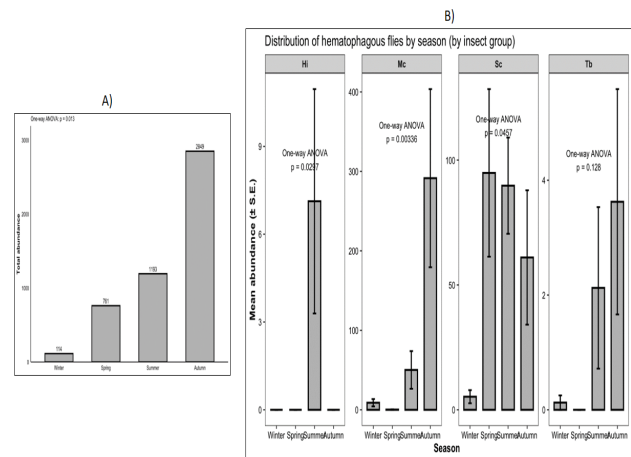


**Fig 5.** Distribution of hematophagous flies by season (HI: *Haematobia irritans*; Mc: *Musca crassirostris*; Sc: *Stomoxys calcitrans*; Tb: *Tabanidae*)

sporadically in the summer, and showed a small increase in the autumn. This result contrasts with findings from El Tarf, where peaks occur in spring [10]. The discrepancy is likely explained by climatic differences. El Tarf's humid Mediterranean climate provides abundant larval habitats, while Sétif's semi-arid climate and harsh winters limit survival [11-13]. Interestingly, the autumnal increase observed in Sétif is more consistent with results from Sudan [14] and Mauritania [15], where populations rise after the rainy season. On the other hand, this pattern differs from that of Croatia [16,17] and El Tarf [10], where peaks occur earlier in the year. Together, these comparisons suggest that rainfall, temperature, and latitude strongly shape Tabanidae activity [17]. Trap efficiency is another factor. Both Nzi and Vavoua traps yielded very low numbers of horseflies. In fact, specialized devices such as the H-Trap have been shown to capture far more individuals-up to 53% of captures compared to only 9% for the Nzi and 4% for the Vavoua [18]. Using only two trap types, therefore, represents a methodological limitation of this study.

The stable fly (*S. calcitrans*) exhibited a more pronounced seasonal trend, with higher numbers during summer and autumn. This agrees with earlier studies in both temperate and tropical regions [19,20]. We also observed that captures rose sharply after rainfall, which underlines the importance of humidity for larval development. In terms of trap performance, Nzi traps consistently outperformed Vavoua traps, particularly at Beni Foua and Ain Roua, confirming earlier reports of their efficiency for this species [21,22]. However, trap results are not the whole story. Environmental conditions, such as vegetation cover, livestock density, and local microclimate, likely also contributed to the variations observed between sites. Because our study covered only one year and four sites, broader patterns should be interpreted with caution.

By contrast, *H. irritans* was rarely captured only 57

individuals in total, all of which were captured in the summer. This low abundance can be explained in two ways. First, the traps we used are poorly suited for this fly, which rarely leaves its cattle host. Higher capture rates are achieved with baited Nzi traps <sup>[23]</sup>, direct collections from cattle <sup>[24]</sup>, or walk-through traps with aspiration <sup>[25]</sup>. Second, environmental constraints play a role. Reproduction of *H. irritans* requires warm, humid conditions <sup>[26]</sup>, and the species undergoes diapause in winter, which limits its activity in temperate climates. In tropical areas, by contrast, populations persist throughout the year <sup>[27]</sup>. In Sétif, the combination of a semi-arid climate and cold winters almost certainly explains why the species was so scarce <sup>[11]</sup>.

In striking contrast, *M. crassirostris* was abundant, especially at mountain sites during summer and autumn. Although Nzi traps caught more individuals than Vavoua, the difference was not statistically significant. This dominance is consistent with reports from Southeast Asia, where *M. crassirostris* accounted for 73% of hematophagous Diptera on dairy farms in Thailand <sup>[28]</sup>. Similar patterns have been reported in India and South Africa, where the species made up 17–20% of captures <sup>[29,30]</sup>. Its absence in winter is consistent with known physiological limits: the species thrives at 21–30°C but declines outside this range <sup>[31,32]</sup>. The present study provides the first molecular confirmation of *M. crassirostris* in Algeria. Its long-standing presence has likely been overlooked mainly due to the absence of entomological surveys. Given its abundance and veterinary importance, this species should be systematically included in national vector monitoring programmes.

The strong differences between sites deserve attention. Mountainous areas, such as Beni Fouda and Ain Roua, supported significantly higher populations than lowland sites. The cooler and more humid conditions clearly favour insect development. In Ain Azel, on the other hand, the population was much lower. This was not only due to its drier climate but also to livestock management practices. Farmers in the area reported frequent use of insecticides, such as thiamethoxam and pyrethroids, as well as citronella oil as a repellent <sup>[33,34]</sup>. By contrast, no chemical interventions were applied at Beni Fouda or Ain Roua. These observations underline that Diptera populations are shaped not only by climate and topography, but also by human activities. Any interpretation of abundance data should therefore take local management into account.

In conclusion, this study documents the diversity and dynamics of hematophagous Diptera in Sétif, Algeria, and provides the first molecularly confirmed record of *M. crassirostris*. Abundance varied with season, altitude, and livestock practices. Despite the limitations (two trap types and a one-year sampling period), these results provide a

baseline for surveillance and vector control in the Sétif region. Further research should focus on the pathogens carried by these ectoparasites, particularly their role in the transmission of bacterial, viral, and protozoan agents affecting cattle. Complementary epidemiological studies on local herds are also needed to clarify the impact of these vectors on animal health and productivity in the region. Such work will provide an essential bridge between entomological surveys and applied veterinary interventions.

## DECLARATIONS

**Availability of Data and Materials:** The data that support the findings of this study are available from the corresponding author (K. Dekkiche) upon reasonable request.

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**Authors' Contributions:** K.D: Conceptualization, field sampling, morphological identification, data collection, Methodology, editing, and initial manuscript drafting. A.B: Supervision, Laboratory analyses, molecular identification, data validation, Methodology and manuscript revision. F.M: Methodology, supervision, critical review of entomological data, morphological identification and editing. A.M: Data analysis, statistical support, and contribution to manuscript editing. L.F: Molecular validation, technical support, and critical review of molecular methods. R.R: Supervision of molecular analyses, data interpretation, manuscript structuring, and final revision

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## RESEARCH ARTICLE

# Reynoutrin Mitigates Metabolic, Immune, and Redox Dysregulation in Acute Dyslipidemia

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## Abstract

Acute dyslipidemia is a key contributor to metabolic disturbances, often driven by lipid imbalance, and systemic inflammation. This study explored the potential of reynoutrin, a bioactive flavonoid glycoside, in counteracting lipid abnormalities and their associated oxidative and inflammatory responses in a rat model of acute dyslipidemia. Reynoutrin (25 and 50 mg/kg) was administered orally for 14 days, followed by the induction of acute dyslipidemia using poloxamer-407 (P407). Biochemical analyses included lipid profile assessment, hepatic lipid-regulating enzyme activity, oxidative stress biomarkers, and inflammatory mediators. The HMG-CoA reductase and fatty acid synthase (FAS) inhibitory activities of reynoutrin were determined. P407 administration resulted in marked elevations in plasma cholesterol, triglycerides, vLDL-C, and LDL-C, with reductions in HDL-C and lipoprotein lipase activity. Reynoutrin treatment mitigated these effects, normalized lipid parameters, and downregulated hepatic HMG-CoA reductase and FAS. It also enhanced LDL-R expression. Reynoutrin significantly reduced hepatic malondialdehyde and nitric oxide while boosting antioxidant enzyme levels. Inflammatory cytokines and NF- $\kappa$ B p65 expression were elevated in dyslipidemic rats but significantly attenuated following reynoutrin administration. Reynoutrin exhibited binding affinities with HMG-CoA reductase, LDL-R PCSK9 binding domain, and FAS KS and TE domains. In conclusion, reynoutrin exhibits potent lipid-lowering, antioxidant, and anti-inflammatory properties, potentially via modulation of lipid metabolism, redox balance, and immune signaling in acute dyslipidemia. These findings suggest its therapeutic promise in managing dyslipidemia-associated complications.

**Keywords:** Dyslipidemia, Reynoutrin, Inflammation, Oxidative stress

## INTRODUCTION

Dyslipidemia is characterized by elevated circulating triglycerides (TG), low-density lipoprotein-cholesterol (LDL-C), and/or reduced high-density lipoprotein-cholesterol (HDL-C). Dyslipidemia represents a pervasive metabolic disorder worldwide, and a key risk factor for cardiovascular disease (CVD) <sup>[1,2]</sup>. The prevalence of dyslipidemia is rising worldwide, largely attributed to sedentary lifestyles, unhealthy diets, and increasing rates of obesity and metabolic syndrome <sup>[3]</sup>, underscoring the urgent need for effective therapeutic and preventive strategies. The public health burden is profound and population-based surveys reveal that prevalence of

dyslipidemia is increasing, closely linked to increased incidence of atherosclerotic cardiovascular events <sup>[1,2]</sup>. Atherosclerosis involves chronic inflammation along with progressive accumulation of fatty plaques within arterial walls, resulting in arterial narrowing, reduced blood flow, and an increased risk of severe cardiovascular events such as myocardial infarction and stroke <sup>[4]</sup>. The primary drivers of atherosclerosis include elevated levels of LDL-C, vLDL-C, and TG, coupled with reduced HDL-C <sup>[5]</sup>.

The underlying causes of dyslipidemia are multifaceted, involving both genetic predispositions and environmental factors. Primary dyslipidemias are often inherited, resulting from genetic mutations affecting lipoprotein synthesis,



transport, or catabolism. Secondary dyslipidemias, more commonly encountered, are acquired due to lifestyle choices, medical conditions, or certain medications [6]. Regardless of etiology, the pathological mechanisms converge on the dysregulation of lipid metabolism, leading to an imbalance between lipid synthesis and clearance. This imbalance promotes the retention and modification of atherogenic lipoproteins within the arterial intima, initiating a cascade of cellular and molecular events that culminate in atherosclerotic plaque formation [7]. While chronic dyslipidemia is well-studied, acute dyslipidemia characterized by rapid and transient elevations in lipids remains underappreciated, despite its ability to trigger metabolic disturbances. Acute dyslipidemia is characterized by a rapid and often profound alteration in lipid profiles, typically involving a sudden surge in circulating TG and cholesterol (CHOL). In acute settings, lipid overload promotes hepatic and systemic dysregulation, including impaired lipoprotein lipase (LPL) activity and dysregulated lipid-regulating enzymes, setting the stage for inflammatory and oxidative consequences [8]. Inflammation and oxidative stress are linked to the pathogenesis and progression of dyslipidemia and its cardiovascular complications [9-11]. Elevated levels of atherogenic lipoproteins trigger an inflammatory response within the arterial wall. This involves the activation of endothelial cells, recruitment of immune cells, including monocytes and T lymphocytes, and the release of pro-inflammatory cytokines and chemokines. These inflammatory mediators perpetuate endothelial dysfunction, promote smooth muscle cell proliferation, and contribute to plaque instability [7]. Concurrently, dyslipidemia induces a state of heightened oxidative stress. This oxidative milieu damages cellular components, including lipids, proteins, and DNA, further exacerbating inflammation and contributing to the progression of atherosclerosis [9-11]. Therefore, attenuation of oxidative damage and inflammation represent a valuable target in dyslipidemias.

Reynoutrin (Reyn; quercetin-3-O-xyloside), a naturally occurring flavonoid glycoside, has attracted considerable scientific interest due to its beneficial effects, including potent antioxidant, anti-inflammatory, and potential lipid-modulating properties [12-14]. Derived from various plant sources, Reyn has been shown to exert its antioxidant effects by scavenging free radicals and enhancing antioxidant defense systems, such as the Nrf2 pathway [12]. Its anti-inflammatory actions are attributed to its ability to inhibit pro-inflammatory mediators and pathways, thereby attenuating inflammatory responses [13]. While Reyn's antioxidant and anti-inflammatory properties are recognized, its direct impact on lipid metabolism pathways, particularly in the context of acute lipid

disturbances, and its molecular interactions with key lipid-regulating enzymes, has not been fully explored. This study investigated the potential of Reyn in counteracting lipid abnormalities and their associated oxidative and inflammatory responses in acute dyslipidemia in rats, and explored its underlying mechanisms of action, including its binding affinities towards key lipid-regulating targets.

## MATERIALS AND METHODS

### Ethical Approval

This study was approved by the ethics committee of Al-Azhar University (Approval no.: AZ-AS/PH-REC/16/25). All animal experiments comply with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8523, revised 1996).

### Animals and Treatments

Thirty adult male Wistar rats (10-week old, 170–180 g) were housed under standard conditions of temperature and humidity on a 12h dark/light cycle with *ad libitum* food and water. To establish an acute hyperlipidemia model, dyslipidemia was induced via intraperitoneal injection of poloxamer-407 (P407; 500 mg/kg; Sigma, USA) as previously described [15,16], while control animals received sterile saline. Reyn (Sigma, USA) was dissolved in 0.5% carboxymethyl cellulose as a vehicle and administered orally for 14 days. The animals were randomly assigned into five groups ( $n = 6$ ), consisting of two normolipidemic and three dyslipidemic cohorts:

Group I (Control): administered vehicle.

Group II (Reyn): administered 50 mg/kg Reyn [13].

Group III (Dyslipidemic): administered vehicle.

Group IV (Dyslipidemic + Reyn 25 mg/kg): received 25 mg/kg Reyn [13].

Group V (Dyslipidemic + Reyn 50 mg/kg): received 50 mg/kg Reyn [13].

On day 15, P407 was administered to the dyslipidemic groups, and blood was drawn from the tail vein at baseline and at 12, 24, and 48 h post-injection for total CHOL (TC) and TG measurement. Following the 48 h sampling, animals were anesthetized using ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), euthanized, and liver was excised. Tissues were homogenized in 10% (w/v) Tris-HCl buffer (pH 7.4), centrifuged, and the supernatants were collected for biochemical and molecular analyses.

### Biochemical Assays

Plasma TC, HDL-C, and TG levels were determined using commercial kits (Biosystems, Spain). vLDL-C and LDL-C levels were calculated as:

$$vLDL = TG/5$$

$$LDL = TC - (HDL + vLDL)$$

LPL, NF- $\kappa$ B p65, and cytokines (IFN- $\gamma$ , IL-4, and TNF- $\alpha$ ) were quantified using ELISA kits from Solarbio and Elabscience (China). Oxidative stress markers including malondialdehyde (MDA), nitric oxide (NO), reduced glutathione (GSH), superoxide dismutase (SOD), and catalase were determined using kits from Bio-Diagnostic (Egypt). Hepatic 3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity was evaluated by assessing the HMG-CoA/mevalonate ratio as reported previously [17].

### qRT-PCR

Gene expression levels of LDL receptor (LDL-R), and fatty acid synthase (FAS) in liver tissue were analyzed via qRT-PCR [18]. Total RNA was extracted using TRIzol (Invitrogen). RNA purity was confirmed by OD260/OD280  $\geq 1.8$ , and cDNA synthesis was carried out by reverse transcription. PCR amplification used SYBR Green Master Mix with primers listed in Table 1. The expression levels were normalized to  $\beta$ -actin, and relative changes were calculated using the  $2^{-\Delta\Delta Ct}$  method [19].

### In Silico Molecular Docking

The binding affinity of Reyn was assessed against HMG-CoA reductase (PDB: 1DQA), the PCSK9-binding domain of LDL-R (PDB: 3GCX), FAS ketoacyl synthase (KS) domain (PDB: 3HHD), and FAS thioesterase (TE) domain (PDB: 1XKT) using PyRx virtual screening software (v0.8) [20] with AutoDock Vina as the docking engine. Target proteins were prepared by removing crystallographic water molecules, adding polar hydrogens, and assigning charges via AutoDock Tools (ADT; v1.5.6). Ligands were geometrically optimized and torsional flexibility parameters were set prior to docking. A grid box encompassing the active site was defined, exhaustiveness was set to 8, and 10 binding poses were generated per ligand-protein pair. The lowest-energy conformation and predicted interactions were analyzed visually in PyMOL (v2.3.2) and interaction diagrams were generated using LigPlot (v2.2.8) [21].

### Statistical Analysis

Data are expressed as mean  $\pm$  standard deviation (SD).

| Gene        | Genbank Accession Number | Sequence (5'-3')                                    | Amplicon Size (bp) |
|-------------|--------------------------|-----------------------------------------------------|--------------------|
| <i>Ldlr</i> | NM_175762.3              | F: CATTTTTCAGTGCCAACCGCC<br>R: TGCCTCACACAGTTTACCC  | 127                |
| <i>FASN</i> | NM_017332.2              | F: CTGGACTCGCTCATGGGTG<br>R: CATTTCTCTGAAGCTTCCGCAG | 111                |
| <i>Actb</i> | NM_031144.3              | F: AGGAGTACGATGAGTCCGGC<br>R: CGCAGCTCAGTAACAGTCCG  | 71                 |

One-way ANOVA followed by Tukey's post hoc test (on GraphPad Prism 8) was used for group comparisons. A P value of  $<0.05$  was considered statistically significant.

## RESULTS

### Reynoutrin Mitigates P407-Induced Dyslipidemia

Administration of P407 led to pronounced hypercholesterolemia (Fig. 1-A) and hypertriglyceridemia (Fig. 1-B), evidenced by marked increases in plasma TC and

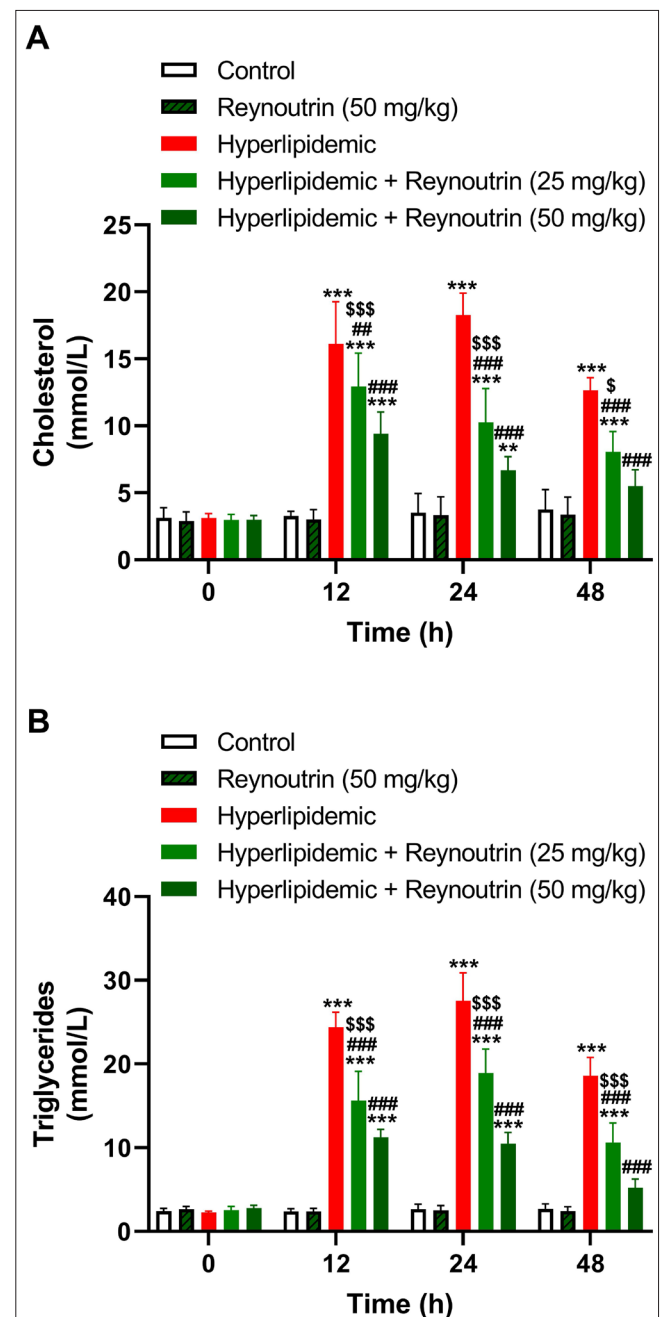


Fig 1. Reynoutrin alleviated plasma cholesterol (A) and TG (B) levels at 12, 24 and 48 h in dyslipidemic rats. Data are Mean $\pm$ SD, (n=6). \*\*P<0.01 and \*\*\*P<0.001 vs Control. \*\*\*P<0.001 vs Hyperlipidemic. \$\$\$P<0.05 and \$\$\$P<0.001 vs Hyperlipidemic + Reynoutrin (50 mg/kg)

TG at 12, 24, and 48 h compared to controls ( $P<0.001$ ). Reyn produced a significant, dose-dependent reduction in TG and TC. Assessment of vLDL-C (Fig. 2-A) and LDL-C (Fig. 2-B) showed substantial elevations following P407 exposure, whereas HDL-C levels (Fig. 2C) were significantly reduced at 48 h. Reyn effectively lowered LDL-C and vLDL-C while restoring HDL-C levels in dyslipidemic rats, with the vLDL-C effect showing dose dependency. Notably, lipid parameters remained unchanged in normolipidemic rats treated with Reyn.

### Reynoutrin Upregulates LPL and LDL-R in Dyslipidemic Rats

The data in Fig. 3 demonstrate that P407 markedly reduced plasma LPL activity (Fig. 3-A) and hepatic LDL-R mRNA (Fig. 3-B) compared to the control group ( $P<0.001$ ). Reyn significantly restored both parameters in dyslipidemic rats, with a dose-dependent effect on LDL-R. Molecular docking simulations (Fig. 3-C) revealed that Reyn binds to the PCSK9-binding domain of LDL-R, engaging in 7 polar interactions and 9 hydrophobic contacts (Table 2). These findings suggest that Reyn not only enhances LDL-R expression *in vivo* but may also directly interact with its regulatory domain to support CHOL homeostasis.

### Reynoutrin Suppresses Hepatic HMG-CoA Reductase in Dyslipidemic Rats

Liver HMG-CoA reductase activity was markedly elevated in dyslipidemic rats compared to controls ( $P<0.001$ ; Fig. 4-A). Reyn exerted an inhibitory effect in the dyslipidemic

group but had no influence on normolipidemic animals. Molecular docking indicated Reyn interactions with 5 residues via polar bonding and with 2 residues through hydrophobic contacts (Fig. 4-B, Table 2).

### Reynoutrin Downregulates Hepatic FAS in Dyslipidemic Rats

Fig. 5 illustrates the effect of Reyn on FAS expression and its molecular interactions with the enzyme catalytic domains. P407 significantly upregulated hepatic FAS mRNA compared to controls (Fig. 5-A;  $P<0.001$ ). Reyn attenuated this increase with the higher dose producing the greatest suppression. Molecular docking analyses demonstrated the binding of Reyn to the TE (Fig. 5-B) and KS (Fig.

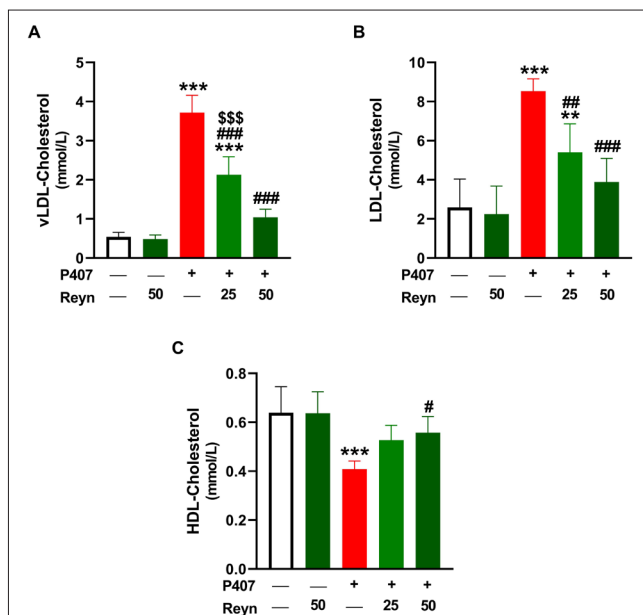


**Fig 2.** Reynoutrin decreased plasma vLDL-C (A) and LDL-C (B), and increased HDL-C levels in hyperlipidemic rats. Data are Mean±SD, ( $n=6$ ). \*\* $P<0.01$  and \*\*\* $P<0.001$  vs Control. # $P<0.05$ , ## $P<0.01$  and ### $P<0.001$  vs Hyperlipidemic. \$\$\$ $P<0.001$  vs Hyperlipidemic + Reynoutrin (50 mg/kg)

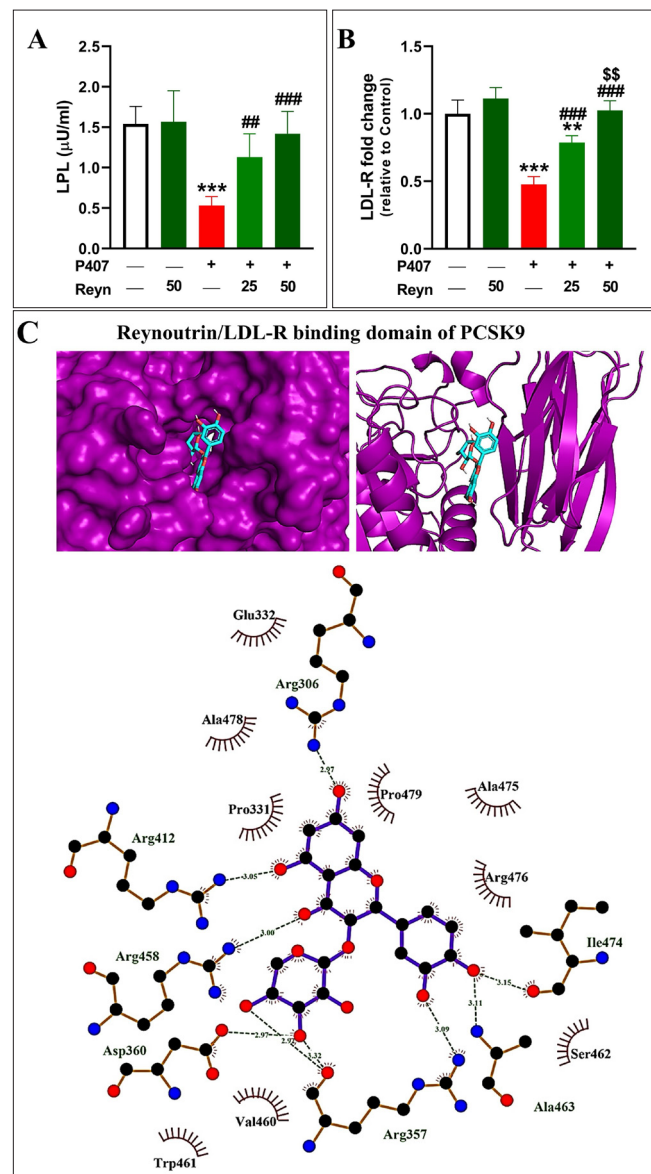


**Fig 3.** Reynoutrin increased plasma LPL (A) and upregulated liver LDL-R mRNA (B) in hyperlipidemic rats. Data are Mean±SD, ( $n=6$ ). \*\* $P<0.01$  and \*\*\* $P<0.001$  vs Control. ## $P<0.01$  and ### $P<0.001$  vs Hyperlipidemic. \$\$\$ $P<0.001$  vs Hyperlipidemic + Reynoutrin (50 mg/kg). (C) Molecular docking of Reyn with LDL-R PCSK9 binding domain

| Table 2. Binding interactions of Reyn with LDL-R PCSK9 binding domain, HMG-CoA reductase, and FAS KS and TE domains |                           |                                                        |                                                                                 |
|---------------------------------------------------------------------------------------------------------------------|---------------------------|--------------------------------------------------------|---------------------------------------------------------------------------------|
| Target                                                                                                              | Binding Energy (kcal/mol) | Polar Interacting Residues                             | Hydrophobic Interacting Residues                                                |
| LDL-R PCSK9 binding domain                                                                                          | -8.6                      | Ile474, Ala463, Arg357, Asp360, Arg458, Arg306, Arg412 | Glu332, Ala478, Pro331, Trp461, Val460, Ala475, Pro479, Arg476, Ser462          |
| HMG-CoA reductase                                                                                                   | -7.6                      | Arg598, Glu677, Arg595, Gln679, Arg641                 | Pro597, Leu596                                                                  |
| FAS KS                                                                                                              | -7.6                      | Arg101                                                 | Val460, Tyr87, Ala464, Asp98, Phe147, Thr463, Gly66, Phe64                      |
| FAS TE                                                                                                              | -7.4                      | Arg2482                                                | Phe2423, Leu2427, Gln2374, Phe2370, Phe2375, Phe2371, Leu2222, Ile2250, His2481 |

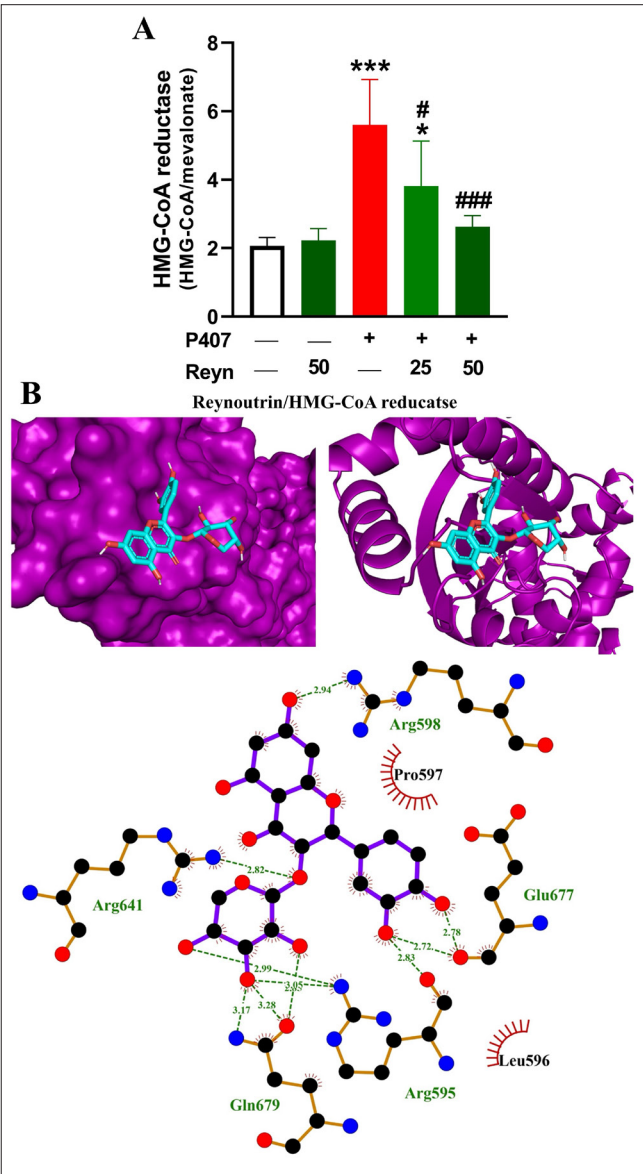


**Fig 4.** Reynoutrin suppressed liver HMG-CoA reductase activity in hyperlipidemic rats (A). Data are Mean±SD, (n=6). \*P<0.05 and \*\*\*P<0.001 vs Control. #P<0.05 and \*\*\*P<0.001 vs Hyperlipidemic. (B) Molecular docking of Reyn with LDL-R PCSK9 binding domain

5-C) domains of FAS, respectively. In the TE domain, Reyn formed a polar bond and engaged in 9 hydrophobic contacts with residues. In the KS domain, Reyn established

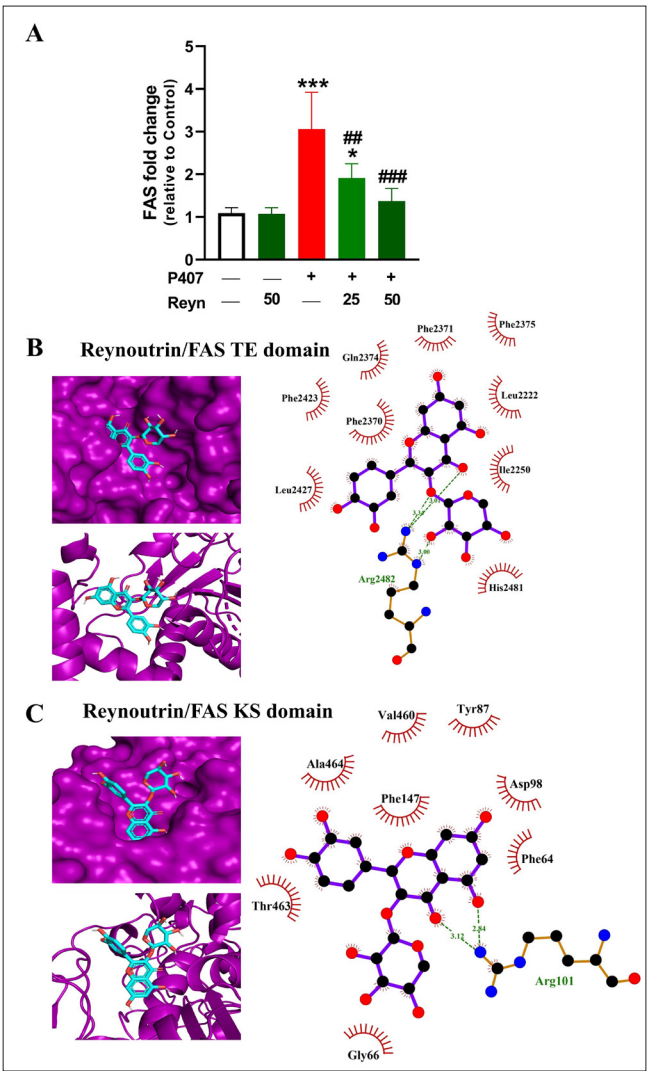


**Fig 5.** Reynoutrin downregulated liver FAS mRNA (A) in hyperlipidemic rats. Data are Mean±SD, (n=6). \*P<0.05 and \*\*\*P<0.001 vs Control. \*\*P<0.01 and \*\*\*P<0.001 vs Hyperlipidemic. (B-C) Molecular docking of Reyn with FAS TE (B) and KS (C) domains

a polar interaction with one residue, alongside hydrophobic contacts involving 8 residues (Table 2).

### Reynoutrin Alleviates Oxidative Stress in Dyslipidemic Rats

Dyslipidemia was linked to significantly increased hepatic

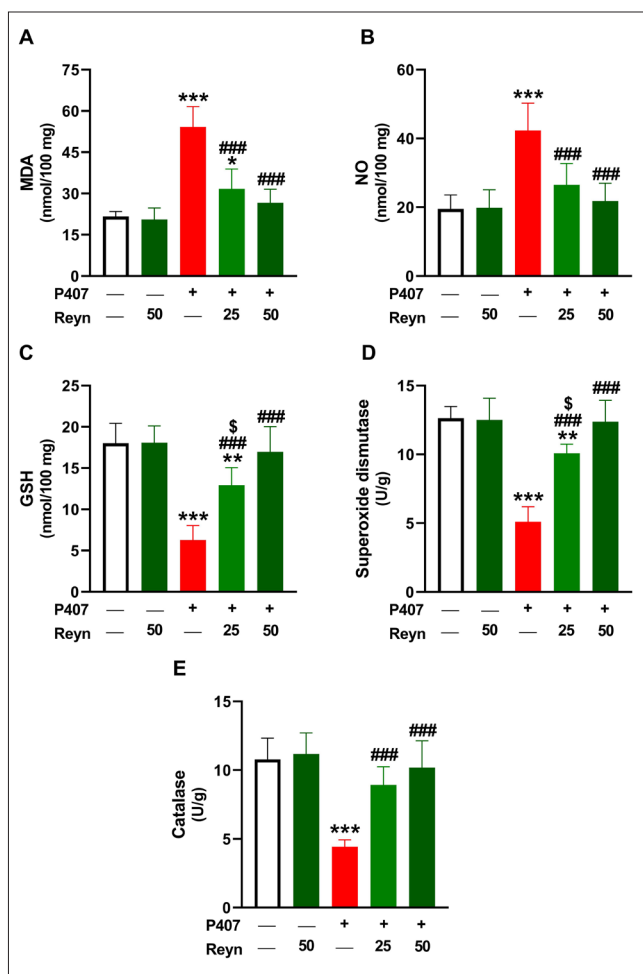


**Fig 6.** Reynoutrin attenuated oxidative stress in hyperlipidemic rats. Reynoutrin decreased liver MDA (A) and NO (B) levels, and increased GSH (C), SOD (D) and CAT (E). Data are Mean $\pm$ SD, (n=6). \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs Control. ###P<0.001 vs Hyperlipidemic. §P<0.05 vs Hyperlipidemic + Reynoutrin (50 mg/kg)

MDA (Fig. 6-A) and NO (Fig. 6-B) levels, alongside reductions in GSH (Fig. 6-C), SOD (Fig. 6-D), and catalase (Fig. 6-E) (P<0.001). Reyn decreased MDA and NO while enhancing GSH, SOD, and CAT. The effects on GSH and SOD exhibited dose dependency. No changes were observed in antioxidant or oxidative markers in normal rats treated with Reyn.

### Reynoutrin Attenuates Inflammation in Dyslipidemic Rats

As depicted in Fig. 7-A, hepatic NF- $\kappa$ B p65 was significantly elevated in dyslipidemic rats compared to controls (P<0.001), while Reyn suppressed this increase. Similarly, plasma TNF- $\alpha$  (B), IL-4 (C), and IFN- $\gamma$  (D) levels were markedly higher in the P407 group (P<0.001), and treatment with Reyn reduced their levels.

## DISCUSSION

The present study investigated the potential of Reyn in

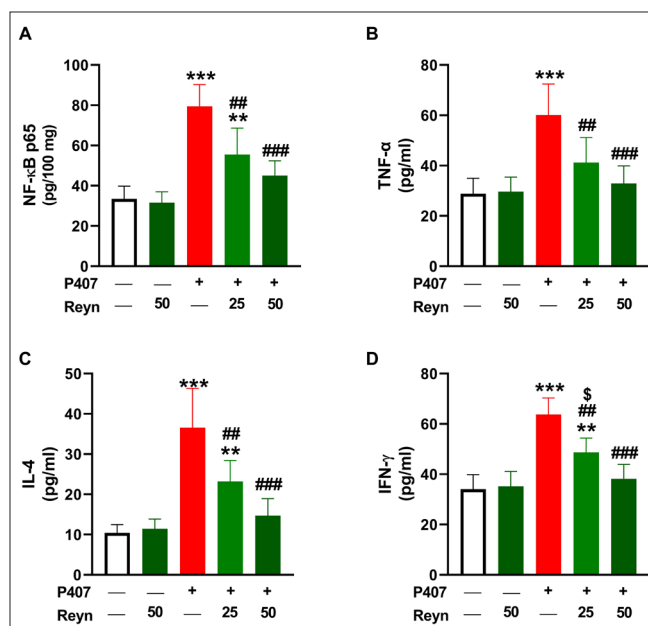


**Fig 7.** Reynoutrin mitigated inflammation in hyperlipidemic rats. Reyn decreased liver NF- $\kappa$ B p65 (A) and plasma TNF- $\alpha$  (B), IL-4 (C), and IFN- $\gamma$  (D). Data are Mean $\pm$ SD, (n=6). \*\*P<0.01 and \*\*\*P<0.001 vs Control. ##P<0.01 and ###P<0.001 vs Hyperlipidemic. §P<0.05 vs Hyperlipidemic + Reynoutrin (50 mg/kg)

mitigating metabolic, immune, and redox dysregulation in experimental acute dyslipidemia. Our findings demonstrate that Reyn effectively counteracts the lipid abnormalities, oxidative stress, and inflammatory responses associated with acute dyslipidemia, suggesting its promising role as a therapeutic agent.

P407 is a non-ionic surfactant widely employed to induce acute dyslipidemia in animal models. P407 achieves this by inhibiting LPL activity, an enzyme crucial for TG hydrolysis, and by stimulating hepatic vLDL production and secretion [22-25]. This dual action leads to a rapid and sustained elevation of TG and CHOL, effectively mimicking the acute metabolic dysregulation observed in certain clinical scenarios and providing a valuable model for studying the immediate consequences of lipid imbalance and its associated inflammatory and oxidative responses [22-25]. Acute dyslipidemia induced by P407 is characterized by a rapid and significant elevation in plasma CHOL and TG, along with adverse shifts in lipoprotein profiles. Our results, showing marked elevations in plasma TC, TG, vLDL-C, and LDL-C, with reductions in HDL-C, are consistent with established literature on P407-induced hyperlipidemia [18,23,24]. The time-course analysis, particularly at 0, 12, 24, and 48 h, confirms the acute and profound nature of these lipid disturbances. Reyn administration significantly mitigated these effects. This suggests that Reyn possesses potent lipid-lowering capabilities, effectively reversing the P407-induced dyslipidemic state. The observed improvements

in plasma lipid profiles are crucial, as elevated atherogenic lipoproteins are directly linked to increased cardiovascular risk [5].

LPL plays a pivotal role in the hydrolysis of TG from circulating lipoproteins, and its inhibition by P407 is a primary mechanism underlying the induced hypertriglyceridemia [22-25]. Our findings indicate that Reyn treatment not only alleviated lipid parameters but also enhanced LPL activity, suggesting a direct or indirect modulatory effect on this crucial enzyme. Furthermore, liver LDL-R is essential for the clearance of LDL-C from the bloodstream [26]. Downregulation of hepatic LDL-R expression is a common feature in dyslipidemia, contributing to elevated LDL-C levels [3,5]. The observed enhancement of LDL-R expression following Reyn administration is a significant finding, indicating that Reyn promotes hepatic uptake and catabolism of LDL-C. This dual action, improving LPL activity and upregulating LDL-R expression, highlights a comprehensive mechanism by which Reyn exerts its lipid-lowering effects, addressing both TG-rich lipoprotein metabolism and CHOL clearance.

Our biochemical analyses revealed that P407 administration resulted in upregulated hepatic HMG-CoA reductase activity and FAS mRNA expression, consistent with the increased lipid synthesis observed in acute dyslipidemia as we previously reported [8,18]. Crucially, Reyn significantly downregulated both hepatic HMG-CoA reductase activity and FAS mRNA expression. This experimental evidence corroborates the molecular docking predictions and provides a direct link between Reyn administration and the inhibition of key enzymes of CHOL and fatty acid synthesis. The suppression of HMG-CoA reductase activity directly contributes to reduced endogenous CHOL production [27], while the downregulation of FAS mRNA expression leads to decreased *de novo* fatty acid synthesis [28]. These findings underscore Reyn's ability to modulate hepatic lipid metabolism at a transcriptional and enzymatic level, offering a comprehensive approach to mitigating dyslipidemia. Moreover, the *in silico* studies provide valuable insights into the potential molecular mechanisms underlying lipid-modulating effects of Reyn. The observed binding affinity of Reyn towards the LDL-R PCSK9 binding domain suggests a direct interaction that could interfere with PCSK9-mediated degradation of LDL-R. By potentially inhibiting PCSK9 binding to LDL-R, Reyn could enhance LDL-R availability on the hepatocyte surface, thereby promoting increased uptake of LDL-C from circulation [29]. This mechanism aligns with the observed upregulation of LDL-R expression and the overall reduction in LDL-C levels. Furthermore, the molecular docking results demonstrate the binding affinity of Reyn to HMG-CoA reductase and FAS KS and TE domains are highly significant. Given its role as the

rate-limiting enzyme in CHOL biosynthesis, inhibition of HMG-CoA reductase is a well-established strategy for lowering CHOL [27]. FAS is critical for *de novo* fatty acid synthesis, and its inhibition can reduce TG accumulation [28]. The binding of Reyn to these key enzymes, mediated via polar and hydrophobic interactions, suggests that Reyn may directly modulate their activity, leading to the observed downregulation of hepatic HMG-CoA reductase and FAS, and consequently, reduced CHOL and fatty acid synthesis. These molecular insights provide a strong foundation for understanding the pleiotropic lipid-lowering actions of Reyn.

Acute dyslipidemia is often accompanied by heightened oxidative stress, characterized by an imbalance between pro-oxidants and antioxidants, leading to cellular damage [9,30]. Our results show that P407 significantly increased hepatic MDA, a marker of LPO, and NO levels, while reducing the levels of the crucial antioxidants enzymes GSH, SOD, and catalase. These findings are consistent with the established role of oxidative stress in the pathogenesis of dyslipidemia and its complications, as previously reported in the same experimental model [8,16,18]. In addition to promoting NF- $\kappa$ B activation and cytokine release [31], as observed herein, elevated ROS oxidize LDL, which in turn activates endothelial cell and attracts T lymphocytes and monocytes [7]. Furthermore, ROS stimulate NF- $\kappa$ B activation and promote cytokine release [31], as observed in this study. Reyn significantly reversed these detrimental changes, reducing MDA and NO levels and boosting antioxidant enzyme activities. This demonstrates the potent antioxidant properties of Reyn, which are crucial for protecting hepatic cells from oxidative damage and maintaining redox balance. The ability of Reyn to enhance endogenous antioxidant defenses contributes significantly to its overall protective effects against dyslipidemia-associated complications. In this context, Reyn has been reported to mitigate oxidative damage in experimental models of type 2 diabetes, diabetic nephropathy, and ischemic heart disease [12-14].

Besides oxidative stress, inflammation is a key component in the progression of dyslipidemia and its associated cardiovascular complications [32]. Our study found that dyslipidemic rats exhibited elevated levels of inflammatory cytokines, including TNF- $\alpha$ , IFN- $\gamma$ , and IL-4, in plasma, along with increased expression of NF- $\kappa$ B p65 in the liver. NF- $\kappa$ B activation is a hallmark of inflammatory processes [31]. The significant attenuation of these inflammatory markers following Reyn administration highlights its potent anti-inflammatory properties. By downregulating NF- $\kappa$ B p65 expression and reducing key pro-inflammatory cytokines, Reyn effectively suppresses the systemic and hepatic inflammatory responses triggered by acute dyslipidemia. This anti-inflammatory action is critical for preventing

endothelial dysfunction, reducing atherosclerotic plaque progression, and mitigating overall cardiovascular risk.

In conclusion, this study provides compelling evidence that Reyn effectively mitigates metabolic, immune, and redox dysregulation in an experimental model of acute dyslipidemia. Our findings demonstrate the effective lipid-lowering effects of Reyn through the alleviation of plasma lipid profiles, enhancement of LPL activity, and upregulation of hepatic LDL-R expression. Furthermore, molecular docking studies and biochemical analyses reveal that Reyn modulates key enzymes involved in lipid metabolism, such as HMG-CoA reductase and FAS. Beyond its lipid-modulating actions, Reyn exhibits significant antioxidant properties by restoring redox balance and potent anti-inflammatory effects by suppressing inflammatory mediators. These multifaceted actions underscore the therapeutic promise of Reyn in managing dyslipidemia-associated complications and highlight its potential as a novel natural compound for cardiovascular health. Future studies should focus on further elucidating the precise signaling pathways involved and validating these findings in clinical settings.

## DECLARATIONS

**Availability of Data and Materials:** The data that support the findings of this study are available from the corresponding author (A. Mahmoud) upon reasonable request.

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## RESEARCH ARTICLE

## Cattle Identification with CLIP-Based Biometric Features

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## Abstract

The individual identification of cattle is crucial for herd management and food safety, as well as for complying with the demands of export markets, particularly those within the European Union. In addition, traditional identification methods such as ear tagging, tattooing, or hot-cold branding have significant limitations in terms of reliability, loss rates, and animal welfare. The study proposes and evaluates a non-invasive biometric identification method using the analysis of distinctive patterns in cow coat colours. The approach we use is the CLIP deep learning model (ViT-L-14) to derive a feature vector, or "biometric signature," from a picture of each cow's coat colour pattern. This method was evaluated on a large dataset (Cows2021) containing 23.350 images representing 301 unique individuals. Utilizing a cross-validation technique (80% training/20% testing), the system exhibits better performance with an accuracy of 94.28%. Additionally, performance metrics revealed precision at 94.67%, recall at 94.28%, and an F1-score at 94.27%; this result confirms the robustness of the model in the face of class imbalances. Consequently, it is believed that the extensive adoption of this method will reduce labour in herd management and improve automatic, reliable, and animal welfare-oriented identification and traceability within the livestock sector, thereby facilitating substantial advancements in precision livestock farming practices.

**Keywords:** Biometric identification, Cattle welfare, Cattle management, CLIP model, Deep learning, Precision livestock farming

## INTRODUCTION

Identification and traceability in livestock is crucial for achieving accuracy in sustainable animal husbandry and ensuring food security. This entails monitoring the whole lifetime of animals, from birth to processing, enabling farmers to successfully oversee health, welfare, and environmental implications. Implementing comprehensive traceability systems enables stakeholders to improve transparency and foster customer confidence in the food supply chain [1,2]. Besides simple herd management, it becomes a strategic concern for biosecurity, epidemiological surveillance, product certification, and customer trust, embodying the notion of "from farm to fork". Traditional animal identification methods such as branding, tattooing, ear notching, collar id, ear-tagging, and even electrical identification methods such as Radio Frequency Identification (RFID) are not reliable enough for cattle identification due to ear-tag loss, label fading, physical damage to tags due to harsh climates, damage

to ears, animal welfare concerns, theft, fraud, and duplication [3-12]. Ear tag identification is also performed manually and is consequently susceptible to human error [13]. These strategies do not produce successful results. However, there are operational, economic, and management challenges with large-scale monitoring of livestock animals [3,5]. Therefore, non-invasive approaches for the identifying of livestock on farms are required [5].

Conventional identifying systems, have been used for an extended period [9,10]. However, traditional methods are being questioned due to their low reliability, risk of loss, wear and tear, forgery, and the damage they cause to animals' ears and bodies. From an ethical and animal welfare perspective, these practices are a demonstrated cause of pain, distress, and infection risk for animals [3-6,8,11,12,14]. Addressing these concerns requires a thorough examination of current practices and a commitment to implementing more humane alternatives. By prioritizing the well-being of animals, we can create a more compassionate approach that aligns with societal values. These challenges have



paved the way for the emergence of a new paradigm based on biometric identification, which aims to utilize unique and unchangeable physiological characteristics as natural identifiers. The literature explores many of these biometric data types. For examples, the analysis of nose prints (the tip of the nose) and retina images has demonstrated a high capacity for discrimination [15,16]. However, its implementation encounters a significant operational constraint: the need for stringent immobilization of the animal and the method's sensitivity to the cleanliness of the nasal surface. Other techniques, such as face recognition or eye analysis, although promising, have obstacles due to differences in brightness, capture angles, age-related morphological changes, and, most importantly, phenotypic uniformity within certain cow breeds, which might confuse the algorithms [17-19]. In this context, the coat color pattern of cattle stands out as particularly robust biometric data: it is unique to each individual, stable over time, covers a large area, and can be captured remotely without direct intervention on the animal [20-23]. However, the potential of this marker has long been underutilized due to the inherent complexity of its patterns and the poor performance of traditional pattern recognition algorithms in the face of such variation. The emergence of deep learning architectures, and particularly pre-trained baseline models on billions of images, offers an unprecedented opportunity to overcome these technological hurdles [24].

This research introduces and substantiates a comprehensive cattle identification approach using the CLIP (Contrastive Language-Image Pre-Training, ViT-L-14) model [25]. The primary contribution of this work is the application of an advanced vision model, not for traditional classification tasks, but for its capacity to encode intricate covering patterns into dense and semantically rich vector representations. These vectors function as a secure digital signature, or "biometric data value," for each animal. This study aims to demonstrate the feasibility and high performance of the proposed approach by developing a system capable of generating signatures, storing them in a database, and utilizing them for identification purposes.

In this respect, the main contributions of this study are threefold: first, it proposes a method of contactless biometric identification that eliminates the stress and risks of infection associated with invasive devices (tags, implants). Secondly, it validates the use of overlay patterns as a permanent and unique biometric feature, offering a reliable alternative to artificial methods that are susceptible to loss or interference. Third, it demonstrates the effectiveness of transfer learning through the CLIP model; its use ensures accurate identification without requiring large annotated datasets and computationally intensive resources typical of traditional deep learning models.

## MATERIAL AND METHODS

### Ethical Statement

This study does not present any ethical concerns.

### Dataset

This study used 23.350 images from the dataset published on <https://datasetninja.com/cows2021#download>. The dataset was specifically designed to improve and evaluate the performance of our identification system. It consists of two separate directories located in the same folder as the execution code: a reference directory called images and a benchmark directory called tests. The reference database was constructed from a directory of images, each containing four images representing unique cattle (Fig. 1); these images were used to generate biometric signatures stored in the animal\_biometric.db database, which forms our repository of known individuals. At the same time, the test set in the test directory consists of 23.350 different images used to query the system and quantitatively evaluate its performance by comparing it with four reference individuals. All images in the dataset are pre-processed, sized at a standard resolution of 224 x 224 pixels, in accordance with the requirements of the CLIP model. To provide a rigorous and statistically significant assessment, the "Identification" dataset of 301 individuals and 23.350 images was used. The data was divided according to a strict protocol: 80% of the images were set as training and the remaining 20% as test sets.

For each image, a 14-dimensional embedding vector was extracted using the CLIP ViT-L-768 model. Then the cosine similarity between the test vector and the set of training vectors was calculated. In contrast to the use of fixed empirical thresholds, the definition is defined as "1. Level Matching" approach, where the identity corresponding to the highest similarity score is preserved. The soundness of the decision is confirmed by the analysis of the ROC curve and the calculation of the AUC score."

### Proposed Approach

This section summarizes the design and functionality



Fig 1. Images in the dataset

of our proposed identification technique. The proposed methodology uses the CLIP (Adversarial Language-Image Pre-Training) deep learning model, specifically its ViT-L-14 variant, to extract features from photographs of cow coat colours<sup>[28]</sup>.

### System Architecture

The proposed identification system has an architecture with two primary stages (Fig. 2):

**1. Mining and Documenting Biometric Signatures:** In this initial phase, a photograph is taken of each cow so that it can be identified. The CLIP model takes the photo of its coat and makes a rich, high-dimensional feature vector from it. This vector, which is the animal's "biometric signature," is stored in a relational database that gives each species a unique ID and the name of the photo file that goes with it. People may then utilize this database to find out who they are.

**2. Identification of a New Cow:** A photograph of the cow's coat pattern is taken so that it may be identified. To get a feature vector from the query image, the CLIP model is employed. After that, a similarity measure is used to compare the vector to all the feature vectors in the database. The algorithm finds the vector that is most like the query vector and calls it the identity if its score is high enough.

### Extracting Visual Features with the CLIP Model

The proposed methodology basically utilizes the CLIP (Contrastive Language-Image Pre-Training) framework developed by OpenAI<sup>[26]</sup>. CLIP learned from a large number of image-text pairs, which helped it learn how to connect visual representations with natural language. This contrastive learning process equips CLIP with a significant ability to understand and express the semantic substance of images, beyond basic object recognition. The ViT-L-14 variation of the CLIP was selected because it worked better, had more complicated architecture generating an integration space of 768 dimensions, and was more accurate and resilient in visual representations<sup>[27]</sup>.

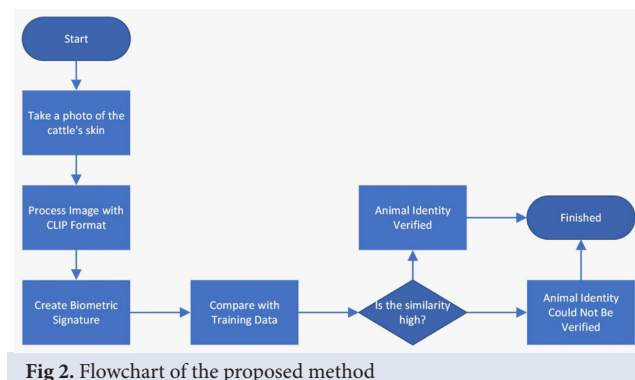


Fig 2. Flowchart of the proposed method

This is how to get visual characteristics from a picture of a cow's coat colour pattern:

**1. Image Loading & Preprocessing:** The texture photo is taken from the right folder and saved in RGB format. The image is resized to a common resolution of 224x224 pixels so that the CLIP model can get the same input every time. This resize standardizes the dimensions of the model's input tensors.

**2. Using the CLIP Model to Code:** The preprocessed picture is then used with the CLIP ViT-L-14 model. The model uses its image encoder to get a vector representation of the picture. This vector shows the coat's typical patterns, textures, and color distributions. It is a high-level abstraction of the image's visual content.

**3. Normalization of the Feature Vector:** The L2 norm is used to normalize the feature vector that the CLIP model created. This normalization ensures that all vectors have a unit magnitude, which is crucial for subsequent comparison based on cosine similarity. Normalization allows us to focus on the orientation of the vectors rather than their magnitude.

The resultant feature vector is a compact digital representation of the coating pattern, prepared for storage in the database or comparison with other vectors for identification purposes.

### Similarity Measurement: Cosine Similarity

Cosine similarity quantifies the resemblance between the feature vector of the query picture and each feature vector (Vbase) inside the database. Cosine similarity quantifies the cosine of the angle between two vectors in a multidimensional space. It is delineated by the accompanying formula:

$$\text{Cosine Similarity}(V_{\text{query}}, V_{\text{database}}) = \frac{V_{\text{query}} \times V_{\text{database}}}{\|V_{\text{query}}\| \times \|V_{\text{database}}\|}$$

a-  $V_{\text{query}} \times V_{\text{database}}$ , this expression represents the scalar product of two vectors.

b-  $\|V_{\text{query}}\| \times \|V_{\text{database}}\|$ , this represents the euclidean norms of the involved vectors.

Cosine similarity produces a value between -1 and 1, where 1 indicates perfect similarity (the two vectors are directed in the same direction), 0 indicates no linear correlation, and -1 indicates perfect contrast.

### Identification and Similarity Thresholds

The identification process relies on calculating the cosine similarity between the query vector and the set of vectors in the database. To ensure maximum resilience in the face of environmental changes, this study opts for a "matching" approach rather than an arbitrary cut-off threshold.

According to this method, the identification decision is determined by the highest similarity score: the queried image is assigned to the identity of the reference vector ( $\text{Similarity}_{\max}$ ), which maximizes cosine similarity. The discriminating capacity of the model is assessed by analysis of the ROC curve with a sub curve area (AUC) of 0.723. This result confirms that the system consistently gives higher scores to positive pairs (in the same individual) than to negative pairs; thus, without the need for manual calibration of a certain threshold, it verifies the reliability of the decision.

## RESULTS

To evaluate the effectiveness of the proposed approach on a representative scale, rigorous experiments were conducted on the full “Identification” subset of the dataset; this subset included 301 cattle, and a total of 23.350 images were found. In contrast to limited pre-tests, this assessment was conducted using the 80% (Training)/20% (Test) section protocol.

Feature vectors were extracted using the CLIP ViT-L-14 model. Identification was performed by the matching method, where the predicted ID matched the vector in the gallery with the highest cosine similarity to the test image (*Table 1*). In the test set, the system achieved an overall accuracy rate of 94.28%. This performance is justified by an F1 score of 94.27%, indicating an optimal balance between precision and recall, as detailed in *Table 2*.

A ROC (Receiver Operating Characteristic) curve was generated to analyze the stability of the system in the verification task (*Fig. 4*). The area under the curve (AUC)

| Table 1. Recognition percentages of the test group (20%) |         |            |       |
|----------------------------------------------------------|---------|------------|-------|
| Image                                                    | Matches | Mismatches | Total |
| Number                                                   | 4402    | 268        | 4670  |
| Percentage (%)                                           | 94.28   | 5.72       | 100   |

| Table 2. Performance Metrics of CLIP ViT-L-14 |            |
|-----------------------------------------------|------------|
| Criteria                                      | Percentage |
| Accuracy                                      | 94.28%     |
| Precision                                     | 94.67%     |
| Recall                                        | 94.28%     |
| F1-Score                                      | 94.27%     |

is 0.723; this value demonstrates the model’s ability to accurately distinguish identities regardless of threshold variations.

In addition, a detailed analysis by class was carried out to assess the consistency of recognition across the 301 individuals. The distribution of accuracy (*Fig. 5*) reveals a strong asymmetry towards maximum performance: 146 individuals (48.5%) were identified with a perfect accuracy of 100%. Conversely, only 23 individuals have a recognition rate of less than 80%. This confirms that the CLIP model is able to extract visual representations that are unique enough for the vast majority of the herd, despite environmental challenges.

Furthermore, in a group of 23 individuals, the recognition rate having an accuracy rate less than 80% indicates that severe clogging, excessive lighting changes, and motion blur are the main causes of misidentification (*Fig. 6*).

Consequently, the experimental results strongly confirm the potential of the proposed approach while identifying clear areas for improvement, particularly regarding robustness to capture conditions and optimization of the decision threshold.

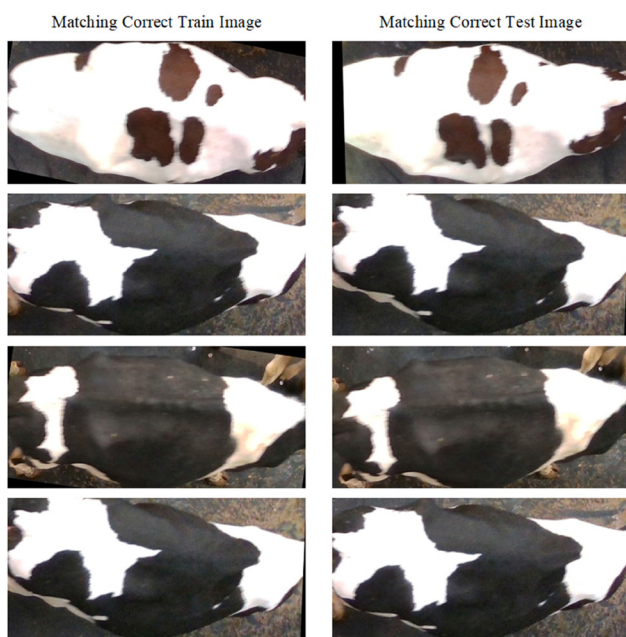


Fig 3. Correctly matched sample images in train and test datasets

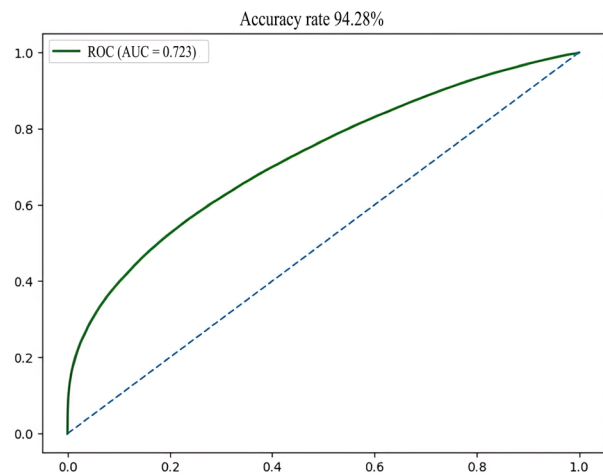


Fig 4. Roc curve

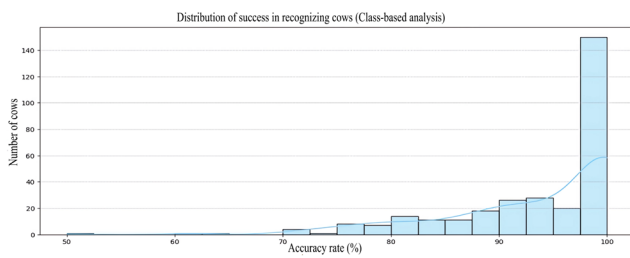


Fig 5. Distribution of recognition success of cows



Fig 6. Incorrectly matched images

## DISCUSSION

The experimental results, obtained in a large dataset containing 301 cows and 23.350 images, demonstrate the robustness of the proposed approach. In contrast to limited preliminary studies, this large-scale analysis achieved a 94.28% Accuracy rate and a 94.27% F1-Score. These performances convincingly confirm the effectiveness of the CLIP model for biometric identification of cattle that does not compromise their body integrity.

The main contribution of this study lies in the successful implementation of zero-shot learning without the need for costly retraining. While the seminal work of Martinez and Kak<sup>[29]</sup> addressed the limitations of classical linear methods, recently, deep learning-based approaches and specialized architectures for livestock identification have been proposed by Sharma et al.<sup>[22]</sup> and Wang et al.<sup>[23]</sup> Despite their high accuracy, these methods necessitate significant computational resources and intricate training processes. Similarly, Jing et al.<sup>[25]</sup> explored vision and language models (Animal-CLIP) for action recognition. Our study complements this emerging literature by showing that the CLIP Standard Model can be used efficiently with minimal computational cost for individual identification; thus, it offers a more accessible and scalable solution for the daily management of herds.

Analysis of errors related to 5.72% of unidentified cases reveals that failures are not random but linked to specific circumstances. Visual inspection of cases with an accuracy rate of less than 80% shows that severe clogging (sludge, equipment) and excessive lighting changes are the main causes, confirming the challenges noted by Andrew et al.<sup>[13]</sup> in their pioneering work on RGB-D imaging. The current ROC-AUC analysis (AUC = 0.72) provides a solid statistical basis for the reliability of the system.

It is important to highlight that the recommended method against misidentification in herd management provides 94.28%. While no biometric system is infallible, this high success rate positions the proposed method as a reliable decision support system. When used in conjunction with human oversight during critical operations, it offers a better alternative to traditional methods and minimizes risks.

In conclusion, the images have environmental challenges that lead to identification errors: loss of emphasis and detail at high light exposure, blurring of motion affecting the sharpness of the pattern, excessive shooting angle limiting visibility, and shadows that alter the appearance of biometric features. These examples validate the primary relationship between system performance and image acquisition quality. It is believed that these challenges encountered during field imaging can be overcome with more advanced camera and imaging methods.

In terms of practical application, the proposed approach considerably simplifies herd management. Unlike traditional models that require expensive retraining at each birth, our system allows for instant database updates simply by adding a reference photo of the new animal, making the technology accessible via standard surveillance cameras.

Despite the findings demonstrate the robustness of the CLIP methodology with a Rank-1 accuracy of 94.28% over 301 subjects, this research presents several limits that should be acknowledged for practical implementation. The examination was conducted post hoc on static pictures. This method models visual fluctuation but fails to replicate the complexities of real-time deployment on a continuous video stream, where optimal frame selection is essential. Secondly, while the model demonstrates resilience to partial occlusions and moderate motion blur, its efficacy under extreme environmental conditions—such as complete obscuration of the coat pattern by excessive mud or near-total darkness—has not been assessed and may necessitate supplementary infrared sensors. Ultimately, an ethical and operational analysis reveals a residual error rate of around 5%. Consequently, this system need to be regarded as a management tool (for instance, for observing behavior or feeding) rather than an unequivocal authority for irrevocable choices (such as slaughter or the implementation of medical treatments). Human validation is advised for these essential procedures to provide comprehensive security.

## DECLARATIONS

**Availability of Data and Materials:** The data supporting this study's findings are available from the corresponding author upon reasonable request. Also the pictures of the 301 individual cattle was published on <https://datasetninja.com/cows2021#download>

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## RESEARCH ARTICLE

# Polymorphism Analysis of Milk Yield Candidate Genes in Turkish Native Goats: Honamlı and Hair Breeds

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## Abstract

This study examined polymorphisms of nine genes (ADFP, AGPAT6, FOLR1, LHX3, PITX1, PITX2, PRLR, PROP1, and STAT5A) in Honamlı and Hair goats and compared allele and genotype frequencies with those reported in other breeds. A total of 200 goats (100 Honamlı, 100 Hair) were genotyped using PCR-RFLP. Allele and genotype frequencies were calculated, and deviations from Hardy-Weinberg equilibrium (HWE) were tested. Polymorphisms were identified in ADFP, AGPAT6, FOLR1, LHX3-1, LHX3-3, PITX1, PITX2-1, PRLR, and PROP1, whereas LHX3-2, PITX2-2, and STAT5A were monomorphic. ADFP showed C-allele/CC predominance with significant HWE deviation in both breeds. AGPAT6 (C-allele/CC) conformed to HWE, while FOLR1 (C-allele/CC) deviated only in pooled data. LHX3-1 (A-allele) was in HWE, whereas LHX3-3 (C-allele) deviated in both breeds. PITX1 (G-allele) was in HWE, while PITX2-1 (T-allele) was in HWE in Honamlı but deviated in Hair and pooled populations. PRLR (T-allele/TT) was in HWE, while PROP1 (C-allele/CC) deviated in Hair and pooled groups. STAT5A was fixed for CC. Turkish goats reveals allele predominance patterns and HWE deviations, suggesting effects of selection and population structure. These results enrich global goat genetic resources and support marker-assisted selection for milk traits.

**Keywords:** Candidate genes, Goat, Honamlı, Hair, Milk yield traits, Polymorphism

## INTRODUCTION

Milk trait is one of the economically important traits of goats. Due to the aspect of the quantitative traits, milk traits are determined by many genes and environmental factors <sup>[1-3]</sup>. There are many genes affecting milk traits in goats. Genetic polymorphisms occurring in genes determining milk traits may result in differences in milk traits of individuals within the population <sup>[2,4]</sup>. Depending on the variations, significant differences may occur in the milk phenotypes such as milk yield, milk fat, and milk <sup>[2,4-9]</sup>.

Adipose differentiation-related protein (ADFP also known as PLIN2) is a member of the perilipin protein family and is involved in the transport and storage of neutral lipids in multiple cell types <sup>[10]</sup>. This protein family regulates body fat distribution and is found on the surface of lipid droplets in different tissues <sup>[11]</sup>. Several single nucleotide polymorphisms (SNP) were reported in the ADFP gene related to milk fat and total solid in Chinese dairy goats <sup>[10]</sup>. 1-acylglycerol-3-phosphate O-acyltransferase 6 (AGPAT6)

is an important gene family that plays a role in the synthesis pathway of triglycerides in different cell types. AGPAT6 is one of the important genes in this family that plays a role in milk fat production in the mammary gland <sup>[12]</sup>. A SNP in the exon 4 of the AGPAT6 gene, 9263C>G, is associated with milk yield, milk protein, milk lactose, and milk fat revealing a favorable G allele in dairy goats <sup>[13,14]</sup>. The folate receptors (FOLR) bind folic acids with high affinity and consists of four members (FOLR1, FOLR2, FOLR3, FOLR4) <sup>[15,16]</sup>, is an important regulator of folate metabolism for milk protein synthesis in the mammary gland. A potential SNP, 7884A>C, in exon 3 of the FOLR1 gene was reported in dairy Xinong Saanen and Guanzhong goat breeds <sup>[4]</sup>. The LHX3 (LIM homebox3) gene, a member of the LIM-homedomain gene family, plays a role in pituitary gland and nervous system development <sup>[8]</sup>. This gene affects milk traits by regulating the expression of genes related to milk production <sup>[17-19]</sup>. Several SNP in the intron2, exon 3, and exon 6 related to milk yield were reported in dairy Xinong Saanen goat.



Pituitary homeobox 1 (PITX1) and PITX2 are involved in several developmental pathways such as cell proliferation, differentiation, hematopoiesis, and organogenesis [9,20] and regulate milk traits in goats [7,9,21]. A novel 201G>A transition in the intron 1 of the PITX1 gene affected milk fat, protein, and milk yield in Guanzhong dairy goat [7]. Similar to PITX1 polymorphism, a novel SNP affecting milk yield, milk fat, and milk lactose was reported in Chinese dairy goats [9]. Prolactin is one of the most important genes that regulate milk traits [4]. Through its receptor, prolactin receptor (PRLR) which belongs to the growth hormone receptor family, this hormone promotes the development of the mammary glands and stimulates it to produce milk, lactose, fat, and proteins [22]. A C>T transition in the 3' UTR of the PRLR gene is associated with average milk yield in dairy goats [4]. Prophet of PIT-1 (PROP1) is expressed in the pituitary gland. It also controls the expression of growth hormone, prolactin, and thyroid-stimulating hormone subunits [2]. Alterations in the PROP1 gene can influence the expression of the POU1F1 gene [23]. Consequently, the PROP1 gene emerges as a promising candidate gene in farm animals. Studies indicate that PROP1 affects milk traits [2,24] and growth traits [25,26] in goats. In Malabadi goat, a SNP in exon 2 of the PROP1 is associated with yield, milk fat, and milk protein gene [2]. Signal transducers and activators of transcription (STATs), a family of transcription factors, regulate the effects of various hormones and cytokines. STAT5 can also be described as mammary gland factor [27]. STAT5A, one of the components of the STAT5 protein, is an important mediator of prolactin signaling and activates the transcription of milk protein genes in response to prolactin. Polymorphism in the STAT5A gene is associated with milk yield and milk fat in goats [5] and cattle [28]. Accumulative findings in the goat show that polymorphisms in these genes affect many milk traits such as milk yield, milk fat, milk protein, and lactose content [2,5,7-10,13,14,22,24].

Türkiye is one of the leading countries in Europe with its goat population. Novel data indicates that Türkiye has 10.8 million head goats from different breeds [29]. And, this number is higher than goat population of the all EU countries [30]. Hair, Honamlı, Angora, Kilis, and Norduz goats are the native goat breeds of Türkiye. However, the predominant goat breed is the Hair goat and extensively bred throughout the Anatolian region. Hair goat is a multipurpose breed and is raised for its meat, milk, and hair [31]. On the other hand, Honamlı goat is raised in a region comprise Burdur, Antalya, and Isparta provinces. Similar to the Hair goat, the Honamlı goat is raised for meat, milk, and hair [31,32]. While numerous studies have examined genetic regions potentially linked to economically important traits in Turkish goat breeds,

research on this subject concerning Honamlı and Hair goat breeds is limited [33-35].

Considering this background, this study aimed to investigate the presence and distribution of polymorphisms in ADFP, AGPAT6, FOLR1, LHX3, PITX1, PITX2, PRLR, PROP1, and STAT5A genes, which can be used as genetic markers in further selection programs in Honamlı and Hair goats, two important native Turkish breeds.

## MATERIAL AND METHODS

### Ethical Statement

All experimental procedures were approved by the Burdur Mehmet Akif Ersoy University Animal Experiments Local Ethics Committee (Approval No: 713, dated 13.01.2021). Hair goats (n=100) and Honamlı goats (n=100) were used as animal materials in the study. All procedures were conducted in accordance with Directive 2010/63/EU on the protection of animals used for scientific purposes.

### Animal Material and Sampling

In the study, a total of 200 goats including Turkish Hair goats (n=100) and Honamlı goats (n=100) were used as animal material. Blood samples from the goats collected aseptically from the jugular vein were placed into 10 mL K<sub>3</sub>-EDTA tubes for DNA isolation.

### DNA Isolation and DNA Quality Controls

Genomic DNA isolation from the collected blood samples was performed using a kit (GeneJET Genomic DNA Purification Kit) according to the instructions of the company. The purify and quantity of genomic DNA were evaluated according to the optical density values at 260/280 nm wavelength in Nanodrop 2000 (Thermo Scientific). The integrity of genomic DNA stained with ethidium bromide was evaluated on the 0.8% agarose gel under UV transilluminator.

### PCR-RFLP

Table 1 shows primer pairs used for amplification of relevant gene regions and PCR conditions. PCR reaction was performed in 25 µL total volume with optimized concentration of 1X buffer, MgCl<sub>2</sub>, dNTP, primers, and Taq DNA polymerase. Annealing temperature was optimized for PCR amplification of each gene region (Table 1). Following PCR amplification, PCR products were evaluated on the 2% agarose gel under UV transilluminator.

PCR products of relevant genes were digested with restriction enzymes represented in Table 1 according to the manufacturers' instructions (Thermo Scientific™). Following RFLP reaction, digested PCR products were visualized on the 3-4% agarose gel and evaluated for detection of genotypes.

**Table 1.** Primer pairs used, PCR product sizes, and restriction patterns of genes in the study

| Gene         | Primers (5'→3')                                              | PCR Product Size (bp) | Enzyme         | Restriction Pattern                                 | Reference                      |
|--------------|--------------------------------------------------------------|-----------------------|----------------|-----------------------------------------------------|--------------------------------|
| ADFP         | F: GTTTCCAATCGACCCAGACG<br>R: TACACAAAACAGCCTCACTGG          | 224                   | <i>Hpy188I</i> | TT: 165+59<br>TC: 165+140+59+25<br>CC: 140+59+25    | [7]                            |
| AGPAT6       | F: ATCTGGCATTTCACACATT<br>R: CTGACTCCATCTAAGAGCCT            | 241                   | <i>NcoI</i>    | CC: 241<br>CG: 241+130+111<br>GG: 130+111           | [13]                           |
| FOLR1        | F: GTCCCTCCACCTGATGTT<br>R: CCTCCTCAGACCAGAATT               | 414                   | <i>SfaNI</i>   | AA: 414<br>AC: 414+229+185<br>CC: 229+185           | [4]                            |
| LHX3 (1-2-3) | F: GCGTAACCAAGTCTGGAGCC<br>R: R:CAACACGAAGAGAGAGCACG         | 205                   | <i>DraI</i>    | TT: 205<br>AT: 205+114+91<br>AA: 114+91             | Primers Designed in this study |
|              | F: CTGCCCTTCCGAATTACCTC<br>R: ATGAGATAGAACTCGTCGCC           | 381                   | <i>HinfI</i>   | CC: 381<br>CT: 381+285+96<br>TT: 285+96             |                                |
|              | F: CCCTTCCTATCCAGCTTGGTG<br>R: GGTCCACCTCGTCCAACCAG          | 187                   | <i>MspI</i>    | TT: 153+34<br>TC: 153+123+64+34<br>CC: 123+64       |                                |
| PITX1        | F: CCGCCTTCCACCTAGCCGCAC<br>R: GCGTCCATGTCCACGTCATCG         | 230                   | <i>MspI</i>    | AA: 230<br>GA: 230+121+109<br>GG: 121+109           | [7]                            |
| PITX2        | F: TCGTCCATGAAGTGCATGAAAGGC<br>R: AAAGGAAGGGAGGTCAGGGTCGTAAT | 432                   | <i>RsaI</i>    | TT: 432<br>TC: 432+319+113<br>CC: 319+113           | [9]                            |
|              | F: TGGCCATGGCTTCGGTCTGGCTCC<br>R: AAAGGAAGGGAGGTCAGGGTCGTAAT | 326                   | <i>SmaI</i>    | CC: 301+25<br>CG: 326+301+25<br>GG: 326             |                                |
| PRLR         | F: AGTGAGAGTTATGGAAGGATG<br>R: AAGGTTAAGCAACTGGTCTT          | 443                   | <i>RsaI</i>    | TT: 443<br>CT: 443+383+60<br>CC: 383+60             | [7]                            |
| PROP1        | F: GATGGATGGATGGGTCTCTG<br>R: TGGTGAAGGTTTGGGTTGG            | 403                   | <i>Hin6I</i>   | CC: 223+152+28<br>CT: 251+223+152+28<br>TT: 251+152 | [2]                            |
| STAT5A       | F: CTGCAGGGTGTCTCTGAGAG<br>R: TGGTACCAGCACTGTTAGCACAT        | 215                   | <i>Eco8II</i>  | CC: 162+53<br>CT: 162+126+53+36<br>TT: 126+53+36    | [5]                            |

**ADFP:** Adipose Differentiation-Related Protein, **AGPAT6:** 1-Acylglycerol-3-Phosphate O-Acyltransferase 6, **FOLR1:** Folate Receptor 1, **LHX3 (1-2-3):** LIM Homeobox 3, **PITX1:** P Pituitary homeobox 1, **PITX2:** Pituitary homeobox 2, **PRLR:** Prolactin Receptor, **PROP1:** Prophet of PIT1, **STAT5A:** Signal Transducer and Activator of Transcription 5A

## Statistical Analyses

For each gene, allele and genotype were calculated frequencies using PopGene32 [36]. The chi-square test ( $\chi^2$ ) was applied to determine whether the populations conformed to Hardy-Weinberg equilibrium.

## RESULTS

### Allelic and Genotypic Distributions

In this study, polymorphisms in ADFP, AGPAT6, FOLR1, LHX3, PITX1, PITX2, PRLR, PROP1, and STAT5A genes were investigated in Honamlı and Hair goat breeds using PCR-RFLP. A total of 200 goats (100 Honamlı and 100 Hair goats) were genotyped, and allelic as well as genotypic distributions were determined (Table 2). Successful

amplification of target gene regions was confirmed by agarose gel electrophoresis.

For the ADFP gene (*Hpy188I*), three genotypes (TT, TC, CC) and two alleles (T and C) were identified in both Hair and Honamlı goats. Similarly, the AGPAT6 gene (*NcoI*) revealed three genotypes (CC, CG, GG) and two alleles (C and G) in both breeds. In the FOLR1 gene (*SfaNI*), AA, AC, and CC genotypes were detected with two alleles (A and C) (Fig. 1). The LHX3-1 (*DraI*) locus showed polymorphism with TT, AT, and AA genotypes, while the LHX3-3 (*MspI*) locus exhibited TT, TC, and CC genotypes (Fig. 1). However, the LHX3-2 (*HinfI*) locus was monomorphic in both breeds.

For the PITX1 gene (*MspI*), three genotypes (AA, GA, GG) and two alleles (A and G) were observed, whereas the PITX2-1 gene (*RsaI*) revealed TT, TC, and CC genotypes

**Table 2.** Allele and genotype frequencies of candidate genes in Hair and Honamlı goat

| Gene    | Breed   | n   | Allele Frequency | Observed Genotypes    | Ho   | He   | $\chi^2$ | p     |
|---------|---------|-----|------------------|-----------------------|------|------|----------|-------|
| ADFP    | Honamlı | 100 | T=0.16 C=0.84    | TT=7 TC=19<br>CC=74   | 0.19 | 0.28 | 10.12    | 0.01* |
|         | Hair    | 100 | T=0.21 C=0.79    | TT=8 TC=27<br>CC=65   | 0.27 | 0.34 | 4.25     | 0.04* |
|         | Total   | 200 | T=0.19 C=0.81    | TT=15 TC=46<br>CC=139 | 0.23 | 0.31 | 13.11    | 0.01* |
| AGPAT6  | Honamlı | 100 | C=0.88<br>G=0.12 | CC=78 CG=19<br>GG=3   | 0.19 | 0.22 | 1.91     | 0.17  |
|         | Hair    | 100 | C=0.82<br>G=0.18 | CC=67 CG=30<br>GG=3   | 0.3  | 0.29 | 0.01     | 0.90  |
|         | Total   | 200 | C=0.85<br>G=0.15 | CC=145<br>CG=49 GG=6  | 0.24 | 0.26 | 0.6      | 0.44  |
| FOLR1   | Honamlı | 100 | A=0.16<br>C=0.84 | AA=5 AC=22<br>CC=73   | 0.22 | 0.27 | 3.54     | 0.06  |
|         | Hair    | 100 | A=0.11<br>C=0.89 | AA=3 AC=16<br>CC=81   | 0.16 | 0.2  | 3.64     | 0.06  |
|         | Total   | 200 | A=0.14<br>C=0.86 | AA=8 AC=38<br>CC=154  | 0.19 | 0.23 | 7.22     | 0.01* |
| LHX3-1  | Honamlı | 100 | T=0.35 A=0.65    | TT=13 AT=44<br>AA=43  | 0.44 | 0.46 | 0.14     | 0.70  |
|         | Hair    | 100 | T=0.39 A=0.61    | TT=19 AT=40<br>AA=41  | 0.4  | 0.48 | 2.7      | 0.10  |
|         | Total   | 200 | T=0.37 A=0.63    | TT=32 AT=84<br>AA=84  | 0.42 | 0.47 | 2.06     | 0.15  |
| LHX3-3  | Honamlı | 100 | T=0.20 C=0.80    | TT=10 TC=21<br>CC=69  | 0.21 | 0.33 | 13.16    | 0.01* |
|         | Hair    | 100 | T=0.28 C=0.72    | TT=13 TC=30<br>CC=57  | 0.3  | 0.41 | 6.84     | 0.01* |
|         | Total   | 200 | T=0.24 C=0.76    | TT=23 TC=51<br>CC=126 | 0.25 | 0.37 | 19.09    | 0.01* |
| PITX1   | Honamlı | 100 | A=0.11<br>G=0.89 | AA=2 GA=18<br>GG=80   | 0.18 | 0.2  | 0.76     | 0.38  |
|         | Hair    | 100 | A=0.22<br>G=0.79 | AA=5 GA=33<br>GG=62   | 0.33 | 0.34 | 0.75     | 0.78  |
|         | Total   | 200 | A=0.16<br>G=0.84 | AA=7 GA=51<br>GG=142  | 0.25 | 0.27 | 0.87     | 0.35  |
| PITX2-1 | Honamlı | 100 | T=0.69 C=0.31    | TT=51 TC=36<br>CC=13  | 0.36 | 0.43 | 2.68     | 0.10  |
|         | Hair    | 100 | T=0.66 C=0.34    | TT=51 TC=31<br>CC=18  | 0.31 | 0.45 | 9.58     | 0.01* |
|         | Total   | 200 | T=0.68 C=0.32    | TT=102 TC=67<br>CC=31 | 0.33 | 0.44 | 11.15    | 0.01* |
| PRLR    | Honamlı | 100 | C=0.08 T=0.92    | CC=1 CT=14<br>TT=85   | 0.14 | 0.15 | 0.31     | 0.58  |
|         | Hair    | 100 | C=0.16 T=0.84    | CC=4 CT=24<br>TT=72   | 0.24 | 0.27 | 1.28     | 0.26  |
|         | Total   | 200 | C=0.12 T=0.88    | CC=5 CT=38<br>TT=157  | 0.19 | 0.21 | 2.14     | 0.14  |
| PROPI   | Honamlı | 100 | C=0.92 T=0.08    | CC=85 CT=14<br>TT=1   | 0.14 | 0.15 | 0.31     | 0.58  |
|         | Hair    | 100 | C=0.83 T=0.17    | CC=71 CT=23<br>TT=6   | 0.23 | 0.29 | 4.41     | 0.04* |
|         | Total   | 200 | C=0.87 T=0.13    | CC=156 CT=37<br>TT=7  | 0.18 | 0.22 | 5.92     | 0.01* |

**ADFP:** Adipose Differentiation-Related Protein, **AGPAT6:** 1-Acylglycerol-3-Phosphate O-Acyltransferase 6, **FOLR1:** Folate Receptor 1, **LHX3 (1-2-3):** LIM Homeobox 3, **PITX1:** P Pituitary homeobox 1, **PITX2:** Pituitary homeobox 2, **PRLR:** Prolactin Receptor, **PROPI:** Prophet of PIT1, **STAT5A:** Signal Transducer and Activator of Transcription 5A

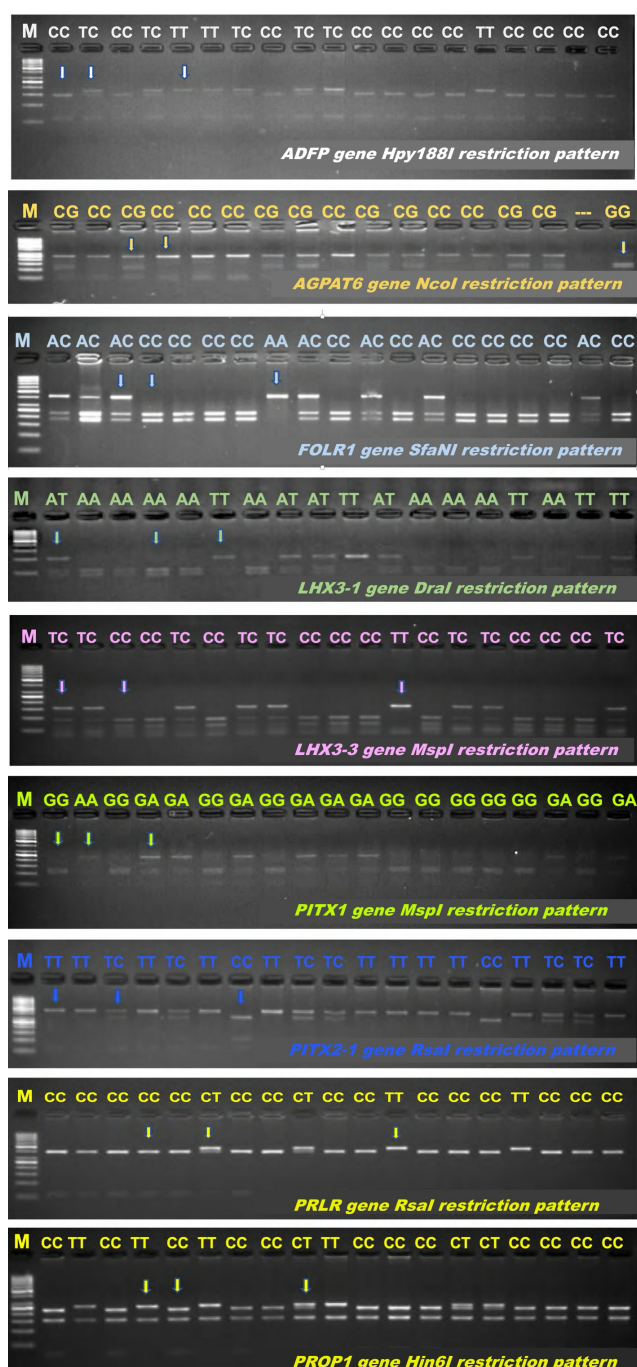


Fig 1. Restriction pattern of ADFP, AGPAT6, FOLR1, LHX3, PITX1, PITX2, PRLR and PROP1 genes. M: 100 bp DNA ladder

with T and C alleles (Fig. 1). The PITX2-2 (*SmaI*) locus was found to be monomorphic. In the PRLR gene (*RsaI*), CC, CT, and TT genotypes were identified, with C and T alleles segregating in both breeds. For the PROP1 gene (*Hin6I*), CC, CT, and TT genotypes were also observed (Fig. 1). Finally, the STAT5A gene (*Eco81I*) was found to be monomorphic in Honamlı and Hair goats.

Allele and genotype frequencies of each locus were also tested for Hardy-Weinberg equilibrium (HWE). While most loci were consistent with HWE, deviations were

observed in some gene loci, suggesting selective pressures or breed-specific effects (Table 2).

## DISCUSSION

In this study, TT, TC, and CC genotypes of the ADFP gene were identified in the 5'UTR region of both Honamlı and Hair goat breeds. The  $\chi^2$  test revealed a significant deviation from Hardy-Weinberg equilibrium ( $P < 0.05$ ), suggesting that selection, genetic drift, or population substructure might be influencing this locus. In contrast, polymorphisms at the same SNP locus were reported in Chinese dairy goats [10]. At the *Hpy188I* site in Xinong Saanen goats, TT, TC, and CC genotypes occurred at frequencies of 0.40, 0.54, and 0.06, with allele frequencies of T 0.67 and C 0.33. In Guanzhong goats, the respective frequencies were 0.32, 0.55, and 0.13 for genotypes, and 0.60 (T) and 0.40 (C) for alleles. For Xinjiang White Cashmere goats, TT and TC genotypes were observed at 0.45 and 0.55, while allele frequencies were 0.22 (T) and 0.78 (C). These findings showed that the T allele was more common in Xinong Saanen and Guanzhong breeds, whereas the C allele was dominant in Xinjiang White Cashmere goats. In contrast, our results demonstrated that the C allele predominates in both Turkish breeds, highlighting breed-specific differences. Although milk characteristics were not examined here, TT was associated with higher milk fat and CC with higher total solids in Xinong Saanen goats [10]. Thus, the dominance of the C allele and CC genotype in Honamlı and Hair goats may have implications for milk composition, which requires further study.

For the AGPAT6 gene, CC, CG, and GG genotypes were observed in both breeds, with the C allele and CC genotype being predominant. Genotype frequencies of 0.694 (CC), 0.198 (CG) and 0.108 (GG) and allele frequencies of 0.793 (C) and 0.207 (G) were reported at the *NcoI* site in Xinong Saanen goats [13]. In Guanzhong goats, frequencies were 0.623, 0.246, and 0.132 for CC, CG, and GG, with allele frequencies of 0.746 (C) and 0.254 (G). Both studies confirmed C allele predominance. In contrast, higher G allele frequencies were reported in the Saanen and Alpine breeds (C 0.36, G 0.64), and similar trends were found in the Saanen goats (C 0.336, G 0.664) (14,34). Our findings are consistent with a previous study supporting the dominance of the C allele in Turkish goats [37]. This study also indicated that GG and CG genotypes were associated with higher milk yield and quality compared to CC. Therefore, the dominance of CC in Honamlı and Hair goats could potentially indicate breed-specific limitations and warrant further association studies [13].

Regarding the FOLR1 gene, Honamlı goats showed genotype frequencies of 0.05 (AA), 0.22 (AC), and 0.73 (CC), while Hair goats showed 0.03, 0.16, and 0.81, respectively. Allele

frequencies were 0.16 (A) and 0.84 (C) in Honamlı, and 0.11 (A) and 0.89 (C) in Hair goats. Pooled data gave allele frequencies of 0.14 (A) and 0.86 (C). A monomorphic exon 3 pattern was reported in Xinong Saanen and Guanzhong goats, while polymorphism was seen in Boer goats with genotype frequencies of 0.07 (AA), 0.63 (AC) and 0.30 (CC) and allele frequencies of 0.38 (A) and 0.62 (C) [4]. While both studies confirm C allele predominance, our populations showed much lower A allele frequencies compared to Boer goats. These differences suggest breed-specific variation in FOLR1 polymorphisms.

For the LHX3 gene, at the LHX3-1 locus, Honamlı goats had genotype frequencies of 0.43 (AA), 0.44 (AT), and 0.13 (TT), while Hair goats showed 0.41, 0.40, and 0.19, respectively. The A allele was predominant in both breeds (0.65 and 0.61). Combined analysis yielded allele frequencies of 0.63 (A) and 0.37 (T), consistent with HWE. Similar results were reported in Guanzhong, Xinong Saanen, and Inner Mongolia White Cashmere goats, although some populations deviated from the HWE [8]. Additionally, A allele dominance was observed in Shami goats [38]. Thus, our results are in line with previous studies. However, at the LHX3-2 locus, our data showed monomorphism, unlike the reported balanced C and T alleles in Chinese goats [8]. At the LHX3-3 locus, C allele predominance was observed (0.80 in Honamlı, 0.72 in Hair), with significant HWE deviations in both breeds ( $P < 0.05$ ). More balanced allele frequencies were reported in Chinese goats [8], suggesting the existence of different selective or demographic forces shaping the diversity in Turkish breeds.

For the PITX1 gene, the G allele (0.77) was reported to be dominant in Guanzhong goats [7]. Similarly, Honamlı and Hair goats in our study showed high G allele frequencies (0.89 and 0.79). The genotype distributions were in HWE, consistent with Guanzhong goats. Since AG and GG genotypes were previously linked with improved milk traits [7], the high G frequency in Turkish breeds may also indicate favorable milk-related traits.

At the PITX2-1 locus, our study revealed TT genotype and T allele predominance (0.69 in Honamlı, 0.66 in Hair), with TT being the most common genotype. This contradicts the study [9] reporting near fixation of the C allele in Guanzhong goats, but is closer to the findings of higher T allele frequencies in Hainan black goats [39]. While Honamlı goats conformed to HWE, Hair goats and pooled data deviated significantly, suggesting possible selective effects. Both Turkish breeds were monomorphic (GG) at the PITX2-2 locus, which was consistent with other studies, indicating conservation of this locus [7,39].

For the PRLR gene, Honamlı and Hair goats showed predominance of the TT genotype and T allele with frequencies of 0.92 and 0.84, respectively. In contrast,

the A allele is dominant in Chinese breed and significant deviations from HWE have been reported [4]. Our data, however, did not deviate from HWE, suggesting different evolutionary pressures in Turkish goats.

For the PROP1 gene, Honamlı and Hair goats displayed high C allele frequencies (0.92 and 0.83), consistent with previous studies [2,7,24]. However, unlike earlier reports, TT genotypes were detected at low frequency in our populations, especially in Hair goats. While Honamlı goats were in HWE, Hair goats showed a slight deviation, suggesting possible breed-specific variation.

Finally, for the STAT5A gene, both Honamlı and Hair goats were monomorphic (CC), while earlier studies in Guanzhong [5] and Garganica [40] goats reported presence of heterozygotes. This suggests reduced variability and possible fixation of the C allele in Turkish breeds.

Within the scope of the existing literature, this study appears to be one of the early examples focusing on polymorphisms in the ADFP, AGPAT6, FOLR1, LHX3, PITX1, PITX2, PRLR, PROP1, and STAT5A genes in Honamlı and Hair goats, two important native genetic resources of Türkiye. The results revealed both polymorphic and monomorphic patterns across different loci, with clear breed-specific differences in allelic and genotypic distributions. Notably, loci such as ADFP, AGPAT6, FOLR1, LHX3, PITX1, PITX2-1, PRLR and PROP1 exhibited substantial variation, while LHX3-2 and STAT5A were found to be monomorphic, suggesting possible fixation due to historical selection or genetic drift. In addition, certain loci such as ADFP, LHX3, and PITX1 showed relatively higher heterozygosity values, indicating their potential as informative genetic markers for milk production traits in Turkish goat breeds. Overall, our findings provide novel insights into the genetic diversity of Turkish native goats and offer a potential basis for ADFP, AGPAT6, FOLR1, LHX3, PITX1, PITX2-1, PRLR, PROP1 and LHX3 gene polymorphism studies aimed at improving milk yield and composition traits in these native breeds. However, since our study did not directly assess production traits, further research integrating polymorphism data with phenotypic performance records will be essential to clarify these associations and harness the genetic potential of Honamlı and Hair goats for sustainable breeding and conservation strategies.

## DECLARATIONS

**Availability of Data and Materials:** Data and Materials are available from the corresponding author (ÖKA).

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**Ethical Statement:** All experimental procedures were approved by the Burdur Mehmet Akif Ersoy University Animal Experiments Local Ethics Committee (Approval No: 713, dated 13.01.2021).

**Competing Interests:** The authors declare that there is no conflict of interest regarding the publication of this paper.

**Declaration of Generative Artificial Intelligence (AI):** The article and tables and figures were not written/created by AI and AI assisted technologies.

**Author Contributions:** ÖKA: designed the study, project administration, supervision, performed molecular analyses, wrote the original draft, conducted the formal analysis, reviewed and edited the paper. AOT: performed molecular analyses, wrote the original draft. ZU: partially wrote the original draft and performed molecular analyses, reviewed the original draft. All authors read and approved the final manuscript.

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RESEARCH ARTICLE

# The Impact of BVDV Infection on the Immune Efficacy of *Brucella abortus* A19 Vaccine

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## Abstract

As *Bovine Viral Diarrhea Virus* (BVDV) is widespread in cattle and often causes immunosuppression, it is essential to evaluate whether it interferes with the effectiveness of widely used live vaccines such as *Brucella abortus* A19. This study evaluated the impact of BVDV infection on the immunoprotective efficacy of the *B. abortus* A19 vaccine using co-infection models in mice and RAW264.7 macrophages. The animal experiments revealed that co-infection led to significantly higher splenic bacterial loads of *B. abortus* A19 on days 14, 42, and 56 post-infection (pi), along with an increased spleen index on days 14, 28, 42, and 56 pi, compared to *B. abortus* A19 single-infection group. Results from the cell model demonstrated that co-infection enhanced intracellular survival of *B. abortus* A19 and significantly upregulated IL-10 mRNA and protein levels while suppressing TNF- $\alpha$  mRNA. In contrast, *B. abortus* A19 single-infection group induced higher expression of IL-12 and IFN- $\gamma$  compared to BVDV single-infection group or co-infected groups. In conclusion, BVDV infection compromises the protective efficacy of the *B. abortus* A19 vaccine by enhancing the intracellular survival of *B. abortus* A19 in macrophages and dysregulating the expression of immune-related cytokines. These findings highlight the importance of BVDV control in vaccination programs and provide new insights into the mechanisms underlying vaccine failure under co-infection conditions.

**Keywords:** Coinfection, Cytokines, Immunity, Intracellular survival dynamics

## INTRODUCTION

Bovine viral diarrhea mucosal disease (BVD-MD) is an infectious disease that affects the global distribution of cattle, caused by BVDV, which is a single stranded RNA virus. In China, BVDV was first isolated from aborted bovine fetuses in 1980 [1]. So far, three different BVDV genotypes have been identified, namely BVDV1, BVDV2, and BVDV3. According to the phenotypic characteristics in cell culture, BVDV strains within each genotype are further classified into two biotypes: cytopathic (CP) and noncytopathic (NCP) [2]. Cattle are the natural host of BVDV, and infections typically manifest as clinical symptoms including fever, diarrhea, miscarriage, stillbirth, and mummified fetuses [3]. NCP type BVDV can cause persistent infection (PI) in cattle through fetal infection during early pregnancy [4]. Although PI cows do not have obvious clinical symptoms, they exhibit a range of adverse features, including impaired growth performance, reduced milk production, decreased survival rates, increased susceptibility to other pathogens, and persistent viral shedding, leading to the continuous circulation and

transmission of BVDV in the herd, causing huge economic losses. [5-7].

*Brucella* is a globally prevalent zoonotic infectious disease caused by bacteria of the *Brucella* genus [8]. *Brucella* has a wide range of hosts, including ungulates, carnivores, rodents, primates, and marine mammals. Infection with this pathogen can induce a series of pathological conditions in the affected host, such as miscarriage, placental inflammation, orchitis, and epididymitis [9]. Currently, vaccination is considered the most cost-effective and effective strategy for preventing and controlling brucellosis [10]. In China, seven *Brucella* vaccines have been officially approved for commercial use, classified according to their bacterial sources: sheep *Brucella* derivative vaccines (strains M5/M5-90, M5-90  $\Delta$  26, Rev. 1, and BA0711), pig *Brucella* derivative vaccines (strain S2), and *Brucella abortus* derivative vaccines (strains A19 and A19  $\Delta$  VirB12) [11]. Among these approved vaccines, *B. abortus* A19 strains are the most widely used in China's livestock industry. Their application can effectively reduce the miscarriage rate of infected animals, thereby alleviating the economic losses of livestock farmers [12].



The success of vaccine immunization largely depends on the host's robust immune system, which is the physiological basis for producing specific antibodies and cellular immune responses. Acute BVDV infection can lead to depletion of lymphocytes in lymphoid organs such as thymus, spleen, and lymph nodes, resulting in immune dysfunction [13,14]. Wang Chuanfeng et al. [15] found that the swine fever vaccine contaminated with BVDV leads to immune failure against swine fever, because BVDV stimulates the production of large amounts of BVDV antibodies in vaccinated pigs while also inhibiting the production of swine fever antibodies. In addition, increasing evidence suggests that BVDV exerts immunomodulatory effects by disrupting immune cells and promoting their migration to tissues where viral replication occurs. These processes lead to a decrease in lymphocytes and neutrophils, ultimately making infected cows more susceptible to secondary infections from other pathogens [16,17]. Therefore, if cows are infected with BVDV before vaccination with the *B. abortus* A19 vaccine, there is a high probability that the immune protection efficiency of the A19 attenuated vaccine will be reduced.

So far, there is limited literature on the co-infection of BVDV and the *B. abortus* A19 vaccine, and information on the interaction between BVDV infection and *B. abortus* A19 vaccination is still limited. This study established a C57BL/6J mouse model co-infected with BVDV and *B. abortus* A19 (BVDV+A19 co-infected). Subsequently, systematic analysis and comparison were conducted on laboratory test data from three experimental groups, namely BVDV single-infection group, *B. abortus* A19 single-infection group, and BVDV+A19 co-infection group. These results provide key experimental evidence for the observed immune failure of BVDV epidemic bovine *Brucella* vaccine in clinical practice.

## MATERIAL AND METHODS

### Ethical Statement

This study was approved by the Biology Ethics Committee of Shihezi University (Approval no: A2025-1073, Date: October 2025).

### Bacterial Strains, BVDV Strain, Cells, Animals, and Culture Conditions

The *B. abortus* A19 vaccine strain was from Xinjiang Center for Disease Control and Prevention. Strains stored at -80°C were inoculated onto BBL™ *Brucella* Agar (BD, Shanghai, China) and incubated at 37°C for 72 h. Single colonies were isolated and propagated in BBL™ *Brucella* Broth (BD, Shanghai, China) at 37°C for 24 h. Bacterial cultures were harvested for subsequent experimental procedures. The CP-type BVDV strain was generously provided by Professor Qiang Fu from Xinjiang Agricultural University.

RAW264.7 cells (maintained in our laboratory) were cultured in DMEM medium supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells in the logarithmic growth phase were seeded into 6-well plates (NEST Biotechnology, Jiangsu, China) at a density of 1×10<sup>6</sup> cells/well and allowed to adhere for 24 h prior to experimentation. Six-week-old female C57BL/6J mice were obtained from Henan Skebes Biotechnology Co., Ltd. and housed in the Animal Hospital.

### Establishment of a Mouse Model of Coinfection

A total of 100 six-week-old mice were randomly divided into four groups, with 25 mice in each group. Group 1 served as the control group, Group 2 as the BVDV single-infection group, Group 3 as the *B. abortus* A19 single-infection group, and Group 4 as the BVDV+A19 co-infected group. The four groups were intraperitoneally injected with 600 µL of 1640 medium, BVDV, 1640 medium, and BVDV, respectively, in sequence. After 14 days of feeding, the four groups were intraperitoneally injected with 200 µL of 1640 medium, 1640 medium, *B. abortus* A19 vaccine strain, and *B. abortus* A19 vaccine strain, respectively, the colony-forming unit (CFU) of the infected *B. abortus* A19 was 5×10<sup>5</sup> CFU/mL. After another 56 days of feeding, each group was intraperitoneally injected with 200 µL of 1640 medium, 1640 medium, *B. abortus* A19 vaccine strain, and *B. abortus* A19 vaccine strain, respectively, with the CFU of the infected *B. abortus* A19 being 1.5×10<sup>6</sup> CFU/mL. Mice in the four groups were reared in isolation in separate rooms with a clean environment, under completely identical feeding conditions, and provided with sufficient rodent food and water. The infection procedure of the mice has been shown (Fig. 1).

### Determination of Spleen Index and Splenic Bacterial Load in Mice

A mouse model of BVDV+A19 co-infection was established. On days 14, 28, 42, and 56 post-modeling, mice were weighed and subjected to aseptic dissection. The spleens of the mice were then weighed, and the spleen index was calculated based on the recorded data. Subsequently, each spleen was placed into a clean EP tube supplemented with 500 µL of 1x phosphate-buffered saline (PBS, Phosphate Buffered Saline). After grinding the spleen, 10-fold serial dilutions of the homogenate



Fig 1. Process diagram for intraperitoneal injection infection of BVDV and the *B. abortus* A19 in mice

were prepared. The diluted samples were spread onto the appropriate medium, followed by incubation for 3-4 days to perform colony counting. The splenic bacterial load of each mouse was calculated using the formula: Bacterial load = Average number of colonies  $\times$  Homogenate volume  $\times$  Dilution factor/Spleen weight. At each time point, 3 mice were included in each group for detection.

### Establishment of a RAW264.7 Cell Model of Coinfection

RAW264.7 cells were thawed and seeded into 10 cm culture dishes. When the cell confluency reached 70-80%, the cells were inoculated with BVDV solution at a multiplicity of infection (MOI) of 1, followed by static adsorption for 2 h. After washing with PBS for 3 times, complete medium was added to the dishes for 24 h of culture. The cells were then isolated and equally divided into two aliquots: one aliquot was passaged into new culture dishes for continuous culture, the other aliquot was lysed with TRIzol reagent and stored at  $-80^{\circ}\text{C}$  for subsequent RNA extraction. This process was repeated until the 10<sup>th</sup> passage (P1-P10). Total RNA was extracted from cells of each passage, and absolute quantitative real-time polymerase chain reaction (qPCR) was performed to determine the BVDV copy number. The complete list of primers used in this study has been shown (Table 1).

### Detection of Cell Viability by CCK-8 Assay

RAW264.7 cells in the logarithmic growth phase were seeded into a 96-well plate (NEST Biotechnology, Jiangsu, China) at a density of 50,000 cells per well. After the cells had adhered to the plate, cell models of single infection with BVDV, single infection with the *B. abortus* A19, and co-infection with BVDV+A19 were established respectively. The constructed cell models were statically cultured for 24 h. Subsequently, 10  $\mu\text{L}$  of CCK-8 detection reagent was added to each well. After incubation for 2 h in the dark, the optical density (OD) value at a wavelength of 450 nm was measured. Cell viability was then calculated based on the measured OD values.

### Detection of Cytokine mRNA and Protein Levels in the Coinfected Cell Model

RAW264.7 cells were seeded into 6-well plates (NEST Biotechnology, Jiangsu, China). When the cell confluency reached 80%, cell models were established, including those with single infection of BVDV, single infection of the *B. abortus* A19, and co-infection of BVDV+A19. At

0 h, 12 h, and 24 h pi, total RNA was extracted from the cells, and cell supernatant samples were isolated. These samples were used for the detection of cytokine mRNA levels (from RNA) and cytokine protein levels (from cell supernatants), respectively. The complete list of primers used in quantitative real-time polymerase chain reaction (qRT-PCR) has been shown (Table 2).

### Detection of Intracellular Survival Ability of A19

RAW264.7 cells were seeded into 6-well plates (NEST Biotechnology, Jiangsu, China). When the cell confluency reached 70-80%, the cells were inoculated with BVDV solution at a MOI of 1, followed by static adsorption for 2 h. Subsequently, the cells were washed 3 times with 1xPBS, and complete medium was added for static culture for 24 h. After this period, the cells were infected with the *B. abortus* A19 strain. At 0 h and 24 h pi, the cells were lysed with 0.1% Triton X-100. The cell lysate was subjected to 10-fold serial dilutions, and the diluted samples were spread onto the appropriate medium. After incubation for 72 h, colony counting was performed, and the results were recorded.

### Statistical Analysis

All results of data analysis are presented as the mean  $\pm$  standard deviation (SD). Data analysis was performed on independent samples using the t-test. Differences were considered as significant when  $P \leq 0.05$ . Statistical analysis was performed using GraphPad Prism 9 software (GraphPad, Inc., La Jolla, CA, USA).

## RESULTS

### BVDV Infection Enhances the Survival and Proliferation Capacities of *B. abortus* A19 Vaccine Strain in Mouse Spleens

Following the successful establishment of intraperitoneal infections with the BVDV strain and the *B. abortus* A19 strain, mice in the BVDV single-infection group, the *B.*

Table 1. BVDV copy number absolute detection primers

| Primers | 5'-3'Sequence         |
|---------|-----------------------|
| 5'-UTR1 | GCTAGCCATGCCCTTAGTAGG |
| 5'-UTR2 | TCCATGTGCCATGTACAGC   |

Table 2. Complete primer list for qRT-PCR

| Primers         | 5'-3'Sequence          |
|-----------------|------------------------|
| <i>Il101</i>    | GCTCTTACTGACTGGCATGAG  |
| <i>Il102</i>    | CGCAGCTCTAGGAGCATGTG   |
| <i>Il12b1</i>   | TGGTTTGCCATCGTTTGTCTG  |
| <i>Il12b2</i>   | ACAGGTGAGGTTCACTGTTTCT |
| <i>Ifng1</i>    | ATGAACGCTACACACTGCATC  |
| <i>Ifng2</i>    | CCATCCTTTTGCCAGTTCCCTC |
| <i>Tnfa1</i>    | GACGTGGAAGTGGCAGAAGAG  |
| <i>Tnfa2</i>    | TTGGTGGTTTGTGAGTGTGAG  |
| $\beta$ -actin1 | TGCTATGTTGCTCTAGACTTCG |
| $\beta$ -actin2 | GTTGGCATAGAGGTCTTTACGG |

*abortus* A19 single-infection group, and BVDV+A19 co-infection group all exhibited clinical signs including lethargy and ruffled fur. Mice were euthanized and dissected on days 14, 28, 42, and 56 pi. Compared with the control group and BVDV single-infection group, mice in the *B. abortus* A19 single-infection group and BVDV+A19 co-infection group showed significant splenomegaly. Notably, the splenic index of mice in the BVDV+A19 co-infection group was significantly higher than that in the control group, BVDV single-infection group, and the *B. abortus* A19 single-infection group at all tested time points (on days 14, 28, 42, and 56 pi) ( $P \leq 0.01$ ) (Fig. 2). On days 14, 42, and 56 pi, the bacterial load of the *B. abortus* A19 in the spleens of mice in the co-infection group was significantly higher than that in the *B. abortus* A19 single-infection group ( $P \leq 0.05$ ) (Fig. 3). Collectively, these results demonstrate that co-infection with BVDV and the *B. abortus* A19 strain significantly exacerbates pathological damage to the mouse spleen and promotes the survival and proliferation of the *B. abortus* A19 in the spleen.

#### Establishment of a BVDV-Infected RAW264.7 Macrophage Model

Total RNA was extracted from passages 1 to 10 of RAW264.7 macrophages infected with BVDV. After reverse transcription, the copy number of BVDV was detected by qRT-PCR. the logarithmic (lg) value of BVDV

copy number in RAW264.7 cells from passage 1 to passage 6 was stably maintained between 5 and 6, showing a relatively steady level. Starting from passage 7, the lg value of BVDV copy number decreased slightly to approximately 4.25, from passage 8 to passage 10, it remained between 4 and 5, with a detectable amount of BVDV copy number. In conclusion, BVDV can stably persist in RAW264.7 cells, indicating the successful establishment of the BVDV-infected RAW264.7 macrophage model (Fig. 4).

#### BVDV and the *B. abortus* A19 Co-Infection Reduces the Viability of RAW264.7 Macrophages

To investigate the effect of BVDV infection on the viability of RAW264.7 cells, the cell viability of the blank control group, BVDV single-infection group, the *B. abortus* A19 single-infection group, and BVDV+A19 co-infection group was detected at 24 h pi using the Cell Counting Kit-8 (CCK-8) assay. Compared with the control group, both single-infection groups induced an extremely significant decrease in the viability of RAW264.7 cells ( $P < 0.001$ ). Notably, the cell viability in the BVDV+A19 co-infection group decreased more remarkably: its viability was not only extremely significantly lower than that of the control group ( $P < 0.001$ ) but also extremely significantly lower than that of either single-infection group ( $P < 0.001$ ). These results indicate that BVDV and the *B. abortus* A19 exert a synergistic effect in inhibiting cell viability (Fig. 5).

#### BVDV Infection Disrupts the Cytokine Secretion Balance in RAW264.7 Cells Induced by the *B. abortus* A19 Vaccine Strain

To investigate the effect of BVDV infection on the immune response of RAW264.7 cells induced by the *B. abortus* A19, the mRNA and protein expression levels of interleukin-10 (IL-10) and interleukin-12 (IL-12) in RAW264.7 cells were detected at 24 h pi using qRT-PCR and enzyme-linked immunosorbent assay (ELISA), respectively.

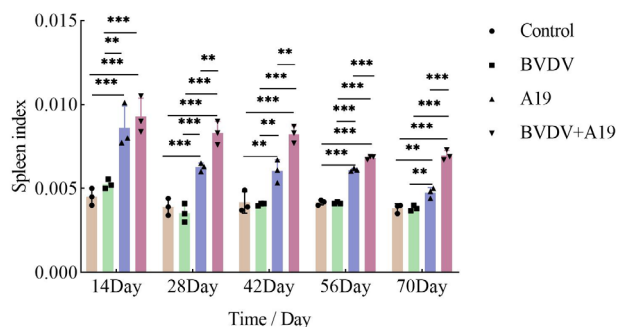


Fig 2. Calculation results of mouse spleen index

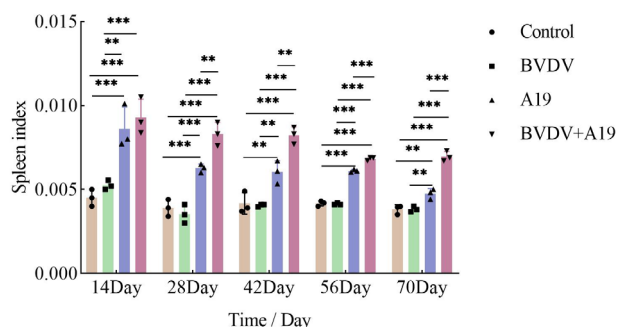


Fig 3. Determination results of bacterial load in mouse spleen

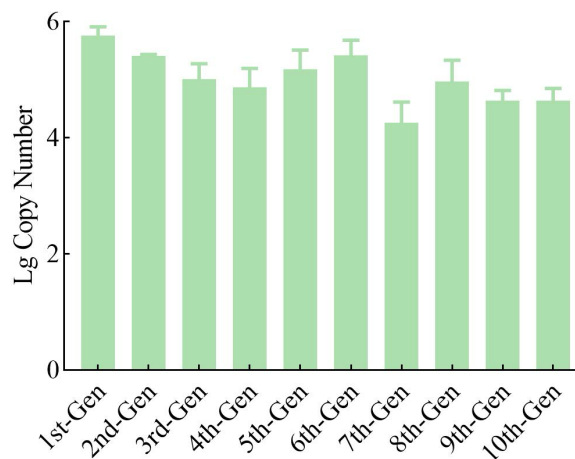


Fig 4. BVDV copy number in RAW264.7 cells upon serial passaging

Compared with the Control group, the relative mRNA expression levels of IL-10 in both the BVDV single-infection group and the BVDV+A19 co-infection group were significantly upregulated ( $P<0.01$ ). Additionally, the relative IL-10 mRNA expression level in the *B. abortus* A19 single-infection group was significantly lower than that in the BVDV single-infection group and the BVDV+A19 co-infection group ( $P<0.01$ ) (Fig. 6-A). At the protein level, the IL-10 concentration in the *B. abortus* A19 single-infection group was significantly downregulated compared with the BVDV+A19 co-infection group ( $P<0.001$ ) (Fig. 6-B). Regarding tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), the relative mRNA expression levels in the BVDV single-infection group and the BVDV+A19 co-infection group were both significantly lower than that in the *B. abortus* A19 single-infection group ( $P<0.001$ ) (Fig. 6-E).

For IL-12, the relative mRNA expression level in the *B. abortus* A19 single-infection group was significantly higher than those in the BVDV single-infection group and the BVDV+A19 co-infection group ( $P<0.05$ ) (Fig. 6-C). Consistently, the IL-12 protein concentration in the *B. abortus* A19 single-infection group was significantly

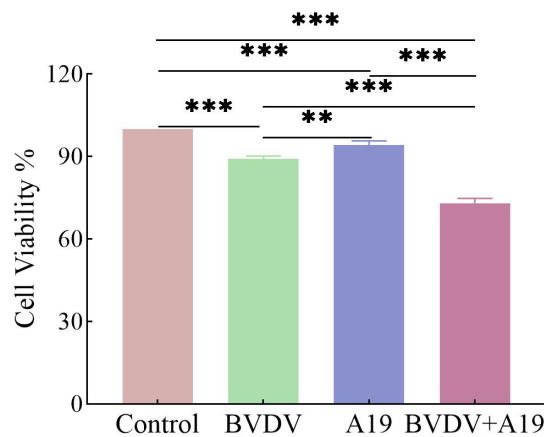


Fig 5. Comparative analysis of cell viability across different treatment groups

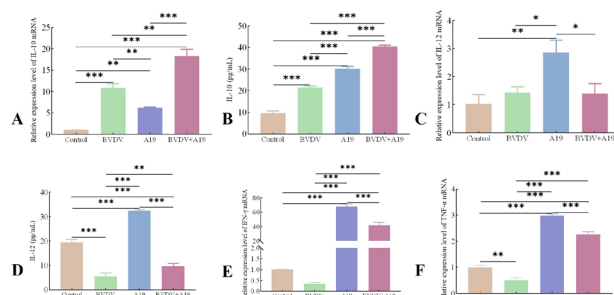


Fig 6. Analysis of mRNA and protein expression levels of cytokines under different treatment conditions. A-E represent the relative expression levels of IL-10 mRNA, protein, IL-12 mRNA, protein, IFN- $\gamma$  mRNA, and TNF- $\alpha$  mRNA, respectively

upregulated compared with the BVDV single-infection group and the BVDV+A19 co-infection group ( $P<0.001$ ) (Fig. 6-D). Furthermore, the relative mRNA expression levels of interferon- $\gamma$  (IFN- $\gamma$ ) in the BVDV single-infection group and the BVDV+A19 co-infection group were significantly lower than that in the *B. abortus* A19 single-infection group ( $P<0.001$ ) (Fig. 6-F).

Collectively, these data demonstrate that BVDV infection can effectively inhibit the expression and secretion of proinflammatory cytokines in RAW264.7 cells, suggesting that BVDV may evade the host innate immune response by impairing the immune activation function of macrophages.

### BVDV Infection Enhances the Intracellular Survival of the *B. abortus* A19 Strain in RAW264.7 Cells

To investigate the effect of BVDV infection on the intracellular survival of the *B. abortus* A19 strain in RAW264.7 cells, colony counting was performed to determine the number of intracellular colonies of the *B. abortus* A19 (MOI=100) at 0 and 24 h pi in RAW264.7 cells. At both 0 h pi and 24 h pi, the number of intracellular the *B. abortus* A19 colonies in the BVDV+A19 co-infection group was significantly higher than that in the *B. abortus* A19 single-infection group ( $P<0.01$ ) (Fig. 7). These results indicate that BVDV may regulate the immune function of cells, thereby creating a favorable environment for the survival and proliferation of the *B. abortus* A19 vaccine strain.

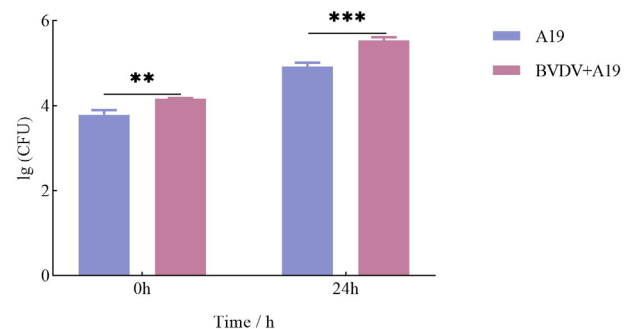


Fig 7. Intracellular survival status of the *B. abortus* A19 and BVDV+A19

## DISCUSSION

This study established a co infection model between bovine viral diarrhea virus (BVDV) and *Brucella* vaccine strain A19, and for the first time confirmed that BVDV infection can significantly reduce the immune-protective efficacy of *Brucella* vaccine. This discovery not only reveals the cross-pathogen interference mechanism of the virus on the immune response of bacterial vaccines, but also provides an important explanation for the previously overlooked

phenomenon of inadequate vaccine protection in current brucellosis prevention and control, that is, the widespread prevalence of BVDV may play a key role in it. Therefore, the results of this study further indicate that incorporating BVDV monitoring and control into the overall prevention and control strategy in brucellosis purification planning is not an auxiliary measure, but a necessary prerequisite for achieving effective immunization and ultimately achieving disease eradication goals.

*In vivo* infection data revealed that, compared with the *B. abortus* A19 single-infection group, mice in the BVDV+A19 co-infection group exhibited significantly higher bacterial loads in the spleen at all tested time points (on days 14, 42, and 56 pi). The spleen is a key immune organ where *Brucella* colonizes and elicits immune responses, and its bacterial load directly reflects the host's ability to clear the pathogen [18]. The sustained elevation of splenic bacterial load in the co-infection group may be attributed to BVDV-mediated suppression of the host immune system: BVDV can directly infect and damage lymphocytes and macrophages, leading to immune cell dysfunction and reduced cell numbers. This impairment severely diminishes the ability of mice to clear the *B. abortus* A19 strain, thereby promoting the survival and proliferation of the *B. abortus* A19 [19, 20].

Additionally, the splenic index of mice in the co-infection group was significantly higher than that in the *B. abortus* A19 single-infection group throughout the entire infection cycle (on days 14, 28, 42, and 56 pi). This elevated splenic index is more likely a consequence of BVDV infection disrupting the host's normal immune regulatory balance, triggering an excessive yet ineffective inflammatory response that ultimately results in lymphocyte necrosis and direct impairment of splenic function. Consistent with this, Liu et al. [21] also confirmed that lymphocyte degeneration and necrosis occur in the spleen of mice in the late stage of BVDV infection.

Together, the observed persistence of high bacterial loads alongside splenic enlargement in co-infected mice suggests that BVDV infection substantially compromises the host's ability to control *B. abortus* A19. This impaired clearance may reflect a dysregulated immune state that permits prolonged bacterial survival *in vivo*. These *in vivo* co-infection phenotypes establish a foundation for further investigation into the cellular and molecular mechanisms involved.

To elucidate the cellular basis of the impaired bacterial clearance observed *in vivo*, we employed the RAW264.7 macrophage model. Consistent with the splenic bacterial load data, BVDV infection significantly enhanced the intracellular survival of *B. abortus* A19 in macrophages. This suggests that BVDV alters the host

cell microenvironment in a way that favors bacterial persistence. A potential explanation is that BVDV infection activates the IFN- $\beta$  pathway in RAW264.7 cells in the early stage to exert antiviral effects, however, in the late stage of infection, the upregulation of SOCS1 (a negative regulator of IFN- $\beta$ ) weakens the sustained antiviral state of the cells, thereby indirectly impairing their bacteriostatic capacity [22]. Alternatively, BVDV infection of host cells may activate the NLRP3 inflammasome, triggering pyroptosis and subsequent cell death, which directly reduces the bacteriostatic capacity of host cells [23].

Moreover, cytokines, as key messenger molecules for intercellular communication, are primarily involved in the regulation of immune homeostasis and inflammatory responses [24]. IL-10 acts on macrophages themselves to inhibit the production of proinflammatory cytokines (e.g., TNF- $\alpha$ , IL-1 $\beta$ ), reduce antigen-presenting capacity, and downregulate the expression of co-stimulatory molecules-processes that facilitate immune evasion and persistent survival of pathogens in the host [25]. In contrast, IL-12 serves as a "bridge" connecting innate and adaptive immunity: it promotes the differentiation of naive CD4+ T cells into Th1 cells, which secrete IFN- $\gamma$  to enhance the ability of macrophages to clear intracellular pathogens [26,27]. Further cytokine analysis in this study revealed key shifts in macrophage response. In BVDV and *B. abortus* A19 co-infected cells, we observed a marked upregulation of the immunosuppressive cytokine IL-10 at both mRNA and protein levels. Concurrently, the expression of key cytokines associated with a pro-inflammatory, Th1-polarized immune response (including IL-12, IFN- $\gamma$ , and TNF- $\alpha$ ) was significantly suppressed compared to infection with *B. abortus* A19 alone. These results indicate that BVDV infection induces increased IL-10 expression in RAW264.7 cells, thereby suppressing the inflammatory and immune responses triggered by RAW264.7 cells.

We therefore propose that BVDV infection drives macrophages toward an immunomodulatory state that is disadvantageous for bacterial clearance. The upregulated IL-10 may inhibit macrophage antimicrobial activity, while the dampened Th1-type cytokine response reduces immune activation. A response critical for activating macrophages to clear intracellular *Brucella* [28]. Collectively, this virus-induced reprogramming of the cytokine network appears to directly promote the intracellular survival and replication of *B. abortus* A19, providing a mechanistic explanation for the enhanced bacterial survival observed both in macrophages and in co-infected mice.

Our findings directly impact brucellosis management in field conditions. The observed immunosuppression suggests that BVDV endemicity may critically undermine

the efficacy of the *Brucella* vaccine, explaining suboptimal control in some vaccinated herds.

We therefore advocate for an integrated control strategy. Screening herds for BVDV before the *Brucella* vaccination should be considered to identify populations at risk of vaccine failure. Moreover, incorporating BVDV vaccination into brucellosis control programs in co-endemic areas could stabilize general herd immunity and potentially enhance the *Brucella* vaccine efficacy.

Based on the broader implications of our findings, we suggest that control programs targeting a single pathogen may have limited success in field settings where co-infections are prevalent. Our work provides a framework for studying similar interactions between immunomodulatory viruses and chronic bacterial pathogens, advocating for a shift toward more holistic, system-based approaches to animal disease management. Ultimately, such integrated strategies promise to improve animal welfare, livestock productivity, and public health outcomes for this major zoonosis.

In summary, this study confirms that BVDV infection can synergistically disrupt the immune protective effect of the *B. abortus* A19 vaccine through multiple mechanisms. These results provide a mechanistic explanation for the suboptimal performance of brucellosis vaccines in field settings and support the development of integrated control strategies that address BVDV co-infection. Adopting such a coordinated approach could enhance livestock health, improve productivity, and reduce the zoonotic risk of brucellosis. However, this study has certain limitations: its results need to be further validated in naturally infected livestock under field conditions to provide more optimal solutions for clinical practice.

## DECLARATIONS

**Availability of Data and Materials:** The datasets generated and/or analyzed during the current study are available from the corresponding author/s (HZ, JG) upon reasonable request.

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

**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:** Conceived of or designed study: H Z, J G; Performed research: YJ C; Analyzed data: YD Z, JH Z; Wrote the paper: YJ C.

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## RESEARCH ARTICLE

# Evaluating the Potential of Three Novel Probiotic Isolates as Antibiotic Alternatives in Improving Growth Performance, Immunity, and Producing Safe Meat in *Salmonella*-Challenged Broiler Chickens

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## Abstract

The overuse of antibiotics in poultry farming underscores the need for safe, effective alternatives to produce clean meat. This study aimed to isolate and evaluate probiotic bacteria with strong antibacterial activity for broiler production. Thirty bacterial isolates were obtained from broiler feces samples, and three isolates were selected and identified using MALDI-TOF MS as *Paenibacillus polymyxa* EB7, *Bacillus licheniformis* EB14, and *Bacillus mycoides* EB26. These isolates were screened for their strong inhibitory activity against pathogens, including *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Among these, *P. polymyxa* EB7 emerged as the most promising, combining high antibacterial and antioxidant activities with exceptional tolerance to acidic pH and bile salts. Importantly, EB7 was sensitive to major antibiotics (tetracycline, azithromycin, erythromycin, and gentamicin) and showed no hemolytic or cytotoxic activity, confirming its safety profile. *In vivo* broiler trials confirmed its effectiveness. Dietary supplementation with EB7 at 200 mg/kg significantly improved growth performance (body weight gain and feed conversion ratio) and mitigated the negative effects of the *Salmonella* challenge. EB7 supplementation improved blood biochemistry by lowering liver and kidney stress markers and oxidative damage (malondialdehyde), while increasing antioxidant enzymes (SOD, GSH, and CAT) and immune markers (IgG and IgA). It also modulated the gut microbiota by reducing pathogenic loads (*E. coli*, *Salmonella*) and increasing beneficial lactic acid bacteria. Furthermore, EB7 helped balance the immune response to *Salmonella* infection by modulating key immune-related genes (TLR4, IL-6, and AvBD6), reducing excessive inflammation while maintaining host defenses. The findings demonstrated that *P. polymyxa* EB7 is a safe, multifunctional probiotic that enhances growth, strengthens immunity, and improves gut health in broilers.

**Keywords:** Probiotics, Human health, Gut microbiota, *Salmonella* challenge, Pathogenic bacteria

## INTRODUCTION

Salmonellosis remains one of the most significant infectious diseases in poultry, responsible for considerable economic losses, public health risk, and persistent challenges in global poultry production <sup>[1]</sup>. *Salmonella enterica* serovars cause poultry salmonellosis, which manifests primarily as enteritis and septicemia, leading to high flock morbidity/mortality and contamination of chicken meat and eggs. Recent systematic reviews report *Salmonella* prevalence in poultry at 12-18%, varying by geographic region and production system <sup>[2,3]</sup>. Outbreaks are not limited to industrial settings but have also been reported in backyard flocks, underscoring widespread vulnerability. In addition to direct flock loss, the economic toll of *Salmonella* on the

poultry industry in major producing countries exceeds \$2.8 billion annually, mainly due to reduced productivity and food safety recalls <sup>[4]</sup>.

*Salmonella* infection in poultry leads to reduced growth, impaired feed conversion, increased susceptibility to secondary infections, and elevated mortality-sometimes up to 50% in poorly managed operations <sup>[2]</sup>. The zoonotic nature of *Salmonella* presents serious foodborne illness risks to consumers, often resulting in large outbreaks and hospitalizations worldwide <sup>[5]</sup>. Contaminated poultry products are a leading source of human salmonellosis, prompting strict regulations and surveillance in many jurisdictions <sup>[1]</sup>.

Historically, antibiotics have been used not only to treat *Salmonella* outbreaks but also as growth promoters and



prophylactics in broiler production. However, misuse and overuse have instigated high levels of antimicrobial resistance (AMR) in *Salmonella*, with recent studies reporting 100% resistance to some commonly used drugs among isolates sampled from chicken environments [1]. This dire scenario has necessitated the urgent search for effective, safe alternatives capable of curbing infection without perpetuating AMR [6,7]. Several strategies, including vaccination, improved biosecurity, organic acids, and notably, probiotics, have emerged as promising tools [8].

Among probiotic candidates, *Bacillus* and *Paenibacillus* species have garnered considerable attention [9]. These spore-forming bacteria are resilient to gastrointestinal conditions, exhibit broad-spectrum antimicrobial activity, and have demonstrated improvements in growth, immunity, and gut health in poultry. Both genera produce extracellular enzymes, competitive exclusion factors, and antimicrobials (such as bacteriocins and lipopeptides) that inhibit not only *Salmonella* but also other pathogens such as *E. coli* and *Staphylococcus* [10].

Recent *in vitro* and *in vivo* studies show that carefully selected *Bacillus* and *Paenibacillus* isolates from chicken feces can yield strong inhibitory effects against *Salmonella*, improve antioxidant status, and support gut barrier functions in broilers [11]. These isolates also possess high survivability in the avian gut though their persistence may be transient, necessitating regular supplementation to maintain their probiotic effect [12].

*Bacillus* and *Paenibacillus* species exhibit antimicrobial activity by producing multiple bioactive compounds, including peptides and organic acids, that reduce *Salmonella* colonization and shedding. Their ability to modulate the immune response, reinforce gut integrity, and suppress oxidative damage adds essential layers of protection against the adverse effects of infection, as evidenced in recent trials. The functionality of these isolates extends to the competitive exclusion of pathogens, the modulation of the microbiota, and the improvement of both nutritional absorption and systemic health in broiler chickens [9-12].

Despite extensive research into probiotic alternatives to antibiotics, several gaps remain. Most notably, the strain-specific effects of *Bacillus* and *Paenibacillus* spp., their synergistic action, and their ability to confer consistent protection against multidrug-resistant *Salmonella* in broilers have not been thoroughly explored, especially using indigenous isolates from regional poultry systems. Few studies focus on the molecular identification, gene profiling, and detailed biological characterization of these candidates as next-generation antibiotic alternatives [9,10].

This study aims to address these gaps by isolating, identifying, and evaluating the safety and efficacy of three

indigenous *Bacillus* and *Paenibacillus* isolates (EB7, EB14, and EB26) as alternatives to antibiotics in combating *Salmonella* infection in broiler chickens. The main aim is to characterize the antioxidant and antimicrobial activities of EB7, EB14, and EB26 against *Salmonella* and other poultry pathogens. The objectives of the study are to assess the probiotic properties and survivability of these isolates in simulated gastrointestinal conditions. Also, to evaluate their influence on broiler health, growth performance, and immune responses during experimental *Salmonella* challenge and to profile their impact on gut microbiota composition and resistance gene transmission. The study seeks to contribute to the development of safe, effective, and sustainable biopreparations to enhance poultry health and reduce reliance on conventional antibiotics.

## MATERIAL AND METHODS

### Ethical Approval

The animal study has been reviewed and approved by ZU-IACUC committee. was performed in accordance with the guidelines of the Egyptian Research Ethics Committee and the guidelines specified in the Guide for the Care and Use of Laboratory Animals (2025). Ethical code number ZU-IACUC/3/F/521/2025.

### Isolation, Screening, and Identification of Selected Isolates

Bacterial isolates were obtained from freshly voided chicken feces collected in sterile containers from poultry farm cages and delivered to the microbiology laboratory within 24 h. About 10 g of fecal samples were homogenized in 90 mL of peptone buffer, yielding a  $10^{-1}$  dilution. Subsequent tenfold serial dilutions were performed to  $10^{-7}$ . Aliquots from each dilution were spread onto Luria-Bertani (LB) agar and incubated for 24 h at 37°C. Isolates displaying pronounced inhibitory effects against *Staphylococcus aureus* and *Pseudomonas aeruginosa* were selected for further analysis [13].

Initial identification of the promising bacterial candidate was based on morphological, biochemical, and physiological profiling in accordance with Bergey's Manual of Systematic Bacteriology. For definitive species-level identification, matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) was employed, utilizing the Microflex LT/SH system (Bruker Daltonics, Bremen, Germany), representing a methodological strength due to its high accuracy (>99%), speed, and reproducibility for probiotic *Bacillus*/*Paenibacillus* identification compared to 16S rRNA sequencing. Bacterial colonies were processed in a suitable extraction buffer, and samples were applied to the target plate, followed by the addition of matrix solution. The generated mass spectra were compared against the Bruker

Biotyper database, allowing rapid and accurate species identification as described by Kluz et al.<sup>[14]</sup>. Indicator pathogens *Salmonella Typhimurium* and *Escherichia coli* were specifically selected due to their prevalence as major enteric pathogens in poultry, causing economic losses *via* diarrhea and mortality, and their relevance to antibiotic-free production challenges<sup>[14]</sup>.

### Safety and Probiotic Properties

To assess the probiotic characteristics of bacterial isolates EB7, EB14, and EB26, acid and bile salt resistance assays were performed based on the method described by Sahadeva et al.<sup>[15]</sup>. For the acid tolerance assay, 1 mL aliquots of bacterial culture were inoculated into 9 mL of LB broth adjusted to pH 2.5, then incubated at 37°C for 3 h. The optical density (OD) of each sample at 650 nm was determined hourly using a spectrophotometer. OD (A650) was adjusted to 0.08±0.05 to normalize bacterial concentrations across samples.

Following the acid-resistance analysis, bile-salt tolerance was tested by inoculating 100 µL of overnight-cultured bacteria into freshly prepared LB broth supplemented with 0.3% bile salts. To evaluate bacterial viability under bile stress, 100 µL samples were withdrawn at 0, 1, 2, 3, and 4 h post-inoculation and plated onto LB agar. Viability was assessed based on the presence (positive) or absence (negative) of colony growth after incubation. The rates of acid tolerance and survival were determined using the following formula:

$$\% \text{ Survival rate} = \text{Absorbance after treatment} / \text{Absorbance before treatment} \times 100$$

To determine the safety of the selected bacterial isolates, antibiotic sensitivity testing was performed. Isolates were plated on a suitable solid nutrient medium at a final concentration of 10<sup>6</sup> colony-forming units (CFU) per gram (CFU/g). Standard antibiotic discs, including tetracycline (30 µg), azithromycin, erythromycin, ceftriaxone, and gentamicin, were then placed on the media. Results were recorded after incubation for 48 h at 42°C.

### Biological Activities

**Antioxidant:** The 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of *Bacillus* suspension (10, 20, 40, 80, 160, and 320 µg/mL) was evaluated according to Abdel-Moneim et al.<sup>[16]</sup>. The reaction was initiated by incubating 0.5 mL of ethanolic DPPH with 1 mL of *Bacillus* suspension in the dark for 30 min, after which the absorbance at 517 nm was measured using a spectrophotometer (JENWAY, UK). The half-maximal inhibitory concentration (IC<sub>50</sub>) value reflects the minimum concentration required to scavenge 50% of the DPPH radical<sup>[17]</sup>. The percentage of DPPH scavenging activity was calculated using the formula:

$$\% \text{ Antioxidant activity} = (\text{Control absorbance} - \text{Sample absorbance}) / \text{control absorbance} \times 100$$

**Antibacterial:** Antibacterial activity was assessed using the distinct bacterial isolate by preparing suspensions at concentrations of 50, 100, and 200 µg/mL. For each concentration, sterile 8-mm filter paper discs were immersed in the corresponding bacterial suspension for 30 min to ensure complete saturation. These treated discs were then placed onto agar plates previously inoculated with pathogenic bacteria relevant to poultry health, including *S. aureus*, *Streptococcus pyogenes*, *Listeria monocytogenes*, *Salmonella typhi*, *E. coli*, and *Klebsiella pneumoniae*. After disc placement, the plates were incubated under optimal conditions for bacterial growth. After incubation, the diameter of the inhibition zones around each disc was measured in millimeters to determine antibacterial efficacy<sup>[18,19]</sup>.

### Experimental Design

A total of 550 Indian River broiler chicks were allocated to eleven experimental groups in a randomized design based on initial body weights on day one. Each group comprised five replicates, with eleven chicks per replicate. This experiment was designed to evaluate the probiotic effects of three soil-derived bacterial isolates (EB7, EB14, and EB26), administered at different concentrations, and their potential to counteract *Salmonella* infection in broiler chickens. The study followed a completely randomized design (CRD) with 11 experimental treatments, as outlined below. Healthy broiler chicks of uniform body weight at 10 days old. The experimental duration was 35 days. The basal diet is formulated to meet the National Research Council (NRC) nutrient requirements for broilers, without antibiotic growth promoters. The composition and nutrient analysis of the basal diet were detailed in the study of Al-Quwaie<sup>[20]</sup>. The treatment groups were as follow: T1; Negative control delivered basal diet without additives (non-infected control), T2; EB7 (low dose delivered basal diet supplemented with *Paenibacillus polymyxa* EB7 at 50 mg/kg feed), T3; EB7 (high dose delivered basal diet supplemented with *P. polymyxa* EB7 at 200 mg/kg feed), T4; EB14 (low dose delivered basal diet supplemented with *Bacillus licheniformis* EB14 at 50 mg/kg feed), T5; EB14 (high dose delivered basal diet supplemented with *B. licheniformis* EB14 at 200 mg/kg feed), T6; EB26 (low dose delivered basal diet supplemented with *Bacillus mycoides* EB26 at 50 mg/kg feed), T7; EB26 (high dose delivered basal diet supplemented with *B. mycoides* EB26 at 200 mg/kg feed); T8; Positive control (infected broilers challenged with *Salmonella* spp. (no supplementation): T9; EB7 + broilers infected with *Salmonella* and treated with EB7 at 200 mg/kg); T10; EB14 + infected broilers and treated with EB14 at 200 mg/kg); T11; (EB26 + infected broilers and treated with EB26 at 200 mg/kg).

EB7 (*P. polymyxa*), EB14 (*B. licheniformis*), and EB26 (*Bacillus mycoides*) were cultured in nutrient broth for 24 h at 37°C, centrifuged, and adjusted to  $10^8$  CFU/mL before dietary inclusion. Each bacterial suspension was uniformly mixed into the formulated diets at designated concentrations (50 mg/kg and 200 mg/kg). On day 15, birds in groups T8-T11 were orally challenged with *S. enterica* ( $10^7$  CFU/mL) to induce intestinal infection [21]. Birds were housed in battery cages featuring three tiers and automated watering systems, with ad libitum access to feed and water throughout the study.

### Growth Performance

Growth performance parameters, including live body weight (LBW), feed intake (FI), body weight gain (BWG), feed conversion ratio (FCR), performance index (PI), and growth rate (GR), were calculated using the methodologies of Saad et al. [22] and Brody and Lardy [23]:

Body weight gain (BWG) = Final body weight (FBW) – Initial body weight (IBW)

Growth rate (GR) =  $(LBW_{35} - LBW_1) / [0.5 \times (LBW_1 + LBW_{35})]$

Performance index (PI) = BWG / FCR

### Biochemical Parameters

At day 35, five chicks from each treatment were anesthetized using an R550 Multioutput laboratory small animal anesthesia machine. Each anesthesia channel operated independently, allowing precise control of gas flow to the induction box in the range of 0-2.0 L/min. Blood samples were collected from the hepatic portal vein for biochemical analyses and transferred into heparinized tubes. The blood samples were centrifuged at 5,000 rpm for 10 min to separate the serum. The activities of liver enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and the AST/ALT ratio were estimated following Cheng et al. [24]. The liver was excised, rinsed in chilled 0.9% saline solution (w/v), weighed, and stored at -70°C. The superoxide dismutase (SOD), glutathione (GSH), catalase (CAT), and malondialdehyde (MDA) contents were assessed according to the protocol of Alatawi et al. [25]. Total antioxidant capacity (TAC) was measured as described by Pappas et al. [26]. Serum concentrations of immunoglobulins (IgG, IgA, and IgM) were quantified using a sandwich ELISA, with OD measured at 450 nm using a microplate reader (BioTek 800, USA), as outlined by Erhard et al. [27].

### Gene Expression

Total RNA was isolated from chick intestinal tissue, and the resulting RNA pellets were resuspended in diethylpyrocarbonate (DEPC)-treated water. RNA purity

and concentration were determined spectrophotometrically by measuring the 260/280 nm absorbance ratio, as described by Saif and Khan [28]. For semiquantitative reverse transcription-PCR, 3 µg of RNA was used as a template. The RNA was denatured at 70°C for 5 min in a Bio-Rad T100TM thermal cycler. cDNA synthesis was performed using 0.5 ng of oligo (dT) primers, 2 µL of 10X RT buffer, 2 µL of 10 mM dNTPs, and 1 µL of 100 U reverse transcriptase. The reaction was incubated at 42°C for 1 h, followed by enzyme inactivation at 70°C for 10 min. Gene expression levels were quantified by real-time PCR using the  $2^{-\Delta\Delta CT}$  method, with actin as the endogenous reference gene. Densitometric analysis was performed to assess mRNA expression, and specific primers (Table 1) were used for amplification. The cycle threshold (Ct) values were used to compare gene expression levels across samples, following established comparative quantification protocols.

### Intestinal Microbial Quantification

Post-mortem, intestinal digesta samples were aseptically collected, homogenized in sterile glass containers, and stored at 4°C until analysis. Microbial enumeration included total viable bacteria, *E. coli*, coliforms, and *Lactobacillus* spp., using selective media as described by Abd El-Wahab et al. [29]. Results were standardized and reported as log10 colony-forming units (CFU/g) per gram of digesta.

### Statistical Analysis

Statistical analysis was performed using SPSS (Version 17.0, IBM, USA). Results were presented as mean  $\pm$  standard deviation (SD). Data were analyzed by one-way analysis of variance (ANOVA), and group means were compared using Fisher's least significant difference (LSD) test when appropriate. Statistical significance was defined as  $P \leq 0.05$ .

## RESULTS

### Isolation, Screening, and Identification of the Selected Isolates

A total of 30 bacterial isolates were recovered from fecal samples and coded as EB1-EB30. These isolates were preliminarily screened for antibacterial activity using a dual-culture agar diffusion method against two indicator pathogens, *S. aureus* and *Pseudomonas aeruginosa*. Three isolates, EB7, EB14, and EB26, were screened for the largest inhibition zones against both bacteria. Following this, all 30 isolates were biochemically profiled using standard Bergey's Manual protocols, and the top three were further identified at the species level by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS). The high antibacterial isolates (EB26, EB14, and EB7, with inhibition zones ranging from 25.9 to

**Table 1.** Primer sequences and characteristics for selected defense-related genes

| Target Gene | Primer Sequence (5'→3')                             | Product Length (bp) | Melting temperature (T <sub>m</sub> °C) |      | GenBank Reference |
|-------------|-----------------------------------------------------|---------------------|-----------------------------------------|------|-------------------|
|             |                                                     |                     | F                                       | R    |                   |
| TLR4        | F: GAACATGCGGCTGAGTGGA<br>R: TGGCTTCTCCACATGGAGAA   | 146                 | 62.2                                    | 60.4 | NM_001030693.2    |
| TLR1LA      | F: ATGTGGCTGAGGTGGTGT'TT<br>R: GCAGGATGACCTTGGAGAA  | 130                 | 55.3                                    | 58.0 | NM_001305826.1    |
| AvBD6       | F: CTTGCAGTGTCTCTGTCTCAGT<br>R: CTCAGCAACCTGCTTCCTG | 110                 | 59.9                                    | 59.9 | NM_204478.3       |
| IL-1B       | F: TGCCAGAAGGAAATGCCAA<br>R: GTCAAGGAGCAGGGTTGG     | 164                 | 58.0                                    | 58.4 | NM_204524.2       |
| IL-6        | F: ACAACACGACTCCCACCAA<br>R: AGGTGAGTGGCTGTCTGTGT   | 112                 | 60.2                                    | 58.7 | NM_204628.2       |
| IFN-γ       | F: GAGCCAGATTGACCAGAGC<br>R: CCTTTTGCCCATCCAGGAGT   | 130                 | 55.8                                    | 58.7 | NM_205149.1       |
| TGF-β1      | F: AGGAATCGGCTGACACAAA<br>R: TTCCAGGTCACTGGTCATCA   | 125                 | 62.2                                    | 57.6 | NM_205149.2       |
| MHC         | F: GCTCAGACACCCGGAGACTT<br>R: GCCCTCGTCGTCTTCTCT    | 140                 | 59.6                                    | 62.0 | NM_205823.1       |
| cLEAP-2     | F: ATTCTGCTTCCCTGAGGCTG<br>R: TCAAGGCAGGTCCACTCTC   | 120                 | 59.9                                    | 59.9 | NM_001277318.1    |
| ACSL1       | F: GATTGCCAGTTCCTTTGTC<br>R: GAGGACAGTGAGGTGCAGG    | 150                 | 62.2                                    | 60.4 | NM_001006323.2    |

TLR4: Toll-like receptor 4; TLR1LA: Toll-like receptor 1 type A; AvBD6: Avian β-defensin 6; IL-1B: Interleukin 1 beta; IL-6: Interleukin 6; IFN-γ: Interferon gamma; TGF-β1: Transforming growth factor beta 1; MHC: Major histocompatibility complex (class II, B); cLEAP-2: Chicken liver-expressed antimicrobial peptide 2; ACSL1: Acyl-CoA synthetase long chain family member 1. F: Forward primer, R: Reverse primer, bp: Base pairs. PCR was performed using SYBR Green-based qPCR with an annealing temperature of 58°C (optimized based on primer T<sub>m</sub> values ranging 55–62°C), initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C/15 s, 58°C/30 s, 72°C/30 s, and melt curve analysis

32.0 mm against *S. aureus* and 21.7 to 26.9 mm against *P. aeruginosa*, were selected.

Based on morphological and biochemical tests, all isolates were Gram-positive, motile, spore-forming, rod-shaped cells observed singly under light microscopy. The colonies on LB agar were flat, round, and translucent with irregular edges and a pale cream color. Based on the results, EB7 corresponded to *P. polymyxa*, EB14 to *B. licheniformis*, and EB26 to *B. mycoides*. Functionally, all three isolates demonstrated multiple beneficial activities: solubilizing tricalcium phosphate within seven days; producing indole-3-acetic acid (IAA) in the presence of tryptophan; fixing nitrogen as confirmed by acetylene reduction assays; utilizing 1-aminocyclopropane-1-carboxylic acid (ACC) as its sole carbon source; and forming dense biofilms on glass surfaces.

MALDI-TOF MS analysis successfully categorized the isolates as follows: EB7 was identified as *P. polymyxa*, EB14 as *B. licheniformis*, and EB26 as *B. mycoides*. Each isolate's spectral profile was matched against an established microbiological reference library with high log-score confidence values (>2.0), indicating precise species-level identification.

*Paenibacillus polymyxa* (EB7) was characterized by its

large mass peak values consistent with polymyxin-type peptide biosynthesis, supporting its broad-spectrum antimicrobial activity and known probiotic functions. The identification of *B. licheniformis* (EB14) matched published spectral libraries, showing characteristic peaks associated with the production of lipopeptides, such as lichenysin, a biosurfactant detected at mass-to-charge (m/z) ratios 1015–1087, often used as a biochemical marker of this species. This bioactivity underpins its strong antibacterial activity. *B. mycoides* (EB26) displayed unique filamentous colony morphology and a distinctive m/z spectral profile typical of spore-forming capability and environmental adaptation.

Overall, the MALDI-TOF MS confirmed that all three isolates belong to spore-forming, Gram-positive genera with established probiotic and biocontrol potential. The differentiation accuracy parallels earlier findings showing that MALDI-TOF MS is particularly reliable for species separation within the *Bacillus subtilis*, *B. licheniformis*, and *Paenibacillus* groups when proper protein extraction and culture conditions are used.

### Probiotic Properties

**Low pH and Bile Salt Survival:** The isolates (EB7, EB14, and EB26) that exhibited the highest zone of inhibition

against *S. aureus* and *P. aeruginosa* also demonstrated strong tolerance to both acidic (pH 2.5) and bile salt (0.3%) conditions, with survival rates indicating their resilience under gastrointestinal tract conditions (Table 2). Specifically, EB7 showed survival rates of 84.1% at pH 2.5 and 77.4% in bile salt (0.3%).

**Antibiotic Resistance:** Among the three tested isolates, EB7 was sensitive to all tested antibiotics, with inhibition zones of 23.0 mm for tetracycline, 25.1 mm for azithromycin, 26.5 mm for erythromycin, and 30.5 mm for gentamicin, each exceeding the Clinical and Laboratory Standards Institute (CLSI) susceptibility cutoff of 19.0 mm. The other isolates, EB14 and EB26, produced inhibition zones below the susceptibility threshold for tetracycline, azithromycin, erythromycin, and gentamicin. For ceftriaxone, none

of the isolates achieved the sensitivity breakpoint, as all measurements fell within the intermediate or resistant range (Table 3).

### Biological Activities

**Antioxidant:** Fig. 1 clearly shows a strong, dose-dependent increase in antioxidant activity (%) for all three isolates (EB7, EB14, and EB26) as the concentration increases from 25 to 200 µg/mL. At every tested concentration, EB7 consistently demonstrated the highest antioxidant activity compared to EB14 and EB26, with statistically significant differences, especially from 50 µg/mL upward (indicated by non-overlapping error bars and greater separation of bars). At the maximum dose (200 µg/mL), EB7 approached or surpassed 90% activity, while EB14 and EB26 reached about 80% and 75%, respectively.

Based on where each curve intersects, EB7 reached IC<sub>50</sub> at a lower concentration (approximately 48 µg/mL), compared to EB14 (~58 µg/mL) and EB26 (~67 µg/mL). Lower IC<sub>50</sub> values indicate higher antioxidant potency. Therefore, EB7 was the strongest antioxidant isolate, requiring the lowest concentration to reach 50% activity, whereas EB14 and EB26 were significantly less potent.

**Antibacterial:** Table 4 reveals significant differences among the isolates (EB7, EB14, and EB26) and concentrations (50, 100 and 200 µg/mL) for all tested pathogenic bacteria. In each case, higher concentrations result in larger inhibition zones, from 50 to 200 µg/mL. EB7 consistently exhibited the strongest antibacterial activity (Table 4). At 200 µg/mL, EB7 produces the largest inhibition zones across all pathogens, such as 32.0 mm for *S. aureus*, 31.2 mm for *S. pyogenes*, and 30.8 mm for *E. coli*, all significantly surpassing EB14 and EB26. EB14 typically showed intermediate activity (for example, 28.3 mm, 27.6 mm, and 27.5 mm for the same three pathogens, respectively), while EB26 was the least effective but still demonstrated notable increases at higher concentrations (30.5 mm for *S. aureus*, 29.4 mm for *S. pyogenes*, and 29.1 mm for *E. coli* at 200 µg/mL).

The statistical letters confirmed that, for each tested pathogen, the differences among the three isolates at the

**Table 2.** Screening the selected isolates based on their inhibition zones against *S. aureus* and *P. aeruginosa*, and survival at low pH and bile salt (0.3%)

| Isolate | Inhibition Zone (mm)  |                       | Survival Rate (%)     |                       |
|---------|-----------------------|-----------------------|-----------------------|-----------------------|
|         | <i>S. aureus</i>      | <i>P. aeruginosa</i>  | pH 2.5                | Bile Salt (0.3%)      |
| EB7     | 32.0±1.2 <sup>c</sup> | 26.9±1.3 <sup>b</sup> | 84.1±2.5 <sup>a</sup> | 77.4±2.1 <sup>a</sup> |
| EB14    | 28.3±0.9 <sup>b</sup> | 26.2±1.0 <sup>b</sup> | 78.3±2.7 <sup>b</sup> | 68.5±2.4 <sup>b</sup> |
| EB26    | 25.9±1.1 <sup>a</sup> | 21.7±1.2 <sup>a</sup> | 69.0±2.8 <sup>c</sup> | 65.9±2.3 <sup>c</sup> |

Data are presented as mean ± SD. Different lowercase letters in the same column indicate the significant differences (P<0.05). *S. aureus*, *P. aeruginosa*

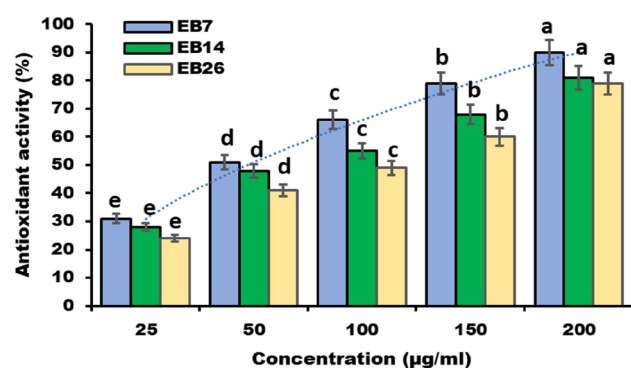


**Fig 1.** Antioxidant activity of EB7, EB14, and EB26 isolates against 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals. Lowercase letters above columns indicate significant differences (P<0.05)

**Table 3.** Antibiotic resistance profiles of selected isolates

| Antibiotic (30 µg) | EB7                   | EB14                   | EB26                  | CLSI Interpretation      |
|--------------------|-----------------------|------------------------|-----------------------|--------------------------|
| Tetracycline       | 23.0±1.1 <sup>a</sup> | 17.9±1.1 <sup>b</sup>  | 16.3±0.7 <sup>b</sup> | S (≥19 mm)               |
| Azithromycin       | 25.1±1.1 <sup>a</sup> | 18.7±1.2 <sup>b</sup>  | 15.1±1.1 <sup>c</sup> | S (≥19 mm)               |
| Erythromycin       | 26.5±1.6 <sup>a</sup> | 21.3±0.8 <sup>b</sup>  | 20.5±1.1 <sup>b</sup> | S (≥19 mm)               |
| Ceftriaxone        | 16.9±0.9 <sup>a</sup> | 15.0±0.5 <sup>ab</sup> | 12.4±0.9 <sup>b</sup> | I (15-18 mm), R (≤14 mm) |
| Gentamicin         | 30.5±1.6 <sup>a</sup> | 23.2±1.4 <sup>b</sup>  | 21.9±1.1 <sup>b</sup> | S (≥19 mm)               |

Data are inhibition zone diameters (mm ± SD; n=3). Statistical letters within a row indicate significant differences (P<0.05). CLSI: Clinical and Laboratory Standards Institute; S: Susceptible; I: Intermediate; R: Resistant

**Table 4.** Antibacterial activity of the selected isolates at concentrations (50, 100, and 200 µg/mL) against pathogenic bacteria

| Pathogen                | EB7 (µg/mL)           |                       |                       | EB14 (µg/mL)          |                       |                       | EB26 (µg/mL)          |                       |                       |
|-------------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|
|                         | 50                    | 100                   | 200                   | 50                    | 100                   | 200                   | 50                    | 100                   | 200                   |
| <i>S. aureus</i>        | 14.2±1.0 <sup>c</sup> | 16.5±0.9 <sup>b</sup> | 32.0±1.3 <sup>a</sup> | 12.1±0.8 <sup>c</sup> | 15.0±1.0 <sup>b</sup> | 28.3±1.1 <sup>a</sup> | 13.8±0.9 <sup>c</sup> | 16.2±1.0 <sup>b</sup> | 30.5±1.2 <sup>a</sup> |
| <i>S. pyogenes</i>      | 12.9±0.9 <sup>c</sup> | 15.4±1.0 <sup>b</sup> | 31.2±1.4 <sup>a</sup> | 11.8±0.8 <sup>c</sup> | 14.5±1.1 <sup>b</sup> | 27.6±1.2 <sup>a</sup> | 13.0±0.8 <sup>c</sup> | 15.7±1.0 <sup>b</sup> | 29.4±1.1 <sup>a</sup> |
| <i>L. monocytogenes</i> | 13.3±0.8 <sup>c</sup> | 15.0±0.9 <sup>b</sup> | 26.7±1.3 <sup>a</sup> | 12.5±0.7 <sup>c</sup> | 14.8±0.9 <sup>b</sup> | 24.2±1.2 <sup>a</sup> | 13.6±0.8 <sup>c</sup> | 15.9±1.0 <sup>b</sup> | 25.3±1.0 <sup>a</sup> |
| <i>S. typhi</i>         | 12.5±0.9 <sup>c</sup> | 14.4±1.0 <sup>b</sup> | 28.6±1.1 <sup>a</sup> | 11.2±0.7 <sup>c</sup> | 13.9±0.8 <sup>b</sup> | 25.9±1.0 <sup>a</sup> | 12.8±0.8 <sup>c</sup> | 15.2±0.9 <sup>b</sup> | 27.1±1.2 <sup>a</sup> |
| <i>E. coli</i>          | 13.0±0.8 <sup>c</sup> | 15.1±1.0 <sup>b</sup> | 30.8±1.2 <sup>a</sup> | 12.0±0.8 <sup>c</sup> | 14.0±1.1 <sup>b</sup> | 27.5±1.2 <sup>a</sup> | 13.5±0.9 <sup>c</sup> | 15.4±1.0 <sup>b</sup> | 29.1±1.3 <sup>a</sup> |
| <i>K. pneumoniae</i>    | 12.7±0.9 <sup>c</sup> | 14.6±1.1 <sup>b</sup> | 28.2±1.3 <sup>a</sup> | 11.5±0.8 <sup>c</sup> | 13.6±0.9 <sup>b</sup> | 25.0±1.1 <sup>a</sup> | 12.9±0.9 <sup>c</sup> | 15.3±0.9 <sup>b</sup> | 26.9±1.1 <sup>a</sup> |

Data are mean inhibition zone diameters (mm ± SD; n = 3). Different superscript letters (a-c) within rows denote significant differences between concentrations (P<0.05). *S. aureus*, *S. pyogenes*, *L. monocytogenes*, *S. typhi*, *E. coli*, and *K. pneumoniae*

same concentration were significant in most cases, with EB7 achieving the highest activity in almost all cases

### In vivo Experiment

#### Growth Performance of Salmonella-challenged Broilers:

The data in Table 5 demonstrate the effects of dietary supplementation with isolates EB7, EB14, and EB26 at two concentrations (50 and 200 mg/kg) on the growth performance of broilers under both normal and *Salmonella*-challenged conditions. Supplementation with EB7 at 200 mg/kg (T3) resulted in the greatest improvements in all growth parameters compared with the control and other treatments. T3 birds exhibited the highest final body weight (FBW: 2418.0 g), body weight gain (BWG: 2370.0 g), performance index (PI: 153.5), and optimal feed conversion ratio (FCR: 1.54), all significantly superior to T1 (negative control) and *Salmonella*-infected groups (T8–T11), as shown by distinct superscript letters. EB14 at 200 mg/kg (T5) also markedly improved metrics (FBW: 2374.0 g, BWG: 2323.0 g, PI: 150.1) versus controls,

performing intermediately between EB7 and EB26 treatments. Lower doses (50 mg/kg; T2, T4, T6) yielded moderate, significant gains over controls, but 200 mg/kg doses excelled consistently; *Salmonella* challenge alone (T8) severely reduced all parameters (lowest FBW/BWG/PI, highest FCR).

Notably, post-challenge treatments with EB7 (T9), EB14 (T10), and EB26 (T11) at 200 mg/kg partially mitigated the harmful effects of *Salmonella* infection, as evidenced by improved BWG and PI and a lower FCR compared to T8. Table 5 clearly demonstrates, supported by statistical groupings, that the probiotic candidates, especially EB7 at 200 mg/kg, can significantly boost broiler growth efficiency and resistance to *Salmonella* challenge.

**Blood Biochemistry Markers:** The results in Table 6 clearly show significant, dose-dependent, and isolate-specific effects of the tested treatments on serum kidney and liver function, oxidative stress markers, and immune parameters in broilers. The *Salmonella* challenge group

**Table 5.** Effect of dietary EB7, EB14, and EB26 at 50 and 200 mg/kg on growth performance parameters of *Salmonella*-challenged broilers

| Treatment | LBW (g)                | FBW (g)                  | BWG (g)                  | FI (g)                   | FCR                     | GR                      | PI                      |
|-----------|------------------------|--------------------------|--------------------------|--------------------------|-------------------------|-------------------------|-------------------------|
| T1        | 45.5±0.3 <sup>c</sup>  | 2240.0±2.5 <sup>bc</sup> | 2195.0±2.6 <sup>bc</sup> | 3665.0±3.0 <sup>bc</sup> | 1.66±0.01 <sup>c</sup>  | 193.0±1.1 <sup>bc</sup> | 135.9±0.8 <sup>bc</sup> |
| T2        | 46.2±0.3 <sup>bc</sup> | 2305.0±2.5 <sup>ab</sup> | 2260.0±2.6 <sup>ab</sup> | 3709.0±3.0 <sup>ab</sup> | 1.60±0.01 <sup>bc</sup> | 194.0±1.1 <sup>ab</sup> | 145.7±0.8 <sup>ab</sup> |
| T3        | 47.9±0.3 <sup>a</sup>  | 2418.0±2.5 <sup>a</sup>  | 2370.0±2.6 <sup>a</sup>  | 3715.0±3.0 <sup>a</sup>  | 1.54±0.01 <sup>a</sup>  | 197.0±1.1 <sup>a</sup>  | 153.5±0.8 <sup>a</sup>  |
| T4        | 46.0±0.3 <sup>bc</sup> | 2292.0±2.5 <sup>ab</sup> | 2250.0±2.6 <sup>ab</sup> | 3701.0±3.0 <sup>ab</sup> | 1.61±0.01 <sup>bc</sup> | 194.0±1.1 <sup>ab</sup> | 144.0±0.8 <sup>ab</sup> |
| T5        | 47.1±0.3 <sup>ab</sup> | 2374.0±2.5 <sup>a</sup>  | 2323.0±2.6 <sup>a</sup>  | 3706.0±3.0 <sup>a</sup>  | 1.57±0.01 <sup>ab</sup> | 196.0±1.1 <sup>a</sup>  | 150.1±0.8 <sup>a</sup>  |
| T6        | 45.7±0.3 <sup>c</sup>  | 2277.0±2.5 <sup>bc</sup> | 2244.0±2.6 <sup>bc</sup> | 3669.0±3.0 <sup>bc</sup> | 1.63±0.01 <sup>c</sup>  | 192.0±1.1 <sup>bc</sup> | 141.9±0.8 <sup>bc</sup> |
| T7        | 46.5±0.3 <sup>bc</sup> | 2310.0±2.5 <sup>b</sup>  | 2260.0±2.6 <sup>b</sup>  | 3683.0±3.0 <sup>b</sup>  | 1.60±0.01 <sup>bc</sup> | 193.0±1.1 <sup>b</sup>  | 143.6±0.8 <sup>b</sup>  |
| T8        | 42.1±0.3 <sup>d</sup>  | 2035.0±2.5 <sup>d</sup>  | 1996.0±2.6 <sup>d</sup>  | 3686.0±3.0 <sup>d</sup>  | 1.84±0.01 <sup>d</sup>  | 186.0±1.1 <sup>d</sup>  | 110.2±0.8 <sup>d</sup>  |
| T9        | 45.2±0.3 <sup>c</sup>  | 2202.0±2.5 <sup>c</sup>  | 2170.0±2.6 <sup>c</sup>  | 3662.0±3.0 <sup>c</sup>  | 1.70±0.01 <sup>c</sup>  | 190.0±1.1 <sup>c</sup>  | 134.4±0.8 <sup>c</sup>  |
| T10       | 45.1±0.3 <sup>c</sup>  | 2196.0±2.5 <sup>c</sup>  | 2161.0±2.6 <sup>c</sup>  | 3644.0±3.0 <sup>c</sup>  | 1.72±0.01 <sup>c</sup>  | 188.0±1.1 <sup>c</sup>  | 132.9±0.8 <sup>c</sup>  |
| T11       | 44.9±0.3 <sup>c</sup>  | 2189.0±2.5 <sup>c</sup>  | 2148.0±2.6 <sup>c</sup>  | 3632.0±3.0 <sup>c</sup>  | 1.75±0.01 <sup>c</sup>  | 187.0±1.1 <sup>c</sup>  | 132.1±0.8 <sup>c</sup>  |

Data are presented as mean ± SE. Different superscript letters within each row indicate significant differences between means (P<0.05). Parameters include live body weight (LBW), final body weight (FBW), body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR), growth rate (GR), and performance index (PI). Treatments included T1: Negative control group, fed a basal diet without additives. T2/T3: Broilers supplemented with EB7 at 50 mg/kg (T2) or 200 mg/kg (T3) diets. T4/T5: Broilers supplemented with EB14 at 50 mg/kg (T4) or 200 mg/kg (T5) diets. T6/T7: Broilers supplemented with EB26 at 50 mg/kg (T6) or 200 mg/kg (T7) diets. T8: Positive control, broilers challenged with *Salmonella* but no probiotic. T9/T10/T11: Broilers challenged with *Salmonella* and treated with EB7 (T9), EB14 (T10), or EB26 (T11) at 200 mg/kg diets

Table 6. Effect of treatments on serum kidney, liver function, oxidative stress, and immunity markers in broilers

| Treatment | AST (U/L)          | ALT (U/L)             | Creat (mg/dL)          | Uric Acid (mg/dL)    | MDA (nmol/mL)        | SOD (U/mL)            | GSH (umol/L)          | CAT (U/mL)            | IgG (mg/dL)           | IgA (mg/dL)          | T3 (ng/dL)             | T4 (ng/dL)         |
|-----------|--------------------|-----------------------|------------------------|----------------------|----------------------|-----------------------|-----------------------|-----------------------|-----------------------|----------------------|------------------------|--------------------|
| T1        | 248±3 <sup>c</sup> | 3.20±0.1 <sup>c</sup> | 0.32±0.01 <sup>b</sup> | 5.4±0.2 <sup>b</sup> | 5.8±0.2 <sup>b</sup> | 32.5±1.2 <sup>d</sup> | 45.2±2.1 <sup>c</sup> | 28.1±1.0 <sup>c</sup> | 958±4.5 <sup>c</sup>  | 177±1.1 <sup>c</sup> | 2.34±0.01 <sup>b</sup> | 134±3 <sup>b</sup> |
| T2        | 242±3 <sup>c</sup> | 2.90±0.1 <sup>c</sup> | 0.34±0.01 <sup>b</sup> | 4.8±0.2 <sup>b</sup> | 4.9±0.2 <sup>b</sup> | 35.9±1.2 <sup>c</sup> | 50.2±2.1 <sup>c</sup> | 31.1±1.0 <sup>c</sup> | 1012±4.5 <sup>c</sup> | 185±1.1 <sup>c</sup> | 2.33±0.01 <sup>b</sup> | 138±3 <sup>b</sup> |
| T3        | 220±3 <sup>a</sup> | 2.10±0.1 <sup>a</sup> | 0.33±0.01 <sup>a</sup> | 4.3±0.2 <sup>a</sup> | 3.8±0.2 <sup>a</sup> | 40.2±1.2 <sup>a</sup> | 56.3±2.1 <sup>a</sup> | 36.5±1.0 <sup>a</sup> | 1091±4.5 <sup>a</sup> | 205±1.1 <sup>a</sup> | 2.32±0.01 <sup>a</sup> | 143±3 <sup>a</sup> |
| T4        | 239±3 <sup>c</sup> | 2.80±0.1 <sup>c</sup> | 0.34±0.01 <sup>b</sup> | 4.9±0.2 <sup>b</sup> | 4.7±0.2 <sup>b</sup> | 34.8±1.2 <sup>c</sup> | 52.0±2.1 <sup>b</sup> | 32.0±1.0 <sup>b</sup> | 1030±4.5 <sup>b</sup> | 186±1.1 <sup>b</sup> | 2.33±0.01 <sup>b</sup> | 137±3 <sup>b</sup> |
| T5        | 225±3 <sup>b</sup> | 2.20±0.1 <sup>b</sup> | 0.33±0.01 <sup>a</sup> | 4.4±0.2 <sup>a</sup> | 4.1±0.2 <sup>a</sup> | 38.7±1.2 <sup>a</sup> | 54.1±2.1 <sup>a</sup> | 35.0±1.0 <sup>a</sup> | 1081±4.5 <sup>a</sup> | 200±1.1 <sup>a</sup> | 2.32±0.01 <sup>a</sup> | 142±3 <sup>a</sup> |
| T6        | 244±3 <sup>c</sup> | 2.90±0.1 <sup>c</sup> | 0.35±0.01 <sup>b</sup> | 5.1±0.2 <sup>b</sup> | 5.0±0.2 <sup>b</sup> | 33.6±1.2 <sup>d</sup> | 48.6±2.1 <sup>c</sup> | 29.7±1.0 <sup>c</sup> | 990±4.5 <sup>c</sup>  | 180±1.1 <sup>c</sup> | 2.33±0.01 <sup>b</sup> | 135±3 <sup>b</sup> |
| T7        | 233±3 <sup>b</sup> | 2.70±0.1 <sup>b</sup> | 0.34±0.01 <sup>a</sup> | 4.6±0.2 <sup>a</sup> | 4.5±0.2 <sup>a</sup> | 36.9±1.2 <sup>b</sup> | 51.5±2.1 <sup>b</sup> | 33.1±1.0 <sup>b</sup> | 1045±4.5 <sup>b</sup> | 189±1.1 <sup>b</sup> | 2.33±0.01 <sup>a</sup> | 138±3 <sup>a</sup> |
| T8        | 269±3 <sup>d</sup> | 3.90±0.1 <sup>d</sup> | 0.37±0.01 <sup>c</sup> | 6.2±0.2 <sup>c</sup> | 7.0±0.2 <sup>c</sup> | 29.2±1.2 <sup>d</sup> | 38.3±2.1 <sup>d</sup> | 25.9±1.0 <sup>d</sup> | 835±4.5 <sup>d</sup>  | 140±1.1 <sup>d</sup> | 2.20±0.01 <sup>c</sup> | 120±3 <sup>c</sup> |
| T9        | 242±3 <sup>b</sup> | 2.50±0.1 <sup>b</sup> | 0.31±0.01 <sup>a</sup> | 4.5±0.2 <sup>a</sup> | 4.0±0.2 <sup>a</sup> | 38.5±1.2 <sup>a</sup> | 55.3±2.1 <sup>a</sup> | 34.2±1.0 <sup>a</sup> | 1066±4.5 <sup>a</sup> | 197±1.1 <sup>a</sup> | 2.31±0.01 <sup>a</sup> | 141±3 <sup>a</sup> |
| T10       | 241±3 <sup>b</sup> | 2.40±0.1 <sup>b</sup> | 0.32±0.01 <sup>a</sup> | 4.4±0.2 <sup>a</sup> | 4.1±0.2 <sup>a</sup> | 38.0±1.2 <sup>a</sup> | 54.9±2.1 <sup>a</sup> | 33.9±1.0 <sup>a</sup> | 1061±4.5 <sup>a</sup> | 196±1.1 <sup>a</sup> | 2.30±0.01 <sup>a</sup> | 140±3 <sup>a</sup> |
| T11       | 240±3 <sup>b</sup> | 2.30±0.1 <sup>b</sup> | 0.32±0.01 <sup>a</sup> | 4.4±0.2 <sup>a</sup> | 4.0±0.2 <sup>a</sup> | 37.9±1.2 <sup>a</sup> | 54.7±2.1 <sup>a</sup> | 33.8±1.0 <sup>a</sup> | 1058±4.5 <sup>a</sup> | 195±1.1 <sup>a</sup> | 2.29±0.01 <sup>a</sup> | 139±3 <sup>a</sup> |

AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, Creat: Creatinine, MDA: Malondialdehyde, SOD: Superoxide dismutase, GSH: Glutathione, CAT: Catalase, IgG: Immunoglobulin G, IgA: Immunoglobulin A, T3: Triiodothyronine, T4: Thyroxine. Different superscript letters (a,b,c,d) within a column indicate significant differences ( $P < 0.05$ ). Values are presented as mean  $\pm$  SE. Treatments included T1: Negative control group, fed a basal diet without additives. T2/T3: Broilers supplemented with EB7 at 50 mg/kg (T2) or 200 mg/kg (T3) diets. T4/T5: Broilers supplemented with EB14 at 50 mg/kg (T4) or 200 mg/kg (T5) diets. T6/T7: Broilers supplemented with EB26 at 50 mg/kg (T6) or 200 mg/kg (T7) diets. T8: Positive control, broilers challenged with *Salmonella* but no probiotic. T9/T10/T11: Broilers challenged with *Salmonella* and treated with EB7 (T9), EB14 (T10), or EB26 (T11) at 200 mg/kg diets

(T8) showed the most notable increases in AST (269±3) and ALT (3.9±0.1), along with higher creatinine and uric acid levels, indicating clear hepatic and renal impairment. All probiotic-supplemented groups, especially at the 200 mg/kg dose (T3, T5, and T7), significantly lowered AST, ALT, and renal indices compared to both the control (T1) and the *Salmonella* group (T8). T3 was the most effective in lowering AST (220±3a) and ALT (2.1±0.1), and in positively affecting creatinine and uric acid levels.

MDA, a key indicator of oxidative damage, was highest in the *Salmonella*-only group (T8: 7.0±0.2) and lowest in the EB7 200 mg/kg group (T3: 3.8±0.2). EB14 and EB26 at 200 mg/kg also demonstrated clear protective effects. The antioxidant activities of SOD, GSH, and CAT were significantly elevated following probiotic supplementation, with the highest increase observed in EB7 (T3) (SOD: 40.2±1.2; GSH: 56.3±2.1a; CAT: 36.5±1.0) in comparison to both unchallenged and *Salmonella*-challenged controls.

**Immunity Markers:** Supplemented groups demonstrated clear improvements in humoral immunity. IgG and IgA reached peak levels in EB7 (T3 and T9), EB14 (T5 and T10), and EB26 (T7 and T11) treated birds at the highest dose, even under *Salmonella* challenge, while the challenge group (T8) showed the lowest values. Probiotics also tended to restore thyroid hormone (T3 and T4) levels toward control levels, counteracting the suppression caused by *Salmonella*. The findings confirmed that probiotic supplementation, especially EB7 at 200 mg/kg, significantly reduced hepatic and renal stress, decreased oxidative damage, and enhanced immunity in broilers, both in healthy conditions and under pathogen challenge. Probiotic effects depend on the isolate and dose, with statistically significant improvements compared to both the control and infection groups.

**Gene Expression Against *Salmonella* Infection:** The *Salmonella* challenge (T8) caused a remarkable increase in TLR4 (3.50-fold), TLR1A (3.20-fold), AvBD6 (3.00-fold), IL-1B (2.90-fold), IL-6 (3.50-fold), IFN- $\gamma$  (3.20-fold), TGF- $\beta$ 1 (2.70-fold), and cLEAP-2 (3.10-fold) compared to control (T1) ( $P < 0.01$  for all, a vs c). This robust innate immune activation and antimicrobial peptide production reflect the acute inflammatory response to infection, accompanied by a significant decrease in major histocompatibility complex (MHC, 0.70-fold), indicating suppression of adaptive antigen presentation. Supplementation with EB7 at 200 mg/kg (T3) before or after challenge (T9) significantly increased the expression of Toll-like receptors (TLRs), avian  $\beta$ -defensins (AvBDs), cytokines (IL-1 $\beta$ , IL-6, IFN- $\gamma$ ), and antimicrobial genes (e.g., TLR4: 2.50±0.14; cLEAP-2: 2.20±0.11), though to a lesser extent than the *Salmonella*-only group-suggesting immunomodulation without excessive inflammation.

**Table 7.** Effects of EB7, EB14, and EB26 treatments on gene expression profiles in *Salmonella*-challenged broilers

| Treatment | TLR4                         | TLR1LA                       | AvBD6                        | IL-1B                        | IL-6                         | IFN- $\gamma$                | TGF- $\beta$ 1               | MHC                          | cLEAP-2                      | ACSL1                        |
|-----------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|
| T1        | 1.00 $\pm$ 0.12 <sup>c</sup> | 1.00 $\pm$ 0.15 <sup>c</sup> | 1.00 $\pm$ 0.11 <sup>c</sup> | 1.00 $\pm$ 0.15 <sup>c</sup> | 1.00 $\pm$ 0.11 <sup>c</sup> | 1.00 $\pm$ 0.15 <sup>c</sup> | 1.00 $\pm$ 0.11 <sup>c</sup> | 1.00 $\pm$ 0.10 <sup>c</sup> | 1.00 $\pm$ 0.14 <sup>c</sup> | 1.00 $\pm$ 0.11 <sup>c</sup> |
| T2        | 1.80 $\pm$ 0.15 <sup>c</sup> | 1.60 $\pm$ 0.14 <sup>c</sup> | 1.70 $\pm$ 0.12 <sup>c</sup> | 1.30 $\pm$ 0.15 <sup>c</sup> | 1.40 $\pm$ 0.13 <sup>c</sup> | 1.60 $\pm$ 0.10 <sup>c</sup> | 1.30 $\pm$ 0.14 <sup>c</sup> | 1.20 $\pm$ 0.12 <sup>b</sup> | 1.50 $\pm$ 0.12 <sup>c</sup> | 1.20 $\pm$ 0.10 <sup>c</sup> |
| T3        | 2.50 $\pm$ 0.14 <sup>a</sup> | 2.20 $\pm$ 0.11 <sup>a</sup> | 2.30 $\pm$ 0.12 <sup>a</sup> | 1.90 $\pm$ 0.14 <sup>a</sup> | 2.30 $\pm$ 0.12 <sup>a</sup> | 2.40 $\pm$ 0.11 <sup>a</sup> | 1.60 $\pm$ 0.10 <sup>a</sup> | 1.80 $\pm$ 0.11 <sup>a</sup> | 2.20 $\pm$ 0.11 <sup>a</sup> | 1.70 $\pm$ 0.13 <sup>a</sup> |
| T4        | 1.60 $\pm$ 0.13 <sup>c</sup> | 1.40 $\pm$ 0.11 <sup>c</sup> | 1.50 $\pm$ 0.14 <sup>c</sup> | 1.20 $\pm$ 0.12 <sup>c</sup> | 1.30 $\pm$ 0.13 <sup>c</sup> | 1.50 $\pm$ 0.10 <sup>c</sup> | 1.20 $\pm$ 0.15 <sup>c</sup> | 1.10 $\pm$ 0.14 <sup>c</sup> | 1.30 $\pm$ 0.14 <sup>c</sup> | 1.10 $\pm$ 0.12 <sup>c</sup> |
| T5        | 2.20 $\pm$ 0.11 <sup>b</sup> | 2.00 $\pm$ 0.11 <sup>b</sup> | 2.10 $\pm$ 0.11 <sup>b</sup> | 1.70 $\pm$ 0.10 <sup>b</sup> | 2.00 $\pm$ 0.13 <sup>b</sup> | 2.30 $\pm$ 0.12 <sup>b</sup> | 1.50 $\pm$ 0.14 <sup>b</sup> | 1.60 $\pm$ 0.13 <sup>a</sup> | 2.00 $\pm$ 0.14 <sup>b</sup> | 1.50 $\pm$ 0.13 <sup>b</sup> |
| T6        | 1.50 $\pm$ 0.11 <sup>c</sup> | 1.30 $\pm$ 0.12 <sup>c</sup> | 1.40 $\pm$ 0.13 <sup>c</sup> | 1.10 $\pm$ 0.13 <sup>c</sup> | 1.20 $\pm$ 0.11 <sup>c</sup> | 1.30 $\pm$ 0.12 <sup>c</sup> | 1.10 $\pm$ 0.11 <sup>c</sup> | 1.00 $\pm$ 0.12 <sup>c</sup> | 1.20 $\pm$ 0.13 <sup>c</sup> | 1.00 $\pm$ 0.15 <sup>c</sup> |
| T7        | 2.00 $\pm$ 0.10 <sup>b</sup> | 1.80 $\pm$ 0.13 <sup>b</sup> | 1.70 $\pm$ 0.13 <sup>c</sup> | 1.40 $\pm$ 0.12 <sup>b</sup> | 1.80 $\pm$ 0.15 <sup>b</sup> | 1.90 $\pm$ 0.11 <sup>b</sup> | 1.30 $\pm$ 0.10 <sup>c</sup> | 1.40 $\pm$ 0.10 <sup>b</sup> | 1.60 $\pm$ 0.14 <sup>b</sup> | 1.30 $\pm$ 0.11 <sup>b</sup> |
| T8        | 3.50 $\pm$ 0.14 <sup>a</sup> | 3.20 $\pm$ 0.12 <sup>a</sup> | 3.00 $\pm$ 0.10 <sup>a</sup> | 2.90 $\pm$ 0.11 <sup>a</sup> | 3.50 $\pm$ 0.14 <sup>a</sup> | 3.20 $\pm$ 0.14 <sup>a</sup> | 2.70 $\pm$ 0.14 <sup>a</sup> | 0.70 $\pm$ 0.12 <sup>a</sup> | 3.10 $\pm$ 0.12 <sup>a</sup> | 1.80 $\pm$ 0.12 <sup>a</sup> |
| T9        | 2.10 $\pm$ 0.13 <sup>b</sup> | 1.80 $\pm$ 0.11 <sup>b</sup> | 2.00 $\pm$ 0.13 <sup>b</sup> | 1.70 $\pm$ 0.12 <sup>b</sup> | 1.90 $\pm$ 0.15 <sup>b</sup> | 2.00 $\pm$ 0.12 <sup>b</sup> | 1.40 $\pm$ 0.14 <sup>b</sup> | 1.60 $\pm$ 0.12 <sup>a</sup> | 1.70 $\pm$ 0.13 <sup>b</sup> | 1.50 $\pm$ 0.14 <sup>b</sup> |
| T10       | 2.00 $\pm$ 0.14 <sup>b</sup> | 1.70 $\pm$ 0.13 <sup>b</sup> | 1.80 $\pm$ 0.11 <sup>b</sup> | 1.60 $\pm$ 0.10 <sup>b</sup> | 1.80 $\pm$ 0.14 <sup>b</sup> | 1.90 $\pm$ 0.11 <sup>b</sup> | 1.40 $\pm$ 0.14 <sup>b</sup> | 1.50 $\pm$ 0.14 <sup>b</sup> | 1.60 $\pm$ 0.12 <sup>b</sup> | 1.40 $\pm$ 0.11 <sup>b</sup> |
| T11       | 1.90 $\pm$ 0.10 <sup>b</sup> | 1.60 $\pm$ 0.11 <sup>c</sup> | 1.70 $\pm$ 0.10 <sup>c</sup> | 1.50 $\pm$ 0.15 <sup>b</sup> | 1.70 $\pm$ 0.13 <sup>b</sup> | 1.80 $\pm$ 0.13 <sup>b</sup> | 1.30 $\pm$ 0.14 <sup>c</sup> | 1.40 $\pm$ 0.13 <sup>b</sup> | 1.50 $\pm$ 0.10 <sup>c</sup> | 1.30 $\pm$ 0.10 <sup>b</sup> |

Different superscript letters (a,b,c) within a column indicate significant differences ( $P < 0.05$ ). Values are presented as mean  $\pm$  SD. Treatments included: T1: Negative control group, fed a basal diet without additives. T2/T3: Broilers supplemented with EB7 at 50 mg/kg (T2) or 200 mg/kg (T3) diets. T4/T5: Broilers supplemented with EB14 at 50 mg/kg (T4) or 200 mg/kg (T5) diets. T6/T7: Broilers supplemented with EB26 at 50 mg/kg (T6) or 200 mg/kg (T7) diets. T8: Positive control, broilers challenged with *Salmonella* but no probiotic. T9/T10/T11: Broilers challenged with *Salmonella* and treated with EB7 (T9), EB14 (T10), or EB26 (T11) at 200 mg/kg diets

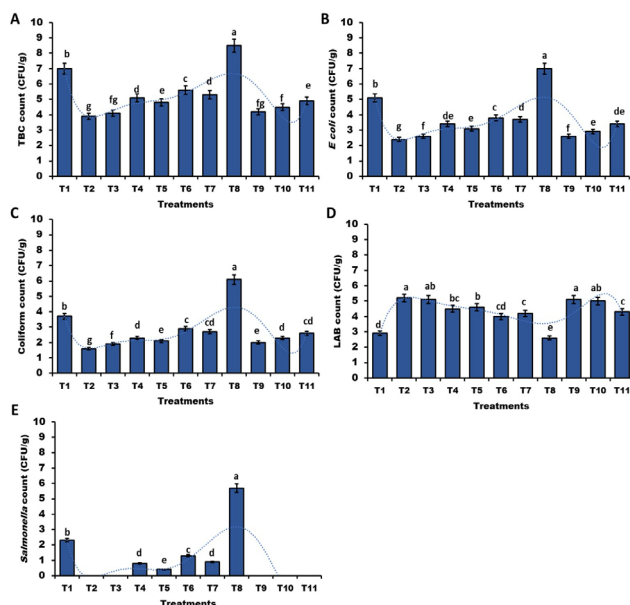


**Fig 2.** Microbial counts (total bacterial count (TBC), *E. coli*, coliform, lactic acid bacteria (LAB), and *Salmonella*) in broiler guts affected by different concentrations of EB7, EB14, and EB26 on *Salmonella*-challenged broilers. Treatments included: T1: Negative control group, fed a basal diet without additives. T2/T3: Broilers supplemented with EB7 at 50 mg/kg (T2) or 200 mg/kg (T3) diets. T4/T5: Broilers supplemented with EB14 at 50 mg/kg (T4) or 200 mg/kg (T5) diets. T6/T7: Broilers supplemented with EB26 at 50 mg/kg (T6) or 200 mg/kg (T7) diets. T8: Positive control, broilers challenged with *Salmonella* but no probiotic. T9/T10/T11: Broilers challenged with *Salmonella* and treated with EB7 (T9), EB14 (T10), or EB26 (T11) at 200 mg/kg diets

Probiotic treatments with EB14 and EB26 induced moderate increases in gene expression, but consistently lower than with EB7 or *Salmonella* alone (Table 7). The results showed strong *Salmonella*-induced upregulation of immune genes, with targeted probiotic treatments

reducing excessive pro-inflammatory responses and supporting a balanced, protective immune profile in broilers. The largest fold-changes were observed for genes related to pathogen recognition (TLRs), cytokines (IL-1 $\beta$ , IL-6, IFN- $\gamma$ ), and antimicrobial peptides (AvBD6 and cLEAP-2), but only EB7 at a high dose achieved significant, strong upregulation while avoiding excessive immunopathology.

**Microbial Count:** Fig. 2 shows that T8 (*Salmonella* challenge) experienced a significant increase in pathogenic bacteria (*E. coli* and *Salmonella*) and total bacterial load (TBC), along with a decrease in beneficial lactic acid bacteria (LAB), indicating severe dysbiosis. All probiotic treatments (T2-T7, T9, and T11) significantly reduced *E. coli*, coliforms, *Salmonella*, and TBC compared to T1 and T8, with the most notable reductions seen in T3 (EB7 high dose) and T9 (*Salmonella* + EB7). LAB counts increased substantially across all probiotic groups, especially in high-dose and combination treatments (T3, T5, T9, and T10), supporting targeted enrichment of beneficial microbiota. Groups treated with EB7 (T3, and T9) showed the lowest pathogenic counts and the highest LAB recovery, closely followed by EB14 (T5, and T10), demonstrating the dose-dependent and strain-specific ability of the probiotics to modulate microbiota. Overall, these results showed that probiotic supplementation, especially at higher doses, effectively restores gut microbial balance, inhibits enteric pathogens, and improves microbiological safety and gut health of broilers.

## DISCUSSION

Salmonellosis is one of the most important infectious diseases affecting poultry worldwide, both in terms of

production losses and public health concerns. Caused mainly by *S. enterica* subspecies *enterica*, this disease causes significant economic damage, including slower growth rates, poor feed efficiency, increased mortality (which can reach 20-50% in endemic areas), and greater vulnerability to other illnesses. Outbreaks in both large-scale and backyard systems have led to major food recalls, higher healthcare costs, and supply chain disruptions, sometimes costing millions of dollars each year in leading poultry-producing countries [30,31].

The persistence of *Salmonella* is fueled by multiple risk factors: large flock sizes, poor biosecurity, mixing of birds from different sources, floor housing, inadequate sanitation, and the widespread use of antibiotics as growth promoters and for infection control. Although various antibiotics have initially been effective, prolonged use has led to a sharp increase in multidrug-resistant *Salmonella* strains and other resistant bacteria in poultry settings. This trend now endangers not only flock health but also consumer safety and the sustainability of poultry industries worldwide [1,30].

As regulatory agencies and producers navigate control measures, including new standards, incentives, and advanced diagnostics, recent scientific and industry consensus has shifted toward prevention. Prebiotics, probiotics, and strict on-farm management are now recognized as essential elements in any sustainable *Salmonella* control plan. *Bacillus* and *Paenibacillus* species, in particular, have become leading probiotic options due to their durability, safety, and ability to fight pathogenic bacteria [7].

The isolation and identification of potent *Bacillus* and *Paenibacillus* strains support a growing body of evidence that these genera are promising probiotic candidates with significant antimicrobial properties for poultry applications. Their distinct antibacterial activity against important pathogens such as *S. aureus* and *Pseudomonas aeruginosa* aligns with findings reported by Alagawany et al. [9]; Zhou et al. [12], in which *Bacillus* and *Paenibacillus* isolates showed broad-spectrum inhibition of poultry pathogens, reinforcing their potential to reduce bacterial load in commercial flocks. The accuracy of MALDI-TOF MS identification, consistent with these studies, confirms the reliability of proteomic methods for species-level microbial classification, aligning with Calderaro and Chezzi [32], who emphasized MALDI-TOF MS as a powerful tool for differentiating closely related *Bacillus* species based on unique spectral profiles.

The triple isolates demonstrated multiple plant growth-promoting traits, including solubilizing phosphate, producing indole acetic acid, nitrogen fixation, and ACC utilization, which not only endorse their environmental

adaptability but also align with observations by previous researchers [12,33], who described the biostimulant and stress mitigation potential of *Bacillus* and *Paenibacillus* strains in agriculture and animal husbandry. These multifunctional properties indicate that such probiotics benefit not only pathogen control but also promote intestinal homeostasis and resilience.

The strong tolerance of the isolates to acidic pH and bile salts emphasizes their ability to survive in the harsh gastrointestinal environment. This key trait is supported by recent studies, including those by Zhang et al. [34] and Alagawany et al. [9], which have confirmed that *Bacillus*-based probiotics can effectively colonize and exert effects within the avian gut. The exceptional survivability of isolate EB7, especially under pH and bile salt challenges, also exemplifies the robustness typical of *P. polymyxa* AM20, a trait linked to improved broiler health and pathogen resistance.

Notably, the antibiotic sensitivity profile of isolate EB7 indicated it is a safe candidate for probiotic use, with susceptibility to major antibiotics. This finding aligns with concerns about the transfer of resistance genes from probiotic strains [35]. It also supports the caution expressed by Khairunnesa et al. [36], who emphasized the importance of confirming antibiotic susceptibility before using probiotic strains harboring resistance. The multidrug resistance observed in other isolates but not in EB7 further supports its use to help prevent the spread of antibiotic resistance while offering therapeutic benefits.

In terms of antioxidant capabilities, the dose-dependent increase in radical scavenging capacity and the significantly lower IC<sub>50</sub> values for EB7 confirm enhanced free radical neutralization—a key feature in maintaining gut and systemic health during infection-related oxidative stress. These findings align with observations by Zhou et al. [12]; Saeed et al. [37], who documented increased antioxidant enzyme activities and serum antioxidant status following dietary supplementation with *Bacillus* and *Paenibacillus* probiotics. Such antioxidant effects can reduce cellular damage and inflammation associated with *Salmonella* infection, supporting immune homeostasis.

The antibacterial assays showed that EB7 not only maintained strong effectiveness against a wide range of pathogens but also demonstrated dose-responsive inhibition, consistent with other recent studies where *Bacillus subtilis* and *P. polymyxa* strains significantly reduced pathogenic bacterial colonization *in vitro* and in poultry models [9,38]. The notable suppression of *Salmonella typhi*, *E. coli*, and *Staphylococcus* spp. highlights the broad-spectrum potential of these isolates, reinforcing their role as potent biological control agents in food animal production.

The *in vivo* efficacy of these isolates in improving growth performance during a *Salmonella* challenge demonstrated their ability to counteract infection-induced growth retardation, within the limits of pathogenic stress. This aligns with the conclusions of Zhang et al.<sup>[34]</sup> and Nam et al.<sup>[39]</sup>, who reported improved feed conversion ratios, body weight gains, and health indices in broilers receiving *Bacillus* and mixed probiotics during a bacterial challenge. The dose-dependent improvements, especially at 200 mg/kg for EB7, highlight the importance of optimal dosing to maximize probiotic benefits.

Serum biochemical improvements, especially in liver and kidney markers, antioxidant enzymes, and immunoglobulin levels, align with extensive literature showing that probiotic supplementation reduces oxidative stress and liver inflammation caused by pathogen exposure<sup>[9,12]</sup>. The increased activities of SOD, GSH, and CAT further demonstrate how these isolates can regulate oxidative balance and inflammatory responses. Additionally, immune markers such as IgG and IgA secretion indicate a direct enhancement of systemic immunity. In parallel, the restoration of thyroid hormone levels reflects improved endocrine homeostasis, suggesting that the treatment confers coordinated immune and thyroid benefits, similar to those reported in previous studies<sup>[36,37]</sup>.

Gene expression profiling demonstrated probiotic-mediated immunomodulation, reducing excessive pro-inflammatory cytokine overexpression while boosting pathogen recognition receptors and antimicrobial peptides. These dynamic immune adjustments are consistent with recent high-resolution transcriptomic studies, which depict *Bacillus* and *Paenibacillus* strains as fine-tuners of innate and adaptive immunity, balancing effective pathogen clearance with controlled tissue damage<sup>[9,35]</sup>. The increased expression of MHC-II and regulatory cytokines after probiotic supplementation also indicates improved antigen presentation and immune resolution, which are essential for maintaining health during infectious challenges.

Microbiological analyses showing decreased pathogenic bacterial loads and increased beneficial LAB reflect the well-known mechanism of competitive exclusion and gut microbial modulation by probiotics, as reported by Zhou et al.<sup>[12]</sup>; Nam et al.<sup>[39]</sup>. This microbial balance directly supports gut integrity and resistance to disease<sup>[40]</sup>. The dose-dependent improvements in LAB populations, especially with EB7 treatment, highlight that potency and strain specificity are crucial factors in probiotic effectiveness.

The superior profiles seen with EB7 confirm its potential for further development as an alternative to antibiotics in broiler production systems. This study not only supports existing knowledge but also addresses significant gaps in the detailed

biological characterization and comparative assessment of local isolates to inform integrated *Salmonella* control strategies in poultry. In conclusion, this study illustrates the potential of selected probiotic isolates-EB7 (*P. polymyxa*), EB14 (*B. licheniformis*), and EB26 (*B. mycoides*) as natural alternatives to antibiotics for enhancing health and resistance to *Salmonella* in broiler chickens. Phenotypic and proteomic analyses demonstrated multifunctional benefits, including robust antibacterial and antioxidant activities, gastrointestinal resilience, safety -exhibiting no virulence or antibiotic sensitivity- and modulation of immunity and gut microbiota. EB7 proved particularly effective, corroborating previous research that indicates *Bacillus/Paenibacillus* supplementation enhances growth, resistance to enteric disease, antioxidant capacity, and immunity for sustainable poultry production. These indigenous strains successfully restored productivity, promoted beneficial microbiota, and reduced pathogen loads in challenged birds, underscoring strain-specific, dose-dependent effects. Consequently, EB7, EB14, and EB26 are deserving of further investigation and commercial development as tailored, biotechnological solutions for poultry health management. While this study demonstrates the promising multifunctional benefits of probiotic isolates EB7 (*P. polymyxa*), EB14 (*B. licheniformis*), and EB26 (*B. mycoides*) as antibiotic alternatives for *Salmonella* resistance and broiler health enhancement, limitations include controlled experimental conditions limiting field extrapolation, short trial durations, reliance on targeted phenotypic and gene expression assays without comprehensive multi-omics profiling, and the absence of molecular screening for antibiotic resistance genes which precludes definitive assessment of horizontal gene transfer risks. Future studies should prioritize long-term field trials, multi-pathogen challenges, dose optimization, synbiotic combinations using metagenomics/metabolomics, and PCR-based resistome profiling for safety validation. For commercial translation, prioritize EB7 for GRAS/FDA approval via comprehensive safety-efficacy dossiers, scale-up production through fermentation-lyophilization at 10<sup>8</sup>-10<sup>9</sup> CFU/kg for feed additives, targeting 3-5% FCR improvement and 10-20% mortality reduction in antibiotic-free poultry markets within 1-2 years.

## DECLARATIONS

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

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

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**Author Contributions:** Conceptualization, EAB, and LAB, formal analysis, EAB, and LAB, investigation, EAB, and LAB, data curation, EAB, and LAB, writing original draft preparation, EAB, and LAB, writing final manuscript and editing, EAB, and LAB, visualization and methodology, EAB, and LAB. All authors have read and agreed to the published version of the manuscript.

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## RESEARCH ARTICLE

# Radiographic Morphometry of the Feline Hip Joint: Clinical Associations and Reference Benchmarks

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## Abstract

Interest in feline orthopedic diseases has increased in recent years, highlighting the need for standardized radiographic criteria for diagnosing hip dysplasia in cats, similar to those used in dogs. This prospective study investigated associations between morphometric parameters and radiographic hip scores in 56 client-owned cats. Standard ventrodorsal pelvic radiographs were obtained, and the Norberg angle (NA) and femoral inclination angle (FIA) were measured bilaterally. Hips were scored using an adapted BVA/KC hip score scheme, with NA analyzed separately and secondary changes categorized. Group differences were analyzed with t-tests/ANOVA or Mann-Whitney U/Kruskal-Wallis as appropriate (Bonferroni where applicable); associations used Spearman's  $\rho$ ; predictors were modeled with multiple linear regression. In non-lame cats, NA values were  $91.46 \pm 6.80^\circ$  (right) and  $92.55 \pm 6.90^\circ$  (left), while FIA values were  $121.71 \pm 5.57^\circ$  (right) and  $124.83 \pm 5.81^\circ$  (left). Lame cats had significantly lower NA and higher FIA than non-lame cats, along with higher primary change and total scores. NA correlated negatively with primary and secondary change scores, secondary change categories (SCcat), and total scores, whereas FIA correlated positively. Across SCcat, NA decreased and primary change scores increased with severity. Younger cats showed higher NA values, while sex and fertility status had secondary effects. In multivariable models, NA was a negative predictor and FIA a positive predictor of total scores bilaterally. These results demonstrate that decreased NA and increased FIA are strongly associated with radiographic osteoarthritic changes, underscoring the role of hip geometry in the pathogenesis of feline hip dysplasia and osteoarthritis.

**Keywords:** Cats, Hip dysplasia, Lameness, Orthopedics, Radiography

## INTRODUCTION

Hip dysplasia (HD) is a developmental disorder of the coxofemoral joint characterized by laxity, incongruity between the femoral head and acetabulum, subluxation, and subsequent degenerative changes <sup>[1-3]</sup>. Although extensively studied in dogs, feline HD has received comparatively limited attention, yet reports indicate that it is more common than previously assumed <sup>[1,4]</sup>. Breed-dependent variation is evident, with purebred cats such as Maine Coons and Scottish Folds showing higher susceptibility than domestic shorthairs <sup>[1,5,6]</sup>. Similar to other species, feline HD is considered heritable with a polygenic mode of inheritance <sup>[1,4,6]</sup>. However, in contrast to dogs, large-scale screening or breeding programs in cats are less frequently reported, and available epidemiological data provide insight into disease distribution <sup>[1,7]</sup>.

Radiography remains the gold standard for diagnosing and phenotypically evaluating HD <sup>[2,8,9]</sup>. Standard ventrodorsal pelvic radiographs are routinely employed to assess

joint congruity and acetabular coverage of the femoral head <sup>[2,5,6]</sup>. Yet, direct application of canine diagnostic thresholds to cats is problematic due to anatomical differences. Cats possess shallower acetabula, meaning the canine benchmark of >50% femoral head coverage may lead to false-positive diagnoses of dysplasia <sup>[2,5,6,9]</sup>. The Norberg angle (NA) is a key radiographic parameter for evaluating hip congruity and reported reference values in cats vary across studies. Small-scale studies have reported NA values between  $\sim 92^\circ$  and  $99^\circ$  in clinically healthy cats, with lower values in dysplastic or osteoarthritic individuals <sup>[10-12]</sup>. However, these studies differed in sample size, breed composition, and age distribution, emphasizing the importance of reporting reference values across defined populations.

Another morphometric parameter of biomechanical importance is the femoral inclination angle (FIA), defined by the orientation of the femoral neck relative to the shaft. Deviations in FIA alter joint loading patterns and may predispose to subluxation, luxation, or degenerative joint



disease<sup>[13-16]</sup>. Although well defined in canine orthopedics, considering that reference ranges or their relationship with HD have been reported in only a few studies in cats<sup>[17,18]</sup>, it is thought that more research is needed in this area. Establishing reliable feline-specific values for both NA and FIA are therefore clinically relevant for diagnosis, prognosis, and surgical planning.

In canine medicine, the British Veterinary Association/Kennel Club (BVA/KC) hip scoring system is widely applied to evaluate radiographic features of hip dysplasia and secondary osteoarthritic changes, providing a structured and semi-quantitative assessment of hip joint morphology<sup>[19]</sup>. Although feline-specific hip scoring systems are still under development, adaptation of established canine schemes offers a pragmatic approach to standardize radiographic assessment of feline hip joints. However, ongoing efforts to establish species-specific consensus cut-off values and reference ranges underscore the importance of continued research in cats.

The present study aimed to (1) investigate associations between morphometric parameters (NA and FIA) and radiographic dysplasia scores in cats, (2) evaluate the influence of demographic factors (age, sex, neuter status, and breed), and (3) generate reference data for feline hips using an adapted BVA/KC scoring system. By independently analyzing the Norberg angle and integrating osteoarthritic features through secondary change classification, this study provides new insights into feline-specific diagnostic markers of HD and contributes reference values that may improve both clinical evaluation and breeding strategies. We hypothesized that lower NA and higher FIA would be associated with greater radiographic hip scores, and that demographic factors and secondary change categories would further influence these associations.

## MATERIAL AND METHODS

### Ethical Statement

This study was approved by the Aydin Adnan Menderes University Animal Experiments Local Ethics Committee (Approval no: 64583101/2025/134 on 14.08.2025). Owners provided informed consent prior to participation.

### Animals

This prospective study enrolled 56 client-owned cats of various breeds, ages, and sexes, presented to the Research and Practice Animal Hospital, Faculty of Veterinary Medicine, Aydin Adnan Menderes University. Cats of any breed, sex, and neuter status were eligible if they had no other orthopedic disorders of the skeletal system, aside from confirmed or suspected HD. Accordingly, the study population included both clinically non-lame cats and cats exhibiting hindlimb lameness attributable to hip

pathology. Cats were excluded entirely from the study if they had a history of pelvic or femoral fractures (including the acetabulum, femoral head, or femoral neck), major trauma, or were younger than 1 year of age. Cats under 1 year of age were excluded because incomplete skeletal maturity may affect hip joint morphology and radiographic measurements, potentially confounding the assessment of HD-related changes. Cats exhibiting lameness not attributable to the hindlimbs or hip joints (e.g., forelimb lameness or neurologic causes) were also excluded to ensure that any observed locomotor impairment could be reliably associated with hip pathology. For each enrolled cat, demographic data including age, breed, sex, and reproductive status were recorded. Age was categorized by rounding to the nearest whole year.

### Clinical and Orthopedic Examination

All cats underwent a comprehensive physical and orthopedic examination performed by the same surgeon (Y.A.O.). General examinations included history, inspection, palpation, percussion, and auscultation. Orthopedic assessment focused on lameness, supplemented by the supination-pronation test, biceps traction test, cranial drawer motion, tibial compression test, and Ortolani maneuver.

### Radiographic Imaging, Morphometric Measurements and Hip Scoring

Cats eligible for inclusion in the study underwent ventrodorsal pelvic radiography. Radiographs were obtained without general anesthesia or sedation, using gentle manual restraint performed by experienced personnel. Positioning was achieved with the hindlimbs extended caudally and the patellae centered to obtain acceptable pelvic symmetry. Sedation was not routinely used in order to minimize pharmacological intervention in client-owned cats, largely due to owner preference or refusal of sedation or anesthesia for diagnostic radiography alone, and because satisfactory positioning could be achieved through gentle manual restraint in the majority of cases. When initial images showed suboptimal positioning or technical imperfections (e.g., pelvic rotation or limb asymmetry), additional radiographs were obtained during the same session to achieve diagnostically acceptable images. Radiographs that remained inadequate despite repositioning were excluded from analysis. No averaging of measurements across multiple radiographic exposures was performed; instead, only the final image meeting predefined quality criteria was included for morphometric and scoring analyses. Digital evaluation (Animalcare, Mindray, Shenzhen, China) and software (Geogebra, Linz, Austria) were used to measure the NA and FIA bilaterally. The NA was defined as the angle formed by a line connecting the centers of both femoral

heads and a line from the femoral head center to the craniolateral acetabular rim (Fig. 1-a). The FIA was measured using the SYMAX method, as the angle between the femoral neck axis and the femoral shaft axis (Fig. 1-b) [8,20]. Each measurement was repeated twice by the same observer, and the mean of the two measurements was used for analysis. Radiographs with inadequate positioning or technical errors were excluded.

Hip joints were evaluated using the British Veterinary Association/Kennel Club (BVA/KC) hip scoring system [19], originally developed for dogs but adapted for feline use. The seven designated anatomical sites (subluxation, cranial acetabular edge, dorsal acetabular edge, cranial effective acetabular rim, acetabular fossa, caudal acetabular edge, femoral head and neck exostoses, and femoral head recontouring) were scored according to the scheme. In this study, the Norberg angle was excluded from the cumulative score and analyzed independently due to the absence of established feline reference ranges and the aim of evaluating its diagnostic role separately. Thus, each hip was scored out of 47 points, yielding a bilateral maximum of 94.

For each hip, primary change (subluxation) and secondary changes (degenerative alterations) were scored and combined to generate total hip scores for right and left hips and cumulatively. To allow categorical assessment of osteoarthritic severity, secondary changes were further stratified into the SCcat. Thresholds were pragmatically defined based on the observed distribution of scores and scaled with reference to the canine BVA scheme: none/minimal (0-5), mild (6-10), moderate (11-15), and severe ( $\geq 16$ ). SCcat categories were used in correlation and group comparison analyses.

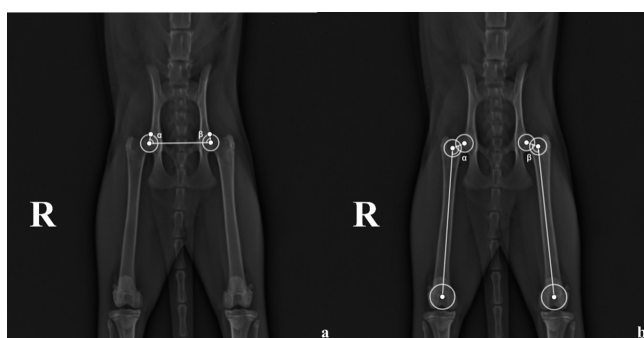


**Fig 1.** Radiographic measurement of the NA and FIA in cats. (a) NA measurement. The centers of the right and left femoral heads were identified by manually placing circular markers using measurement software. A reference line connecting the centers of both femoral heads and a second line extending from each femoral head center to the corresponding craniolateral acetabular rim were manually drawn. The Norberg angles were automatically calculated by the software and are displayed as  $\alpha$  (right hip) and  $\beta$  (left hip); (b) FIA measurement. The FIA was measured using the SYMAX method. The femoral neck axis and femoral shaft axis were manually defined on the same radiograph, after which the software automatically calculated the angles, displayed as  $\alpha$  (right femur) and  $\beta$  (left femur). Circular markers and reference lines indicate operator-defined anatomical reference points and axes

## Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics version 25.0 (IBM Corp., Armonk, NY, USA). Data were first transferred into Microsoft Excel (Microsoft Corp., Redmond, WA, USA) for recording and preprocessing. The distribution of continuous variables was assessed using the Shapiro-Wilk test, complemented by visual inspection of histograms and Q-Q plots. Descriptive statistics were expressed as mean $\pm$ standard deviation (SD) for normally distributed variables and as median with minimum-maximum values for non-normally distributed variables. Categorical variables were summarized as frequencies and percentages.

Lameness status was classified as “lame” or “not lame” and included as a grouping variable in subsequent analyses. Comparisons between right and left hips were conducted using paired-samples t-tests for normally distributed parameters and Wilcoxon signed-rank tests otherwise. Group comparisons according to sex, neuter status, and lameness were carried out with independent-samples t-tests or Mann-Whitney U tests, while differences across age groups, breeds, and SCcat categories were assessed with one-way ANOVA or Kruskal-Wallis tests, followed by Bonferroni-adjusted post hoc tests where appropriate. Associations between morphometric measures (NA and FIA) and radiographic scores (primary and secondary change scores, total score, SCcat) were evaluated using Spearman’s correlation coefficients.

Multiple linear regression analyses were used to identify predictors of right and left total hip scores. Independent variables entered into the models included age, sex, neuter status, NA, FIA, and breed, with tabby cats set as the reference category in dummy coding. Although hip scores were not normally distributed, regression analyses were retained because assumptions apply to model residuals rather than raw outcomes. Residual plots confirmed approximate normality and homoscedasticity. Multicollinearity was assessed via tolerance and variance inflation factor (VIF) values ( $<10$ ), and model robustness was further evaluated with Cook’s distance and leverage statistics to rule out influential outliers. A P-value of  $<0.05$  was considered statistically significant for all analyses.

## RESULTS

### Association of Lameness with Hip Morphometric Parameters and Hip Scores

Table 1 presents the comparison of hip morphometric variables and hip scores between lame and non-lame cats. On both sides, lame cats had statistically significantly lower NA values ( $P=0.036$  and  $P=0.001$  for right and left hips, respectively) and statistically significantly higher FIA values ( $P=0.006$  and  $P=0.042$  for right and left

hips, respectively). Primary change (PC) scores were also statistically significantly higher in lame cats compared with non-lame cats ( $P=0.048$  and  $P=0.012$  for right and left hips, respectively), whereas SC scores did not differ significantly between groups ( $P>0.05$ ). Furthermore, SCcat classifications and total hip scores for each hip and cumulatively were consistently higher in lame cats ( $P<0.05$ ; [Table 1](#)).

### Correlation Analyses

Correlation matrices for the right and left hips are presented in [Table 2](#) and [Table 3](#), respectively.

For the right hip ([Table 2](#)), the NA was negatively correlated

with several hip score components, including PC score, SC score, SCcat, hip score, and the cumulative score ( $\rho=-0.272$  to  $-0.464$ ,  $P<0.05$ ; [Table 2](#)). The FIA was positively correlated with hip score components, with significant associations for PC score, total PC score, the right hip score, and the cumulative score ( $\rho=0.315$ -.429,  $P<0.05$ ). Strong positive associations were observed among hip score components themselves, particularly between PC and SC scores ( $\rho=0.337$ ,  $P=0.011$ ) and across total and cumulative scores ( $\rho=0.671$ -.875,  $P<0.001$ ; [Table 2](#)).

For the left hip ([Table 3](#)), the NA showed strong negative correlations with hip score components, including PC score, SC score, SCcat, and total hip score ( $\rho=-0.675$  to  $-0.530$ ,  $P<0.001$ ; [Table 3](#)). The FIA correlated positively with several components, with significant associations for PC score ( $\rho=0.431$ ,  $P=0.001$ ), SCcat ( $\rho=0.274$ ,  $P=0.041$ ), and the total hip score ( $\rho=0.305$ ,  $P=0.022$ ). Strong positive correlations were also observed among hip score components, particularly between PC and SC scores ( $\rho=0.523$ ,  $P<0.001$ ) and across total hip scores ( $\rho=0.761$ -.918,  $P<0.001$ ; [Table 3](#)).

### Group Comparisons (Age, Breed, and Demographics)

**Age Comparisons:** Hip morphometric parameters and hip scores according to age groups are presented in [Table 4](#). Both right and left NA values were higher in younger cats compared with older cats ( $P=0.048$  and  $P=0.011$ , respectively; [Table 4](#)). Specifically, cats aged 1-2 years had significantly greater NA values than cats aged 4-6 years ([Table 4](#)). The FIA values showed a slight decrease with age, although these differences were not statistically significant ( $P>0.05$ ). Primary, secondary, and total hip scores did not vary significantly across age groups ([Table 4](#)).

**Breed Comparisons:** Breed-related comparisons of hip morphometric parameters and hip scores are shown in [Table 5](#). No significant breed-related differences were detected in NA and FIA values, or hip score components

**Table 1.** Hip morphometric variables and hip scores according to lameness status (means $\pm$ SD for angles; medians [min-max] for scores)

| Variables        | Hip   | Lameness Status   |                   | P-value |
|------------------|-------|-------------------|-------------------|---------|
|                  |       | Non-Lame (n=37)   | Lame (n=19)       |         |
| NA               | Right | 91.46 $\pm$ 6.80  | 83.41 $\pm$ 9.34  | 0.036   |
|                  | Left  | 92.55 $\pm$ 6.90  | 84.40 $\pm$ 8.88  | 0.001   |
| FIA              | Right | 121.71 $\pm$ 5.57 | 127.84 $\pm$ 4.66 | 0.006   |
|                  | Left  | 124.83 $\pm$ 5.81 | 128.30 $\pm$ 5.32 | 0.042   |
| PC               | Right | 2 (0-10)          | 4 (0-11)          | 0.048   |
|                  | Left  | 2 (0-9)           | 4 (1-8)           | 0.012   |
| SC               | Right | 6 (0-15)          | 7 (1-14)          | 0.326   |
|                  | Left  | 8 (1-18)          | 10 (4-25)         | 0.118   |
| SCcat            | Right | 2 (1-3)           | 2 (1-4)           | 0.041   |
|                  | Left  | 2 (1-3)           | 2 (1-4)           | 0.030   |
| Total            | Right | 9 (0-23)          | 11 (5-25)         | 0.038   |
|                  | Left  | 8 (1-23)          | 12 (4-26)         | 0.030   |
| Cumulative Score |       | 19 (5-40)         | 23 (9-42)         | 0.041   |

NA: Norberg angle; FIA: Femoral inclination angle; PC: Primary change score, SC: Secondary changes score; SCcat: Categorized secondary change score

**Table 2.** Right hip correlation matrix among morphometric measures and hip scores (n=56)

| Variables           | 1 | 2     | 3        | 4       | 5       | 6        | 7       | 8        | 9        |
|---------------------|---|-------|----------|---------|---------|----------|---------|----------|----------|
| 1. Right NA         |   | 0.016 | -0.433** | -0.299* | -0.272* | -0.364** | -0.328* | -0.464** | -0.387** |
| 2. Right FIA        |   |       | 0.330*   | 0.250   | 0.211   | 0.429**  | 0.228   | 0.315*   | 0.338*   |
| 3. Right PC         |   |       |          | 0.337*  | 0.401** | 0.836**  | 0.315*  | 0.757**  | 0.607**  |
| 4. Right SC         |   |       |          |         | 0.610** | 0.329*   | 0.783** | 0.855**  | 0.642**  |
| 5. Right SCcat      |   |       |          |         |         | 0.412**  | 0.390** | 0.520**  | 0.481**  |
| 6. Total PC         |   |       |          |         |         |          | 0.452** | 0.671**  | 0.783**  |
| 7. Total SC         |   |       |          |         |         |          |         | 0.710**  | 0.875**  |
| 8. Right Hip Score  |   |       |          |         |         |          |         |          | 0.775**  |
| 9. Cumulative Score |   |       |          |         |         |          |         |          |          |

NA: Norberg angle; FIA: Femoral inclination angle; PC: Primary change score, SC: Secondary changes score; SCcat: Categorized secondary changes. \*  $P<0.05$ , \*\*  $P<0.01$ , two-tailed

**Table 3.** Left hip correlation matrix among morphometric measures and hip scores (n=56)

| Variables           | 1 | 2      | 3        | 4        | 5        | 6        | 7        | 8        | 9        |
|---------------------|---|--------|----------|----------|----------|----------|----------|----------|----------|
| 1. Left NA          |   | -0.233 | -0.675** | -0.562** | -0.546** | -0.590** | -0.441** | -0.645** | -0.530** |
| 2. Left FIA         |   |        | 0.431**  | 0.210    | 0.274*   | 0.405**  | 0.237    | 0.305*   | 0.344**  |
| 3. Left PC          |   |        |          | 0.523**  | 0.499**  | 0.795**  | 0.431**  | 0.806**  | 0.693**  |
| 4. Left SC          |   |        |          |          | 0.688**  | 0.430**  | 0.823**  | 0.918**  | 0.785**  |
| 5. Left SCcat       |   |        |          |          |          | 0.477**  | 0.612**  | 0.590**  | 0.572**  |
| 6. Total PC         |   |        |          |          |          |          | 0.452**  | 0.654**  | 0.783**  |
| 7. Total SC         |   |        |          |          |          |          |          | 0.761**  | 0.875**  |
| 8. Left Total Score |   |        |          |          |          |          |          |          | 0.862**  |
| 9. Cumulative Score |   |        |          |          |          |          |          |          |          |

NA: Norberg angle; FIA: Femoral inclination angle; PC: Primary change score, SC: Secondary changes score; SCcat: Categorized secondary changes. \* P<0.05, \*\* P<0.01, two-tailed

**Table 4.** Hip morphometric measures and hip scores by age groups (means±SD for angles; medians [min-max] for scores)

| Variables        | Hip   | Age (year)              |                         |                          |                          |                          |                         | P-value |
|------------------|-------|-------------------------|-------------------------|--------------------------|--------------------------|--------------------------|-------------------------|---------|
|                  |       | 1 (n=3)                 | 2 (n=9)                 | 3 (n=13)                 | 4 (n=12)                 | 5 (n=11)                 | ≥6 (n=8)                |         |
| NA (°)           | Right | 95.13±4.22 <sup>a</sup> | 92.38±5.77 <sup>a</sup> | 88.27±3.74 <sup>ab</sup> | 83.71±11.85 <sup>b</sup> | 85.4±9.02 <sup>b</sup>   | 85.86±5.71 <sup>b</sup> | 0.048   |
|                  | Left  | 93.7±6.2 <sup>a</sup>   | 94.93±5.75 <sup>a</sup> | 90.38±5.47 <sup>ab</sup> | 84.38±9.18 <sup>b</sup>  | 85.68±10.27 <sup>b</sup> | 85.35±5.79 <sup>b</sup> | 0.011   |
| FIA (°)          | Right | 127.98±3.77             | 127.34±5.59             | 125.81±3.77              | 124.81±4.59              | 121.2±6.82               | 125.57±7.03             | 0.164   |
|                  | Left  | 131.66±2.44             | 128.28±5.6              | 126.34±4.57              | 126.22±4.54              | 123.15±7.56              | 124.42±6.67             | 0.186   |
| PC               | Right | 5 (1-6)                 | 2 (0-7)                 | 4 (0-7)                  | 5 (0-11)                 | 3 (0-10)                 | 4 (1-10)                | 0.492   |
|                  | Left  | 4 (4-9)                 | 3 (0-7)                 | 2 (0-8)                  | 5 (1-9)                  | 2 (0-7)                  | 1.5 (0-4)               | 0.111   |
| SC               | Right | 7 (5-12)                | 7 (1-9)                 | 6 (1-10)                 | 8 (1-15)                 | 6 (0-11)                 | 8 (1-13)                | 0.667   |
|                  | Left  | 7 (5-18)                | 7 (1-14)                | 6 (3-14)                 | 13 (2-25)                | 9 (1-17)                 | 7.5 (3-16)              | 0.087   |
| SCcat            | Right | 2 (1-3)                 | 2 (1-2)                 | 2 (1-2)                  | 2 (1-3)                  | 2 (1-3)                  | 2 (1-3)                 | 0.867   |
|                  | Left  | 2 (1-3)                 | 2 (1-3)                 | 2 (1-3)                  | 3 (1-4)                  | 2 (1-3)                  | 2 (1-3)                 | 0.118   |
| Total Score      | Right | 12 (6-18)               | 9 (4-14)                | 9 (5-16)                 | 12 (3-25)                | 10 (0-20)                | 12 (3-23)               | 0.403   |
|                  | Left  | 9 (9-22)                | 9 (1-14)                | 8 (3-19)                 | 16 (2-26)                | 10 (3-21)                | 7.5 (4-17)              | 0.130   |
| Cumulative Score |       | 21 (15-40)              | 19 (5-28)               | 18 (9-26)                | 27 (5-42)                | 17 (8-37)                | 21 (7-40)               | 0.087   |

NA: Norberg angle; FIA: Femoral inclination angle; PC: Primary changes, SC: Secondary changes; SD: Standard deviation; SCcat: Categorized secondary changes. Different superscript letters (a, b) within a row indicate statistically significant differences between groups

(all P>0.05; [Table 5](#)). Descriptively, Persian and Scottish Fold cats tended to have lower NA values, whereas British Shorthair cats showed intermediate values ([Table 5](#)).

**Demographic Factor Comparisons:** Comparisons according to sex and fertility status are presented in [Table 6](#). Sex-related differences were observed for FIA values, with males showing higher values than females (P=0.013 and P=0.025, respectively). Fertility status was associated with NA values, as sterile cats had statistically significantly higher NA values compared with intact cats (P=0.027 and P=0.033, respectively). Hip score components did not differ significantly between sexes or fertility groups (P>0.05; [Table 6](#)).

#### Morphometric Parameters According to SCcat

Morphometric parameters and PC scores according to

SCcat categories are summarized in [Table 7](#). NA values decreased significantly with increasing severity of secondary changes in both hips, with the lowest values observed in the moderate group (P=0.001 and P=0.041 for left and right hips, respectively). FIA values tended to be higher in the moderate group, but these differences were not statistically significant (P>0.05). PC scores were significantly higher in the moderate group compared with the none and mild groups (P<0.001 and P=0.014 for left and right hips, respectively). Only one left hip was classified as severe, preventing statistical evaluation of this category ([Table 7](#)).

#### Regression Analyses

Results of multiple linear regression analyses predicting right and left total hip scores are presented in [Table 8](#).

**Table 5.** Hip morphometric measures and hip scores by breeds (means±SD for angles; medians [min-max] for scores)

| Variables        | Hip   | Breed        |             |             |             |             |             |             |             |             | P-value |
|------------------|-------|--------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|---------|
|                  |       | ANK (n=3)    | BRI (n=13)  | CHI (n=4)   | MIX (n=10)  | PER (n=3)   | CAL (n=4)   | SCO (n=10)  | SIA (n=2)   | TAB (n=7)   |         |
| NA (°)           | Right | 90.10±9.71   | 87.95±9.61  | 90.63±6.70  | 90.13±6.96  | 82.90±5.16  | 86.38±5.91  | 84.52±11.72 | 90±5.94     | 85.47±3.42  | 0.715   |
|                  | Left  | 92.17±12.09  | 89.42±10.73 | 92.90±6.25  | 94.51±5.61  | 93.17±5.48  | 90.85±6.99  | 86.12±10.60 | 87.55±2.47  | 91.93±7.86  | 0.648   |
| FIA (°)          | Right | 124.81±10.77 | 124.60±4.96 | 125.19±4.95 | 124.99±6.52 | 127.90±3.87 | 122.77±5.73 | 127.29±4.73 | 118.83±7.43 | 124.40±5.66 | 0.714   |
|                  | Left  | 125.28±8.68  | 126.27±5.02 | 126.61±4.04 | 125.73±7.04 | 129.5±6.77  | 123.13±2.78 | 128.31±4.55 | 120.51±4.52 | 124.35±8.17 | 0.666   |
| PC               | Right | 5 (0-5)      | 4 (0-7)     | 1.5 (0-5)   | 4 (0-10)    | 5 (4-8)     | 3.5 (1-4)   | 1 (0-11)    | 3.5 (0-7)   | 4 (1-10)    | 0.491   |
|                  | Left  | 2 (0-4)      | 4 (0-9)     | 2.5 (0-5)   | 1 (0-9)     | 2 (1-8)     | 1.5 (0-5)   | 3.5 (0-8)   | 5 (3-7)     | 2 (0-6)     | 0.308   |
| SC               | Right | 1 (0-11)     | 8 (5-12)    | 6 (3-9)     | 3.5 (1-13)  | 6 (4-10)    | 6.5 (4-15)  | 6 (2-14)    | 4.5 (2-7)   | 8 (1-10)    | 0.404   |
|                  | Left  | 8 (7-12)     | 9 (1-18)    | 7 (1-25)    | 5.5 (2-16)  | 8 (3-11)    | 10 (3-17)   | 10 (2-14)   | 9.5 (9-10)  | 10 (4-15)   | 0.862   |
| SCcat            | Right | 1 (1-3)      | 2 (1-3)     | 1.5 (1-2)   | 1 (1-3)     | 2 (1-2)     | 2 (1-3)     | 2 (1-3)     | 1.5 (1-2)   | 2 (1-2)     | 0.360   |
|                  | Left  | 2 (2-3)      | 2 (1-3)     | 2 (1-4)     | 1.5 (1-3)   | 2 (1-3)     | 2.5 (1-3)   | 2.5 (1-3)   | 2 (2-2)     | 2 (1-3)     | 0.861   |
| Total Score      | Right | 6 (0-16)     | 12 (5-18)   | 7 (4-14)    | 6.5 (3-23)  | 12 (10-15)  | 9 (7-19)    | 6 (3-25)    | 8 (2-14)    | 12 (5-20)   | 0.355   |
|                  | Left  | 11 (8-12)    | 10 (3-23)   | 9 (1-26)    | 6 (2-17)    | 8 (4-16)    | 11.5 (3-21) | 12 (2-19)   | 12 (10-14)  | 10 (4-17)   | 0.755   |
| Cumulative Score |       | 17 (8-28)    | 21 (17-40)  | 16 (5-40)   | 14 (7-40)   | 20 (19-26)  | 19 (13-40)  | 18.5 (5-42) | 20 (12-28)  | 22 (12-37)  | 0.663   |

NA: Norberg angle; FIA: Femoral inclination angle; PC: Primary changes; SC: Secondary changes; SD: Standard deviation; SCcat: Categorized secondary changes; ANK: Ankara; BRI: British Shorthair; CHI: Chinchilla; MIX: Mix; PER: Persian; CAL: Calico; O: Scottish Fold; SIA: Siamese; TAB: Tabby

**Table 6.** Hip morphometric measures and hip scores by demographic factors (sex and fertility status; means±SD for angles; medians [min-max] for scores)

| Variables        | Hip   | Sex         |               | P-value | Fertility      |               | P-value |
|------------------|-------|-------------|---------------|---------|----------------|---------------|---------|
|                  |       | Male (n=27) | Female (n=29) |         | Sterile (n=29) | Intact (n=27) |         |
| NA (°)           | Right | 88.24±7.54  | 86.64±8.83    | 0.461   | 89.21±8.98     | 85.48±6.91    | 0.027   |
|                  | Left  | 91.74±8.63  | 89.70±8.71    | 0.371   | 92.83±6.63     | 86.89±9.62    | 0.033   |
| FIA (°)          | Right | 126.94±5.43 | 123.24±5.28   | 0.013   | 125.26±6.13    | 124.77±5.13   | 0.746   |
|                  | Left  | 127.80±5.61 | 124.34±5.64   | 0.025   | 125.94±5.76    | 126.09±6.03   | 0.923   |
| PC               | Right | 4 (0-10)    | 4 (0-11)      | 0.530   | 4 (0-10)       | 4 (0-11)      | 0.875   |
|                  | Left  | 3 (0-9)     | 2 (0-9)       | 0.967   | 2 (0-9)        | 3 (0-9)       | 0.862   |
| SC               | Right | 6 (1-13)    | 7 (0-15)      | 0.675   | 6 (0-11)       | 6 (0-15)      | 0.947   |
|                  | Left  | 7 (2-18)    | 9 (1-25)      | 0.850   | 10 (1-18)      | 8 (2-25)      | 0.850   |
| SCcat            | Right | 2 (1-3)     | 2 (1-3)       | 0.927   | 2 (1-3)        | 2 (1-3)       | 0.820   |
|                  | Left  | 2 (1-3)     | 2 (1-4)       | 0.965   | 2 (1-4)        | 2 (1-3)       | 0.986   |
| Total Score      | Right | 9 (3-23)    | 11 (0-25)     | 0.411   | 10 (0-25)      | 10 (3-20)     | 0.974   |
|                  | Left  | 10 (2-23)   | 10 (1-26)     | 0.863   | 9 (2-26)       | 10 (1-23)     | 0.994   |
| Cumulative Score |       | 20 (5-40)   | 19 (5-42)     | 0.818   | 19 (7-42)      | 22 (5-37)     | 0.761   |

NA: Norberg angle; FIA: Femoral inclination angle; PC: Primary changes; SC: Secondary changes; SD: Standard deviation; SCcat: Categorized secondary changes

For the right hip, the model was statistically significant,  $F(13,42)=4.842$ ,  $P<0.001$ , explaining 47.6% of the variance (Adjusted  $R^2=0.476$ ). Significant predictors were male sex ( $B=2.466$ ,  $P=0.035$ ), fertility status ( $B=-2.517$ ,  $P=0.049$ ), right NA ( $B=-0.320$ ,  $P<0.001$ ), right FIA ( $B=0.448$ ,  $P<0.001$ ), and Scottish Fold breed compared with tabby ( $B=-5.470$ ,  $P=0.010$ ; [Table 8](#)).

For the left hip, the model was also significant,  $F(13,42)=3.524$ ,  $P=0.001$ , accounting for 37.4% of the variance

(Adjusted  $R^2=0.374$ ). Significant predictors included fertility status ( $B=-3.719$ ,  $P=0.029$ ), left NA ( $B=-0.495$ ,  $P<0.001$ ), and left FIA ( $B=0.330$ ,  $P<0.001$ ). No breed effects reached significance in this model ([Table 8](#)).

## DISCUSSION

The findings support our hypothesis that morphometric parameters, particularly reduced NA and increased FIA, are closely associated with higher radiographic hip scores,

**Table 7.** Morphometric parameters and primary changes according to categorized secondary changes (SCcat) in cats (means±SD for angles; medians [min-max] for scores)

| Variables | Hip   | SCcat                   |                         |                          |              | P-value |
|-----------|-------|-------------------------|-------------------------|--------------------------|--------------|---------|
|           |       | None (14/21)            | Mild (24/29)            | Moderate (17/6)          | Severe (1/0) |         |
| NA (°)    | Left  | 94.73±5.30 <sup>b</sup> | 91.95±5.91 <sup>b</sup> | 82.91±9.84 <sup>a</sup>  | 104          | 0.001   |
|           | Right | 91.88±7.12 <sup>b</sup> | 87.69±6.03 <sup>b</sup> | 77.45±13.70 <sup>a</sup> | —            | 0.041   |
| FIA (°)   | Left  | 124.32±5.13             | 125.10±5.57             | 128.67±6.32              | 139.9        | 0.162   |
|           | Right | 123.90±5.77             | 125.01±5.22             | 128.97±6.16              | —            | 0.210   |
| PC        | Left  | 1 (0-4) <sup>a</sup>    | 3 (0-7) <sup>a,b</sup>  | 5 (1-9) <sup>b</sup>     | 9            | <0.001  |
|           | Right | 2 (0-8) <sup>a</sup>    | 4 (0-10) <sup>a,b</sup> | 5.5 (4-11) <sup>b</sup>  | —            | 0.014   |

NA: Norberg angle; FIA: Femoral inclination angle; PC: Primary changes, SD: Standard deviation; SCcat: Categorized secondary changes. Group sizes are presented as left/right hips. Different superscript letters (a, b) within a row indicate statistically significant differences between groups

**Table 8.** Multiple linear regression predicting right and left total hip scores from morphometric and demographic variables (n=56)

| Model     | Predictors                       | B      | SE    | β      | t      | P-value |
|-----------|----------------------------------|--------|-------|--------|--------|---------|
| Right hip | Age                              | 0.085  | 0.452 | 0.024  | 0.19   | 0.851   |
|           | Sex (Male vs. Female)            | 2.466  | 1.132 | 0.238  | 2.18   | 0.035   |
|           | Fertility (Sterile vs. Intact)   | -2.517 | 1.245 | -0.243 | -2.02  | 0.049   |
|           | Right NA                         | -0.320 | 0.073 | -0.501 | -4.37  | <0.001  |
|           | Right FIA                        | 0.448  | 0.106 | 0.482  | 4.21   | <0.001  |
|           | Breed: Scottish Fold (vs. Tabby) | -5.470 | 2.030 | -0.404 | -3.00  | 0.010   |
|           | Other Breeds                     | NS     |       |        |        |         |
| Left hip  | Age                              | 0.060  | 0.582 | 0.014  | 0.103  | 0.919   |
|           | Sex (Male vs. Female)            | -0.386 | 1.448 | -0.032 | -0.267 | 0.791   |
|           | Fertility (Sterile vs. Intact)   | -3.719 | 1.648 | -0.310 | -2.256 | 0.029   |
|           | Left NA                          | -0.495 | 0.097 | -0.697 | -5.091 | <0.001  |
|           | Left FIA                         | 0.330  | 0.144 | 0.368  | 4.25   | <0.001  |
|           | Breeds (vs. Tabby)               | NS     |       |        |        |         |

B: Unstandardized regression coefficient; SE: Standard error of the coefficient; β: Standardized regression coefficient; t: t-statistic; NA: Norberg angle; FIA: Femoral inclination angle; NS: non-significant

while demographic characteristics and SCcat played a more modest role. By adapting the BVA/KC scheme and analyzing NA independently of the cumulative score, the present study provides a structured methodological framework for evaluating feline hip morphology. This separation was necessary given the lack of validated feline reference ranges and the inconsistencies in previous studies [6,10-12]. The approach allowed NA to be evaluated as a direct predictor of radiographic change, providing preliminary feline-specific reference data and illustrating the broader challenge of translating canine-based frameworks into feline practice.

Current feline-specific scoring systems remain limited to registry-based schemes. The Orthopedic Foundation for Animals (OFA) and the Swedish PawPeds programme grade hips on broad ordinal scales (0-4) [7,21]. While these systems are valuable for population-level surveillance and

selective breeding, their coarse categories risk overlooking subtle morphologic variation. Moreover, their reliability is limited by high observer dependence, with agreement rates often inconsistent -particularly in mid-range grades-highlighting that consensus alone cannot serve as a reliable gold standard [22]. In contrast, the adapted BVA/KC system applied in the present study enabled detailed evaluation of individual anatomical landmarks, including acetabular edges, the acetabular fossa, and femoral head and neck exostoses. This component-based framework offered a more nuanced and continuous assessment of both early and advanced changes, extending beyond the limitations of broad categorical grading. Although extrapolation from canine models requires caution, the findings suggest that a structured, multi-parameter approach can provide more clinically informative insights into feline hip morphology.

The inclusion of hindlimb lameness evaluation strengthened

the functional interpretation of morphometric findings. Cats presenting hindlimb lameness consistently exhibited morphometric deviations, indicating that locomotor impairment is more closely associated with joint incongruity and early degenerative changes than with advanced osteoarthritic remodeling (*Table 1*). The additional link between SCcat categories, cumulative hip scores, and lameness further supports the clinical relevance of radiographic scoring. Given that systematic evaluations of lameness in relation to feline hip scoring are scarce, these clinical-radiographic links should be confirmed with objective gait measures in larger, prospective cohorts.

In the present study, both the NA and FIA were closely associated with radiographic scoring outcomes (*Table 1*, *Table 2*, *Table 3*, *Table 4*, *Table 5*, *Table 6*, *Table 7*, and *Table 8*), highlighting their value as quantitative descriptors of hip conformation in cats. These measures provide clinically informative benchmarks for interpreting feline hip morphology [6,11]. Previous reports show wide variation in FIA and alignment values, largely attributable to differing measurement methods [17,18], and some studies did not assess FIA at all [17]. In the present study, the SYMAX method was applied, which has been described as a reliable approach for femoral alignment assessment [8], thereby contributing new comparative data. While FIA has been extensively characterized in canine orthopedics [13-15], evidence in cats remains sparse, and methodological inconsistency limits comparability across studies. From this perspective, our results provide preliminary benchmarks rather than diagnostic thresholds, emphasizing the need for standardized measurement protocols and larger datasets to establish robust feline-specific reference ranges.

In the present study, demographic characteristics appeared to play a secondary role in hip morphology, yet some patterns provide important context. The higher NA values observed in younger cats (*Table 4*) support the concept of age-related loss of hip congruity and mirror earlier findings that hip laxity and degenerative changes accumulate with age [6,7]. Sex-related differences were also noted, with males tending toward higher FIA values than females (*Table 6*), a pattern that may reflect subtle dimorphic variation in pelvic biomechanics rather than a primary risk factor [18]. Fertility status emerged as a particularly interesting factor: sterilized cats exhibited more favorable NA values compared with intact cats (*Table 6*). Although the influence of gonadectomy on feline hips has not been systematically examined, studies in both cats and dogs suggest hormonal status may alter musculoskeletal development and risk of orthopedic disease [1,6,7,23,24]. This potential protective association in cats is novel but should be interpreted cautiously until confirmed in larger, controlled studies. Breed-related differences were not statistically significant (*Table 5*); however, the consistently

lower NA in Scottish Folds and their identification as a negative predictor in regression (*Table 8*) align with registry-based data highlighting increased susceptibility in purebred populations, particularly Maine Coons and Scottish Folds [1,4-7].

The SCcat classification was introduced as an analytical tool to facilitate stratified evaluation of secondary radiographic changes in relation to morphometric parameters, rather than as a diagnostic grading system. Cats placed in higher SCcat categories consistently showed lower NA and higher PC scores (*Table 7*), supporting the internal validity of this pragmatic approach. Although the limited number of severe cases restricted statistical evaluation, the observed trends suggest that SCcat, when combined with morphometric analysis, may help distinguish early alterations from more advanced osteoarthritic remodeling. Compared with existing systems such as the OFA registry [7] and the PawPeds programme [21], which rely on broad categorical grading, SCcat integrates both primary and secondary radiographic features and anchors them to specific anatomical changes [19]. This combined framework may therefore offer a more nuanced and clinically informative method for evaluating hip pathology in cats.

Beyond the identification of predictors, the present study contributes preliminary reference values for feline hip morphometry and radiographic scoring (*Table 1*, *Table 2*, *Table 3*, *Table 4*, *Table 5*, *Table 6*, *Table 7*, and *Table 8*). Establishing such benchmarks is important, as they provide a framework for distinguishing normal variation from pathological change and for monitoring disease progression or treatment outcomes. Given the scarcity of feline-specific data in the feline orthopedic literature, these values address a notable gap and may serve as a foundation for both clinical decision-making and future research in feline hip evaluation.

Several limitations should be acknowledged. The sample size was limited and derived from a single referral center, with restricted representation of some breeds and very few severe SCcat cases, which may limit generalizability. Importantly, radiographs were obtained without anesthesia or sedation, which represents a methodological limitation. Although this approach reflects routine clinical practice in client-owned cats, ventrodorsal positioning, hindlimb extension, and patellar centralization may vary in non-sedated animals and could systematically influence measurements of the NA and FIA. Sedation or general anesthesia was not routinely employed due to owner preference and the desire to minimize pharmacological intervention in a clinical setting, and because diagnostically acceptable positioning could be achieved in the majority of cases with gentle manual restraint by experienced personnel. Consequently, measured values

reflect clinically obtainable radiographs rather than idealized anesthetized positioning, which may increase measurement variability but enhances the applicability of the findings to real-world veterinary practice. In the present study, lameness assessment was based on standardized clinical orthopedic examination performed by a single experienced surgeon and included observation of gait, stance, weight-bearing, and targeted orthopedic tests to localize hindlimb involvement. However, lameness was assessed subjectively, and objective gait analysis techniques such as force-plate analysis or motion capture were not employed. This limitation is common in clinical veterinary settings and should be considered when interpreting associations between lameness status and radiographic findings. Inter- and intra-observer repeatability was not formally assessed, although duplicate measurements from the same radiographs were averaged to reduce random measurement error. Another limitation is the use of the BVA/KC scoring system, which was originally developed for dogs and has not been formally validated for cats, although it provided a structured framework for assessment. Similarly, the SCcat stratification was pragmatically defined based on score distribution and should be interpreted as an exploratory analytical framework, pending validation against clinical outcomes in larger, longitudinal datasets. The multiple linear regression models included several independent variables relative to the sample size, raising the possibility of overparameterization. Although multicollinearity diagnostics and residual analyses did not indicate model instability, these findings should be interpreted cautiously. Future studies with larger sample sizes may benefit from simplified model structures, such as binary breed classification (purebred vs. non-purebred) or penalized regression approaches, to improve model parsimony and robustness. In addition, the referral-based population studied here may not fully reflect the general feline population, potentially introducing selection bias. Finally, the cross-sectional design precludes conclusions regarding disease progression, and functional assessments such as objective gait analysis were not performed. Future studies should therefore include larger, multicenter populations with longitudinal follow-up, incorporate validated feline-specific scoring criteria, and integrate functional outcome measures to refine diagnostic thresholds.

In conclusion, this study demonstrates that the NA is the most reliable radiographic parameter for assessing feline hip dysplasia, showing strong associations with primary and secondary changes, SCcat categories, and total hip scores. Clinically non-lame cats exhibited NA values of  $91.46 \pm 6.80^\circ$  (right) and  $92.55 \pm 6.90^\circ$  (left), whereas lame cats showed lower NA values ( $83.41 \pm 9.34^\circ$  right;  $84.40 \pm 8.88^\circ$  left), supporting the clinical relevance of

reduced acetabular coverage. The FIA also contributed as a supportive predictor, particularly in regression analyses, with non-lame cats showing FIA values of  $121.71 \pm 5.57^\circ$  (right) and  $124.83 \pm 5.81^\circ$  (left) and lame cats demonstrating higher FIA values ( $127.84 \pm 4.66^\circ$  right;  $128.30 \pm 5.32^\circ$  left). While demographic factors such as sex, fertility status, and breed exerted secondary but noteworthy influences, NA and FIA remained the most clinically informative morphometric measures. By adapting the BVA/KC scoring system and analyzing NA separately, this work provides practical reference benchmarks for feline hip morphometry and radiographic scoring. These benchmarks may aid veterinarians in distinguishing normal variation from clinically significant dysplasia, guide breeding decisions, and support management strategies. Future multicenter and longitudinal studies, integrating functional assessments, are warranted to validate these findings and refine diagnostic thresholds.

## DECLARATIONS

**Availability of Data and Materials:** The data that support the findings of this study are available from the corresponding author (Y.A.O.), upon reasonable request.

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**Ethical Approval:** This study was approved by the Aydin Adnan Menderes University Animal Experiments Local Ethics Committee (Approval no: 64583101/2025/134 on 14.08.2025).

**Conflict of Interests:** All authors declare that they have no conflicts of interest.

**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.

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## RESEARCH ARTICLE

# Detection of Various Prohibited Substances in Thoroughbred Racehorses at Three Major Racetracks in Türkiye: A Nine-Year Experience

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**Abstract**

The use of prohibited substances raises significant ethical and global issues for horseracing. To mitigate these problems and maintain control, prohibited substance controls are conducted on samples taken from thoroughbred horses before and after races. However, limited information is available on prohibited substances detected as a result of these controls. In this study, 39.935 samples from races held at three racetracks in Türkiye between 2015 and 2023 were analysed qualitatively and/or quantitatively using gas chromatography-mass spectrometry, liquid chromatography-mass spectrometry, high-resolution mass spectrometry, inductively coupled plasma mass spectrometry, and a biochemistry autoanalyser. Overall, 219 violations caused by 314 findings were reported. Of these findings, 140 were International Screening Limit, 86 were prohibited for use at any time, 60 were threshold, and 28 were substances within the scope of International Residue Limit. Consequently, violations detected at three major racetracks in Türkiye over nine years were evaluated, and useful information was shared with scientists working in this field, race veterinarians, horse owners, and trainers.

**Keywords:** Horseracing, anti-doping, Türkiye, prohibited substance, racetracks

## INTRODUCTION

Doping in racehorses is described as the administration of any substance, other than normal food, intended to alter a horse's speed, strength, or stamina in running. In horse sports, prohibited substances not only include doping agents used to enhance performance but also legitimate therapeutic agents (off-label use of veterinary or human drugs necessary for the horse's health) that may indirectly influence performance. For this reason, regulatory rules have been developed by the International Federation of Horseracing Authorities (IFHA) to control the substances used in horseracing. Türkiye has adopted and applies the rules set by the IFHA without exception <sup>[1]</sup>.

The prohibited substances in horseracing and their exceptions are defined in Article 6A of the "International Agreement on Breeding, Racing and Wagering (IABRW)" published by IFHA <sup>[1]</sup>. Accordingly, substances capable of acting on one or more of the cardiovascular, respiratory,

digestive, urinary, reproductive, musculoskeletal, hematologic, immune (except licensed vaccines against infectious agents), and endocrine systems in mammals, as well as their synthetic counterparts, masking agents, oxygen carriers, and substances that directly or indirectly affect or manipulate gene expression, are classified as prohibited substances. These are divided into four categories: substances subject to International Screening Limits (ISL) <sup>[2-4]</sup>, substances with threshold values <sup>[1]</sup>, international residue limited (IRL) substances <sup>[5]</sup>, and substances that are prohibited for use at any time <sup>[1]</sup>.

In this context, the urine and plasma limits of ISL substances permitted for therapeutic use by the IFHA was defined, and the rules for their use were established. When a substance exceeding the ISL is detected during screening analyzes for the control of prohibited substances, a qualitative confirmatory analysis, usually performed by mass spectrometry is carried out to confirm the presence of the prohibited substance. Quantification



is not required for this confirmation. In addition, analyzes using different types of mass spectrometers (screening and confirmation) based detection of the parent compounds or metabolites. Under these rules, the detection of a substance above the ISL constitutes a direct violation. Even if substances are detected below the ISL, their presence in combination with another substance sharing the same mechanism of action or with a masking agent also constitutes a violation <sup>[3,4]</sup>.

Endogenous substances in horses (e.g., testosterone, carbon dioxide) or substances present in plants traditionally grazed or used as horse feed (e.g., arsenic, cobalt) are defined under the category of threshold substances. During screening analyzes, if a substance in this group is suspected of exceeding the threshold, quantitative analyzes are performed. If exceedance is confirmed, it is reported as a violation <sup>[1,6,7]</sup>.

Internationally harmonized residue limits are applied to control certain feed contaminants and environmental substances. The substances in this category are controlled at the screening level, like those of ISL substances <sup>[5]</sup>.

Detection of these substances themselves, their metabolites, isomers, metabolite of the metabolites, or prodrug forms, as well as any scientific evidence indicating their administration, causes a violation <sup>[1]</sup>.

Anti-doping laboratories generally analyze prohibited substances in accordance with IFHA rules and the technical criteria established by the Association of Official Racing Chemists (AORC) <sup>[8-10]</sup>. However, published reports on the findings of these laboratories are quite limited. The most comprehensive report on the use of prohibited substances in racing covers the 12 years <sup>[11]</sup>. IFHA has published the results of all laboratories for specific periods <sup>[12]</sup>. These reported violation rates vary among studies: in Illinois, a violation rate of 0.45% was observed over a five-year period <sup>[13]</sup>, in Louisiana, 1.01% of 52.909 samples analysed <sup>[14]</sup>, in Iran, 31.4% of 656 samples analysed <sup>[15,16]</sup>, in Cyprus, 161 violations (1.52%) were reported as a result of analyzing 94.800 samples <sup>[17]</sup>, in the Czech Republic, twelve different prohibited substances were detected, alone or in combination, in 2.03% of 641 samples <sup>[18]</sup>, and in Italy 549 violations (0.4%) were reported as a result of analyzing 104.770 samples <sup>[19]</sup>. Furthermore, the results of similar studies are evaluated in another study <sup>[20]</sup>.

This study aimed to retrospectively analyze prohibited substance findings in urine and blood samples collected from three racetracks in Izmir, Bursa, and Kocaeli, Türkiye, between 2015 and 2023, and to provide information for scientists, racing veterinarians, horse owners, and trainers.

## MATERIALS AND METHODS

### Ethics Statement

This study does not require ethics committee approval, as it is based on previously published analysis results.

### The Laboratory and Samples

Urine and blood samples collected before and after thoroughbred horse races held at the racetracks in Izmir, Bursa, and Kocaeli were divided into two groups: "A" and "B." The portion of each sample designated as the "A sample" was sent to the Istanbul Pendik Veterinary Control Institute Doping Laboratory, while the "B sample" was retained at the respective racetrack for confirmatory analysis.

Each sample underwent an initial screening analysis, followed by confirmatory testing for those in which the presence of prohibited substances was suspected. If the presence of a prohibited substance was confirmed, the finding was reported as a positive result. Following such notifications, the corresponding "B sample" was transferred by the relevant racecourse to another authorized doping laboratory (Veterinary Control Central Research Institute Doping Laboratory, Ankara) to confirm the presence of the prohibited substance.

Samples received at the laboratory were analysed according to established procedures <sup>[21,22]</sup> within 1-15 days of receipt. Negative samples were stored for one month, while positive samples for six months.

### Instrumentation and Methodology

Analysis of prohibited substances in blood and urine samples were carried out in accordance with the guidelines of the Association of Official Racing Chemists (AORC) <sup>[8]</sup>, IFHA <sup>[1]</sup>, Commission Implementing Regulation (EU) 2021/808 and ISO/IEC 17025:2017 standards. Mass spectrometer calibrations (in both positive and negative ion modes) and instrument cleaning were performed for each sample batch before analysis. After calibration and cleaning, a system suitability test was conducted to verify the performance and stability of the instrument before beginning the analytical sequence.

### Urine Samples Analysis

Urine samples collected post-race and submitted to the laboratory were divided and subjected to both enzymatic and alkaline hydrolysis. A 15 mL aliquot was taken for enzymatic hydrolysis and subsequently divided into three portions. Each portion was extracted using different solid-phase extraction (SPE) procedures. The resulting extracts were individually analysed on mass spectrometric instruments selected according to the chemical characteristics of the compounds (LC-MS/MS <sup>[23-25]</sup>,

**a** The number of samples analysed, **b** The number of single-substance violations, **c** The number of multiple-substance violations, **d** The % percentage of violation received according to the number of samples taken

**Table 2.** Distribution of detected findings between 2015 and 2023 according to IFHA groups, mechanisms of action, and quantities of single and multiple substance findings

| Name                                       | Effect              | Distribution of Single and Multiple Substance Findings by Years |   |      |   |      |   |      |   |      |   |      |   |      |   |      |   |      |    | Number of Finding |
|--------------------------------------------|---------------------|-----------------------------------------------------------------|---|------|---|------|---|------|---|------|---|------|---|------|---|------|---|------|----|-------------------|
|                                            |                     | 2015                                                            |   | 2016 |   | 2017 |   | 2018 |   | 2019 |   | 2020 |   | 2021 |   | 2022 |   | 2023 |    |                   |
| Substances prohibited for use at any time  |                     | S                                                               | M | S    | M | S    | M | S    | M | S    | M | S    | M | S    | M | S    | M | S    | M  |                   |
| Altrenogest                                | Oestrus suppression |                                                                 |   |      |   |      |   |      |   | 1    |   |      |   |      |   |      |   |      |    | 1                 |
| Capsaicin                                  | Topical analgesic   |                                                                 |   |      |   |      |   |      |   |      |   |      |   |      | 2 |      |   |      |    | 2                 |
| Cocaine                                    | Stimulant           |                                                                 |   |      |   |      |   |      |   |      |   |      |   | 1    |   |      |   | 1    |    | 2                 |
| Diisopropylamine                           | Vasodilator         |                                                                 |   |      |   |      |   |      |   | 3    |   | 5    |   |      |   |      |   |      |    | 8                 |
| Dyphylline                                 | Muscle relaxant     | 2                                                               | 1 | 1    |   |      |   |      |   |      |   | 1    | 2 | 1    | 1 | 1    |   |      |    | 10                |
| Etodolac                                   | NSAID               |                                                                 |   |      |   |      |   |      |   |      |   |      |   |      | 1 | 2    | 2 | 1    |    | 6                 |
| Etofenamat/Flufenamic acid                 | NSAID               | 1                                                               | 1 |      |   |      |   | 1    |   |      |   | 2    |   |      |   | 2    | 4 |      |    | 11                |
| Heptaminol                                 | Stimulant           |                                                                 |   |      |   |      |   |      |   |      |   | 1    |   |      |   |      |   |      |    | 1                 |
| Levamisole                                 | Stimulant           |                                                                 | 2 |      |   |      | 2 |      |   |      |   |      |   |      |   |      |   |      | 1  | 5                 |
| Neostigmine                                | Anticholinesterase  | 1                                                               |   |      |   |      |   |      |   |      |   |      |   |      |   |      |   |      |    | 1                 |
| Nikethamide                                | Stimulant           | 1                                                               | 1 |      |   |      |   |      |   |      |   |      | 1 |      |   |      |   |      |    | 3                 |
| Pemoline                                   | Stimulant           | 1                                                               | 2 |      |   |      |   |      |   |      |   |      |   |      |   |      |   |      |    | 3                 |
| Pentoxifylline                             | Vasodilator         |                                                                 |   |      |   |      |   | 2    |   | 1    |   | 2    |   | 1    |   |      |   |      |    | 6                 |
| Procaine                                   | Local anaesthetic   |                                                                 |   | 2    | 1 |      | 2 | 1    | 2 |      | 1 | 1    |   | 2    |   |      |   |      |    | 12                |
| Pyrilamine                                 | Antihistamine       |                                                                 |   | 1    |   |      |   |      |   |      |   |      |   |      |   |      | 2 |      |    | 3                 |
| Ranitidine                                 | Histamine-2 blocker |                                                                 |   |      | 1 |      | 1 |      |   | 1    |   |      | 2 |      |   | 1    |   |      |    | 6                 |
| Sildenafil                                 | Vasodilator         |                                                                 |   |      |   |      |   |      |   |      |   |      |   | 1    |   | 1    |   |      |    | 2                 |
| Tenoxicam                                  | NSAID               |                                                                 |   |      |   |      |   |      |   |      |   | 1    |   |      | 1 |      |   |      |    | 2                 |
| Trenbolone                                 | Anabolic Steroid    |                                                                 |   |      |   |      |   |      |   |      |   |      |   |      |   | 1    |   |      |    | 1                 |
| Verapamil                                  | Antiarrhythmic      |                                                                 |   |      |   | 1    |   |      |   |      |   |      |   |      |   |      |   |      |    | 1                 |
| Total                                      |                     |                                                                 |   |      |   |      |   |      |   |      |   |      |   |      |   |      |   |      |    | 86                |
| Threshold substances                       |                     | S                                                               | M | S    | M | S    | M | S    | M | S    | M | S    | M | S    | M | S    | M | S    | M  |                   |
| Arsenic                                    | Stimulant/toxic     |                                                                 |   |      |   | 15   |   | 2    |   |      |   | 3    |   | 4    |   | 4    |   | 2    |    | 30                |
| Cobalt                                     | Erythropoiesis      |                                                                 |   |      |   | 8    | 1 | 3    |   | 7    |   | 3    |   | 2    | 1 | 2    | 1 | 1    | 1  | 30                |
| Total                                      |                     |                                                                 |   |      |   |      |   |      |   |      |   |      |   |      |   |      |   |      |    | 60                |
| International screening limited substances |                     | S                                                               | M | S    | M | S    | M | S    | M | S    | M | S    | M | S    | M | S    | M | S    | M  |                   |
| Ambroxol                                   | Mucolytic           |                                                                 |   |      |   |      |   |      | 2 |      |   |      | 1 |      |   |      | 2 |      |    | 5                 |
| Betamethasone                              | Corticosteroid      |                                                                 |   |      |   |      |   |      | 1 | 2    | 1 |      | 1 | 1    |   |      |   |      |    | 6                 |
| Clenbuterol                                | Bronchodilator      |                                                                 |   |      |   |      |   |      |   |      |   |      |   | 1    |   |      |   |      |    | 1                 |
| Dexamethasone                              | Corticosteroid      |                                                                 |   |      |   |      | 1 |      |   |      |   |      | 1 |      |   | 1    |   |      | 2  | 5                 |
| Diclofenac                                 | NSAID               |                                                                 |   |      |   |      | 1 |      |   |      | 1 |      |   |      |   |      |   |      |    | 2                 |
| Flunixin                                   | NSAID               | 1                                                               |   | 1    | 1 | 2    | 3 |      | 1 | 1    |   |      |   |      | 1 |      | 5 |      | 13 | 29                |
| Furosemide                                 | Diuretic            | 1                                                               |   |      |   |      |   |      |   |      |   |      |   |      |   |      |   |      |    | 1                 |
| HEPS                                       | Sedative            |                                                                 |   |      |   |      |   |      |   | 1    | 1 |      |   |      |   |      |   |      |    | 2                 |
| Hydroxy lidocaine                          | Local anaesthetic   |                                                                 |   |      |   |      |   |      | 1 |      | 1 |      | 1 |      | 1 | 1    | 2 |      | 3  | 10                |
| Hydroxy xylazine                           | Sedative            |                                                                 |   |      |   |      |   | 2    |   |      |   |      |   |      |   |      |   |      | 3  | 5                 |
| Ketoprofen                                 | NSAID               | 2                                                               |   |      | 2 |      | 1 |      |   |      |   |      | 1 |      | 1 |      | 2 |      | 1  | 10                |

| Table 2. Continue                                                                                                                                                                                                                                                                                                                                                                        |                 |                                                                 |   |      |   |      |   |      |   |      |   |      |   |      |   |      |   |                   |      |     |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------|-----------------------------------------------------------------|---|------|---|------|---|------|---|------|---|------|---|------|---|------|---|-------------------|------|-----|
| Name                                                                                                                                                                                                                                                                                                                                                                                     | Effect          | Distribution of Single and Multiple Substance Findings by Years |   |      |   |      |   |      |   |      |   |      |   |      |   |      |   | Number of Finding |      |     |
|                                                                                                                                                                                                                                                                                                                                                                                          |                 | 2015                                                            |   | 2016 |   | 2017 |   | 2018 |   | 2019 |   | 2020 |   | 2021 |   | 2022 |   |                   | 2023 |     |
| International screening limited substances                                                                                                                                                                                                                                                                                                                                               |                 | S                                                               | M | S    | M | S    | M | S    | M | S    | M | S    | M | S    | M | S    | M | S                 | M    |     |
| Meloxicam/Hydroxymethyl meloxicam                                                                                                                                                                                                                                                                                                                                                        | NSAID           |                                                                 |   |      |   |      |   |      |   |      |   |      | 2 |      | 2 |      | 7 |                   | 1    | 12  |
| Methocarbamol*                                                                                                                                                                                                                                                                                                                                                                           | Muscle relaxant | 1                                                               |   |      |   |      | 1 |      |   |      |   |      |   | 1    |   |      |   |                   | 3    | 6   |
| Methylaminoantipyrine                                                                                                                                                                                                                                                                                                                                                                    | NSAID           | 3                                                               |   | 1    | 3 |      | 1 |      | 2 | 1    | 1 |      | 4 |      | 2 |      | 7 |                   | 4    | 29  |
| Naproxen                                                                                                                                                                                                                                                                                                                                                                                 | NSAID           | 1                                                               |   | 9    |   |      |   |      |   |      |   |      |   |      |   |      |   |                   |      | 10  |
| N-butyl scopolamine                                                                                                                                                                                                                                                                                                                                                                      | Parasympathetic | 1                                                               |   |      |   |      |   |      |   |      |   |      | 1 |      | 1 |      |   |                   |      | 3   |
| Phenylbutazone                                                                                                                                                                                                                                                                                                                                                                           | NSAID           |                                                                 |   |      |   |      |   |      |   |      | 1 |      |   |      |   |      |   |                   |      | 1   |
| Triamcinolone acetonide                                                                                                                                                                                                                                                                                                                                                                  | Corticosteroid  |                                                                 |   |      |   |      |   |      |   |      |   |      |   |      | 1 |      | 2 |                   |      | 3   |
| Total                                                                                                                                                                                                                                                                                                                                                                                    |                 |                                                                 |   |      |   |      |   |      |   |      |   |      |   |      |   |      |   |                   |      | 140 |
| Residue Limits Substances                                                                                                                                                                                                                                                                                                                                                                |                 | S                                                               | M | S    | M | S    | M | S    | M | S    | M | S    | M | S    | M | S    | M | S                 | M    |     |
| Atropin                                                                                                                                                                                                                                                                                                                                                                                  | Anticholinergic |                                                                 | 1 |      | 1 |      | 1 |      | 1 |      |   |      |   |      |   |      |   |                   |      | 4   |
| Caffeine                                                                                                                                                                                                                                                                                                                                                                                 | Stimulant       | 1                                                               | 1 | 4    |   |      |   | 1    | 2 |      |   | 1    |   |      |   | 1    |   |                   | 3    | 14  |
| Morphine                                                                                                                                                                                                                                                                                                                                                                                 | Opiod analgesic |                                                                 |   |      |   | 1    |   |      |   |      | 1 | 5    |   | 3    |   |      |   |                   |      | 10  |
| Total                                                                                                                                                                                                                                                                                                                                                                                    |                 |                                                                 |   |      |   |      |   |      |   |      |   |      |   |      |   |      |   |                   |      | 28  |
| General total                                                                                                                                                                                                                                                                                                                                                                            |                 |                                                                 |   |      |   |      |   |      |   |      |   |      |   |      |   |      |   |                   |      | 314 |
| Ambroxol: Metabolites of bromhexine, HEPS: Metabolites of acepromazine, Hydroxy lidocaine: Metabolites of lidocaine, Hydroxymethyl meloxicam: Metabolites of meloxicam, Hydroxy xylazine: Metabolites of xylazine, Methylamino antipyrine: Metabolites of dipyrone, Flufenamic acid: Metabolites of etofenamate<br>* Asian screening limited. S: Single Substance. M: Multiple Substance |                 |                                                                 |   |      |   |      |   |      |   |      |   |      |   |      |   |      |   |                   |      |     |

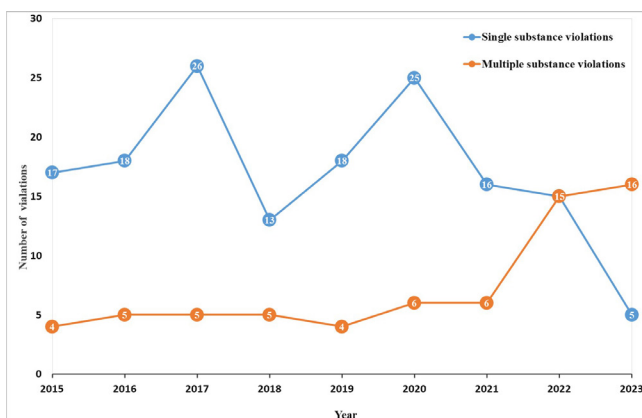


Fig 1. Distribution of violations due to multiple/single findings by years

findings were ISL, 86 were “substance prohibited for use at any time,” 60 were “threshold substances,” and 28 were within the scope of the IRL. Among these substances, 111 were non-steroidal anti-inflammatory drugs (NSAIDs), 58 were stimulants, 22 were local anaesthetics, 16 were vasodilators, 14 were corticosteroids, and 10 had opioid analgesic effects (Table 2).

According to the results, arsenic and cobalt were the most frequently detected substances within the threshold substance category, each detected in 30 cases. All arsenic violations were caused by single-substance findings,

whereas 4 of the cobalt cases involved multiple-substance violations; in the remaining cases, cobalt was detected alone (Table 2).

In the present study, all of the 28 violation findings caused by the substances covered by the IRL were due to atropine, caffeine, and morphine. Seventeen of these findings were caused by single-substance violations, while the others were multiple-substance violations. In this category, caffeine caused the most violations with 14 findings, followed by morphine with 10 findings and atropine with 4 findings (Table 2).

Fifty-two of these findings caused multiple-substance violations, while 34 caused single-substance violations. These violations were mostly caused by NSAID (etofenamate/flufenamic acid 11 finding, etodolac 6 finding), local anaesthetic (procaine 12 finding), muscle relaxant (diphylline 10 finding), and vasodilator (diisopropylamine 8 finding) effective substances (Table 2).

Thirty-five of the ISL substance findings resulted in single-substance violations, and the others (105 findings) resulted in multiple-substance violations (Table 1). Some of these identified violations resulted from the detection of multiple prohibited substances in combination, while others resulted from the identification of a single prohibited substance. Due to the continuous changes in substance/formulation findings detected in multiple-substance

Table 3. Distribution of multiple substance violations involving procaine, meloxicam and flunixin by year

| Multiple Substance Violations Containing Procaine                  |   |                                                  | 2015 | 2016 | 2017 | 2018 | 2019 | 2020 | 2021 | 2022 | 2023 | Total |
|--------------------------------------------------------------------|---|--------------------------------------------------|------|------|------|------|------|------|------|------|------|-------|
| Multiple Substance Violations Containing Meloxicam and Metabolites |   |                                                  |      |      |      |      |      |      |      |      |      |       |
| Procaine                                                           | + | Methylaminoantipyrine, ketoprofen                |      | 1    |      |      |      |      |      |      |      | 1     |
|                                                                    |   | Ranitidine                                       |      | 1    |      |      |      |      |      |      |      | 1     |
|                                                                    |   | Methocarbamol, flunixin                          |      |      | 1    |      |      |      |      |      |      | 1     |
|                                                                    |   | Ketoprofen                                       |      |      | 1    |      |      |      |      |      |      | 1     |
|                                                                    |   | Flunixin, atropin                                |      |      |      | 1    |      |      |      |      |      | 1     |
|                                                                    |   | Methylaminoantipyrine, flunixin                  |      |      |      | 1    |      |      |      |      |      | 1     |
|                                                                    |   | Methylaminoantipyrine                            |      |      |      |      | 1    |      |      |      |      | 1     |
|                                                                    |   | Ranitidine, hydroxy lidocaine, methocarbamol     |      |      |      |      |      | 1    |      |      |      | 1     |
| Total                                                              |   |                                                  |      | 2    | 2    | 2    | 1    | 1    |      |      |      | 8     |
| Hydroxymethyl meloxicam/ Meloxicam                                 | + | Methylaminoantipyrine, ambroxol                  |      |      |      |      |      | 1    |      |      |      | 1     |
|                                                                    |   | Nikethamide, dyphylline, methylaminoantipyrine   |      |      |      |      |      | 1    |      |      |      | 1     |
|                                                                    |   | Etodolac, capsaicine                             |      |      |      |      |      |      | 1    |      |      | 1     |
|                                                                    |   | Flunixin, ambroxol                               |      |      |      |      |      |      |      | 1    |      | 1     |
|                                                                    |   | Flunixin, methylaminoantipyrine                  |      |      |      |      |      |      |      | 1    |      | 1     |
|                                                                    |   | Flunixin, triamcinolone acetonide                |      |      |      |      |      |      |      | 1    |      | 1     |
|                                                                    |   | Etofenamate, cobalt                              |      |      |      |      |      |      |      | 1    |      | 1     |
|                                                                    |   | Hydroxy lidocaine, methylaminoantipyrine         |      |      |      |      |      |      |      | 1    |      | 1     |
|                                                                    |   | Methylaminoantipyrine, flunixin                  |      |      |      |      |      |      |      | 1    |      | 1     |
|                                                                    |   | Dexamethasone, levamisole                        |      |      |      |      |      |      |      |      | 1    | 1     |
|                                                                    |   | Ketoprofen, meloxicam, methylaminoantipyrine     |      |      |      |      |      |      |      | 1    | 1    | 2     |
| Total                                                              |   |                                                  |      |      |      |      |      | 2    | 1    | 7    | 2    | 12    |
| Multiple substance violations containing flunixin                  |   |                                                  |      |      |      |      |      |      |      |      |      |       |
| Flunixin                                                           | + | Hydroxy lidocaine                                |      |      |      |      |      |      |      |      | 2    | 2     |
|                                                                    |   | Cobalt                                           |      |      |      |      |      |      |      |      | 1    | 1     |
|                                                                    |   | Dexamethasone                                    |      |      |      |      |      |      |      |      | 1    | 1     |
|                                                                    |   | Diclofenac, caffeine                             |      |      |      | 1    |      |      |      |      |      | 1     |
|                                                                    |   | Caffeine                                         |      |      |      |      |      |      |      |      | 1    | 1     |
|                                                                    |   | Hydroxymethyl meloxicam, ambroxol                |      |      |      |      |      |      |      | 1    |      | 1     |
|                                                                    |   | Hydroxymethyl meloxicam, methylaminoantipyrine   |      |      |      |      |      |      |      | 1    |      | 1     |
|                                                                    |   | Hydroxymethyl meloxicam, triamcinolone acetonide |      |      |      |      |      |      |      | 1    |      | 1     |
|                                                                    |   | Hydroxy xylazine                                 |      |      |      |      |      |      |      |      | 3    | 3     |
|                                                                    |   | Methocarbamol                                    |      |      |      |      |      |      |      |      | 2    | 2     |
|                                                                    |   | Methocarbamol, methylaminoantipyrine             |      |      |      |      |      |      |      |      | 1    | 1     |
|                                                                    |   | Methocarbamol, procaine                          |      |      | 1    |      |      |      |      |      |      | 1     |
|                                                                    |   | Methylaminoantipyrine                            |      |      |      |      |      |      |      | 1    | 2    | 3     |
|                                                                    |   | Methylaminoantipyrine, hydroxymethyl meloxicam   |      |      |      |      |      |      |      | 1    |      | 1     |
|                                                                    |   | Procaine, atropine                               |      |      |      | 1    |      |      |      |      |      | 1     |
|                                                                    |   | Procaine, methylaminoantipyrine                  |      |      |      | 1    |      |      |      |      |      | 1     |
|                                                                    |   | Total                                            |      |      | 0    | 0    | 1    | 3    | 0    | 0    | 0    | 5     |

**Table 4.** Distribution by year of some painkillers and NSAIDs resulting in single-substance violations

| Substances                  | 2015 | 2016 | 2017 | 2018 | 2019 | 2020 | 2021 | 2022 | 2023 |
|-----------------------------|------|------|------|------|------|------|------|------|------|
| Betamethasone/dexamethasone |      |      |      |      | 2    |      | 1    |      |      |
| Etodolac                    |      |      |      |      |      |      |      | 2    |      |
| Tenoxicam                   |      |      |      |      |      | 1    |      |      |      |
| Hydroxy xylazine            |      |      |      | 2    |      |      |      |      |      |
| Morphine                    |      |      | 1    |      |      | 5    | 3    |      |      |
| Etofenamate/flufenamic acid | 1    |      |      | 1    |      | 2    |      | 2    |      |
| Flunixin                    | 1    | 1    | 2    |      | 1    |      |      |      |      |
| Ketoprofen                  | 2    |      |      |      |      |      |      |      |      |
| Methylaminoantipyrine       | 3    | 1    |      |      | 1    |      |      |      |      |
| Naproxen                    | 1    | 9    |      |      |      |      |      |      |      |

violations, such violations were generally identified once. However, the combinations of flunixin + hydroxy lidocaine and flunixin + methocarbamol were each detected twice, whereas the flunixin + hydroxyxylazine and flunixin + methylaminoantipyrine combination was detected three times. However, significant changes over time were detected in both multiple and single-substance findings (*Table 3*, *Table 4*).

## DISCUSSION

When interpreting the results, it is worth noting that the Pendik Veterinary Control Institute Doping Laboratory started its activities in October 2015; racetracks were closed for 3 months because of the suspension of horseracing in 2020 due to the Covid-19 pandemic, and elemental analysis of horse urine and blood began in 2017. According to the findings, the violation rate was calculated as 0.55% in 39.935 samples. This rate is lower than those reported from racetracks in Iran <sup>[15]</sup>, the Czech Republic <sup>[18]</sup>, Cyprus <sup>[17]</sup>, and Louisiana (USA) <sup>[14]</sup>, but higher than those reported from racetracks in Illinois (USA) <sup>[13]</sup> and Italy <sup>[19]</sup>. These differences are thought to be associated with national regulatory frameworks, analytical capacity of laboratories, regional differences in food sources, and substance use practices.

In this current study, all detected threshold substance violations were caused by arsenic and cobalt (*Table 2*). Arsenic has been used as a tonic in horses and is considered a potent doping agent due to its potential performance-enhancing properties <sup>[6,31]</sup>. Cobalt, a well-known chemical inducer of hypoxia-like responses, has been utilised clinically to stimulate erythropoiesis in patients with chronic anaemia and to promote physiological adaptation to low-oxygen conditions <sup>[7,32]</sup>. Until 2015, licensed formulations containing arsenic were available in several countries; however, their approval

was subsequently suspended worldwide because of carcinogenic effects <sup>[29]</sup>. Despite this, arsenic has been reported to occur in homoeopathic products and various plant sources <sup>[29,33]</sup>. In contrast, there are cobalt-containing approval formulations worldwide; moreover, cobalt may also be present as a component of premixes used in horse nutrition <sup>[7]</sup>.

Nevertheless, arsenic and cobalt violations have been reported in thoroughbred racehorses <sup>[29,34]</sup>. Consistent with previous studies, the results of the present study indicate that, following 15 arsenic violations in 2017, the number of detections declined in subsequent years; however, the continued detection of arsenic in 2018 (2 violations), 2020 (3 violations), and 2021-2022 (4 violations) suggests that this substance may have been administered either illegally or unknowingly.

Similarly, cobalt accounted for a high number of violations in 2017 (8 violations), concomitant with arsenic, and despite a reduction in arsenic detections, cobalt violations increased again in 2019 (7 violations) before declining from 2021 onwards (*Table 2*). This pattern suggests that during the period in which arsenic violations decreased (2018-2019), there may have been a shift towards alternative substances with erythropoietic properties, such as cobalt.

Furthermore, the frequent detection of arsenic and cobalt violations within short time intervals during specific years suggests that racehorses may have been feeds or formulations with unknown composition may be responsible. This detection indicates that threshold substance violations may arise not only from intentional administration but also from indirect or inadvertent exposure pathways. In addition, the detection of a high number of violations in 2017, when the laboratory first began arsenic and cobalt analyses, can be considered an important indicator that substances not included in the

analytical scope may have been widely used (*Table 2*).

Diisopropylamine (DIPA) is classified as a substance that has always been prohibited for use in racehorses [1]. Known to exhibit physiological effects similar to those of cobalt, DIPA was first detected in this study in 2019, with 3 violations identified in 2019 and 5 in 2020 [35]. Notably, during the period in which DIPA was detected, the frequency of cobalt violations decreased compared with previous years (7 violations in 2019 and 2 in 2020). This detection suggests that DIPA may have been preferred as an alternative to cobalt use (*Table 2*).

In residue limited substances category, caffeine was the most frequently detected substance (14 findings), followed by morphine (10 findings) and atropine (4 findings) (*Table 2*). Caffeine is a substance that has stimulant effects on the central nervous system and the musculoskeletal system. In addition, it has mild analgesic, bronchodilator/vasodilator, and diuretic properties [36]. Morphine, a substance derived from *Papaver somniferum*, is widely used in horses as a narcotic analgesic and anaesthetic [37]. Atropine is defined as a prototypical muscarinic receptor antagonist [38]. Because these substances may occur at variable concentrations in feeds used for horse nutrition [37-41], IFHA has established substance-specific IRLs for blood and urine [5]. In addition, licensed pharmaceutical formulations containing each of these substances are available in various countries, indicating multiple potential sources of exposure.

In this study, the detection of morphine (8 violations between 2020 and 2021) within a specific time period, followed by the cessation of these violations, suggests that a particular formulation may have been administered. However, the proximity of Izmir and Bursa to Afyon, where legal poppy (*Papaver somniferum*) cultivation is carried out, together with the presence of well-developed livestock and feed supply infrastructures in these provinces, also suggests the possibility of feed-related exposure [42]. Within this study, the detection of five morphine findings at the Bursa racetrack and four at the Izmir racetrack supports this interpretation.

Similarly, caffeine was detected as a single substance in some cases and in combination with analgesics and muscle relaxants in others, while atropine was detected exclusively in multiple-substance violations (*Table 2*).

These findings suggest that some of the detected substances may be associated with indirect or inadvertent exposure through feed. Nevertheless, based on the available analytical data, it is not possible to definitively distinguish whether the detected IRL substances, including caffeine, atropine, and morphine, originated from nutritional sources or from deliberate pharmaceutical administration.

As in previous studies, the most common reason for violations in this study was the items within the scope of ISL [20]. One of the main reasons for single-substance violations within the scope of the ISL may be the racing of horses under treatment without adherence to the detection times established by the IFHA [43]. Another possible reason could be that doping laboratories do not report findings below the ISL. This practice can create the idea that these substances cannot be detected by laboratories, thus leading to uncontrolled drug use. On the other hand, horse owners and veterinarians may not be sufficiently informed about the detection times of drugs [43].

In multiple-substance violations where metamizole and flunixin were detected, low concentrations of local anaesthetics, corticosteroids, and other NSAIDs were also detected. The fact that the detection times [43] of these detected substances are very close to each other can be considered as an indication that substances were administered at the same time, and it can also be considered as an indicator of the possibility of some non-defined cocktail formulations being used.

The majority of violations involving substances prohibited for use at any time were associated with NSAIDs (etofenamate, flufenamic acid, and etodolac), local anaesthetics (procaine), bronchodilators (dyphylline), and vasodilators (DIPA). Although rare, substances not approved for veterinary use, including verapamil, sildenafil, cocaine, and neostigmine, were also detected. In addition, nikethamide in 2015, verapamil in 2018, heptaminol in 2020, and cocaine in 2021 were detected only once. Similarly, findings of DIPA, pentoxifylline, and pyrilamine were observed during specific periods but were not detected afterwards (*Table 2*). These findings may suggest a tendency to favour alternative compounds believed to be undetectable, instead of substances already detected by the laboratory.

In this present study, etodolac and dyphylline, which have very short detection times, caused violations by being detected at low concentrations on 6 and 10 occasions, respectively [44,45]. While three etodolac findings were detected as single-substance violations and three as multiple-substance violations, five dyphylline findings were identified as single-substance violations, and the remaining five as multiple-substance violations in combination with NSAIDs (*Table 2*). These results suggest that substances with short detection times may have been administered in a manner consistent with their pharmacokinetic properties.

Our findings reveal that single and multiple-substance violations vary over the years and that, particularly from 2019 onwards, there has been a marked and sustained increase in multiple-substance violations (*Fig. 1*). This

situation suggests not only an increase in the frequency of violations but also a structural transformation in the nature.

Within the scope of the study, certain multiple-substance findings were detected more frequently during specific periods. Multiple-substance violations involving procaine, which began to be detected in 2016, had almost ceased by the end of 2020. Following the cessation of these violations, the detection of combinations containing meloxicam/hydroxymeloxicam began, and after these findings ended in 2022, multiple-substance violations containing flunixin started to be detected (*Table 3*). This data suggests that users may have shifted to alternative substances or formulations to avoid previously detected compounds. These findings indicate that multiple-substance violations are not random but rather that specific substance combinations were deliberately used or prepared. Although multiple-substance violations have been reported in previous studies, the temporal changes and substance profiles observed in this study indicate, unlike the existing literature, the presence of a more systematic approach to multiple-substance use <sup>[16,20]</sup>.

Findings related to single-substance violations indicate that the detection profile of certain NSAIDs and analgesic agents has changed over time. Until 2018, these violations were caused by etofenamate/flufenamic acid, ketoprofen, methylaminoantipyrine, and naproxen; after 2018, they were more frequently caused by betamethasone/dexamethasone, etodolac, etofenamate/flufenamic acid, hydroxylidocaine, morphine, and tenoxicam (*Table 4*). These findings indicate a significant shift in the use of NSAID/analgesic prohibited substances from 2018 onwards.

In conclusion, this nine-year retrospective evaluation provides a comprehensive overview of prohibited substance findings in thoroughbred racehorses in Türkiye. The results demonstrate that both single and multiple-substance violations persist over time, with a notable increase in multiple-substance detections in recent years. Temporal changes in detected substances emphasise the importance of ongoing monitoring approaches, harmonised regulatory thresholds, and increased awareness of potential feed-related contamination. These findings contribute region-specific data to the international literature and support ongoing efforts to ensure fair competition and protect horse welfare.

## DECLARATIONS

**Availability of Data and Materials:** The data that support the findings of this study are available from the corresponding author (E. Kabil) upon reasonable request.

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**Author Contribution:** E.K: Laboratory analysis and controls, article writing, statistical calculations E.F.G: Laboratory analysis, method validation, control of article writing, E.G: Laboratory analysis, L.Y: Laboratory analysis, T.A.Z: Laboratory analysis, M.H.T: Laboratory analyzes, B.I: Laboratory analysis, M.T: Laboratory analysis.

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## RESEARCH ARTICLE

# Early Behavioral Indicators of Mortality Risk in Pyrethroid-Exposed Bees Using Explainable Artificial Intelligence

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## Abstract

Pollinator populations, which play a critical role in maintaining global ecosystem health, have been experiencing marked declines worldwide due to widespread pesticide usage. However, early behavioral indicators of lethal stress induced by chemical exposure remain insufficiently characterized, largely because conventional ecotoxicological assessments predominantly focus on mortality-based endpoints. In this study, we evaluated the potential to predict mortality risk at an early stage using behavioral markers, based on 1.506 behavioral observation records collected from seven bee species exposed to lambda-cyhalothrin. To this end, we implemented explainable artificial intelligence models, including Random Forest, XGBoost, and LightGBM, and interpreted the model outputs using SHAP analysis. Among these models, Random Forest and XGBoost demonstrated the strongest performance in distinguishing high mortality risk, achieving an accuracy of 0.873 on an independent test dataset. SHAP-based model interpretation revealed a temporal behavioral progression associated with elevated mortality risk: cramps and apathy emerged as early warning indicators (2–4-hour window), uncoordinated movement represented the intermediate phase, and the dorsal recumbent position characterized the terminal collapse stage. These findings demonstrate that behavioral early-warning signals of lethal pesticide stress can be reliably detected prior to mortality and highlight the potential of explainable artificial intelligence as a robust decision-support tool for pollinator health monitoring and pesticide risk assessment.

**Keywords:** Behavioral biomarkers, Explainable AI, Pollinator health, Pyrethroid ecotoxicity, Sublethal effects

## INTRODUCTION

Animal pollinators are essential biotic agents that support the reproductive processes of flowering plants, thereby sustaining ecosystem functioning and enhancing agricultural productivity. Among them, bees represent one of the most widespread and efficient pollinator groups globally. With more than 20.000 recognized species, bees contribute to the reproductive success of nearly 90% of flowering plant species and significantly enhance the yield of numerous agricultural crops <sup>[1]</sup>. Approximately one-third of global food production directly depends on bee-mediated pollination <sup>[2]</sup>, and the annual economic value of these pollination services is estimated to exceed 200 billion USD <sup>[3,4]</sup>. Despite their ecological and economic importance, the pollination services provided by bees are facing increasing global threats. A growing body of evidence indicates a persistent and rapid decline in pollinator populations, particularly among bee species, driven by intensified anthropogenic pressures <sup>[5,6]</sup>. Although multiple interacting drivers contribute to these declines -including habitat loss, climate change, parasites,

and pathogens- widespread pesticide use in agricultural landscapes has emerged as a pervasive and acute stressor. Exposure to neurotoxic agrochemicals has been shown to severely impair bee navigation, foraging behavior, immune function, and reproductive biology, thereby accelerating colony collapse and contributing to the decline of wild bee populations <sup>[5,7]</sup>.

Pesticide exposure, one of the most significant threats to bee health, remains insufficiently integrated into comprehensive environmental management strategies. Current regulatory frameworks continue to rely predominantly on acute, mortality-based endpoints, which hinders the incorporation of early behavioral warning signals into risk assessment processes. Yet, exposure to sublethal pesticide doses can disrupt navigation, social organization, and colony-level functioning well before mortality or colony collapse becomes apparent <sup>[8]</sup>. Michelangeli et al.<sup>[6]</sup> emphasized that such sublethal effects may play a critical, yet frequently overlooked, role in long-term population declines and argued for the more systematic integration of behavioral indicators



into ecological risk evaluation. However, quantitative and temporally explicit analyses of behavioral progression under toxic stress remain limited. Most existing studies focus on static behavioral markers or isolated reaction patterns, making it difficult to capture the sequential and emergent neuro-motor disruptions induced by chemical exposure.

In this context, the early detection of pesticide-induced ecological impacts not only increases the sensitivity of environmental risk assessment frameworks but also substantially improves the timing and effectiveness of interventions aimed at safeguarding pollinator health. To overcome the limitations of traditional observational approaches and systematically analyze early behavioral signals, data-driven computational methods such as Artificial Intelligence (AI) and Machine Learning (ML) have increasingly been integrated into ecotoxicological research in recent years<sup>[9,10]</sup>. These approaches not only enhance predictive performance but also possess the capacity to learn multivariate behavioral patterns and model their temporal progression under toxic stress<sup>[11]</sup>. However, the inherently “black-box” nature of many ML algorithms -where decision-making processes are not directly interpretable- poses challenges for biologically grounded inference and limits their reliability for regulatory decision-makers. To address this issue, Explainable Artificial Intelligence (XAI) techniques, particularly SHAP (SHapley Additive exPlanations) and LIME (Local Interpretable Model-agnostic Explanations), provide model-agnostic and model-specific interpretability frameworks that elucidate not only what the model predicts but also why and how<sup>[7]</sup>. By enabling transparent attribution of relative feature contributions to model outcomes, XAI approaches allow pesticide-related behavioral disruptions to be interpreted in a mechanistically meaningful way. Thus, XAI establishes a new methodological axis in ecotoxicology by coupling predictive accuracy with biological interpretability, which is critical for the early identification of environmental stress signals.

This study introduces an innovative modeling framework that integrates XAI approaches into ecotoxicology to enable the early detection of behavioral stress responses induced by pesticide exposure. We analyzed a dataset comprising 1.506 individual behavioral observations representing lambda-cyhalothrin exposure across seven bee species -*Andrena vaga*, *Bombus terrestris*, *Colletes cunicularius*, *Osmia bicornis*, *Osmia cornuta*, *Megachile rotundata*, and *Apis mellifera*- using powerful tree-based machine learning algorithms, including Random Forest, XGBoost, and LightGBM. Following mortality risk classification, model decision pathways were interpreted using SHAP analyses, which allowed us to explicitly quantify the directionality and relative importance of

behavioral indicators contributing to elevated mortality risk. This integrative approach not only achieved high predictive accuracy but also enabled the characterization of the behavioral progression underlying pyrethroid-induced lethality.

## MATERIAL AND METHODS

### Ethical Statement

This study did not involve any procedures requiring ethical approval.

### Data Source and Experimental Context

This study is based on the re-analysis of an open-access dataset comprising 1.506 individual behavioral observations that document the time-resolved effects of lambda-cyhalothrin exposure on seven bee species: *Andrena vaga*, *Bombus terrestris*, *Colletes cunicularius*, *Osmia bicornis*, *Osmia cornuta*, *Megachile rotundata*, and *Apis mellifera*. The data were originally collected by Jütte et al.<sup>[12]</sup> under standardized cage test conditions, with behavioral and mortality assessments recorded at 2, 4, 24, 48, 72, and 96 hours post-exposure. Detailed experimental procedures, including rearing conditions, exposure protocols, and scoring criteria, are described comprehensively in the original publication. The dataset used in this study is publicly available through the OpenAgrar Repository: [https://www.openagrar.de/receive/openagrar\\_mods\\_00092232](https://www.openagrar.de/receive/openagrar_mods_00092232).

### Mortality Rate and Behavioral Variable Encoding

At each observation time point, colony-level mortality was calculated using the number of dead and surviving individuals. Specifically, the mortality rate was obtained by dividing the number of dead individuals by the total number of individuals observed (dead + alive).

To assess the ability of behavioral indicators to predict mortality outcomes, this continuous mortality measure was converted into a binary risk variable based on toxicological decision thresholds:

- Low Risk: Mortality <30%
- High Risk: Mortality ≥30%

This threshold was selected to represent biologically meaningful colony-level stress while still enabling early detection of behavioral deterioration preceding terminal mortality, consistent with growing emphasis on sublethal and functional endpoints in ecotoxicological risk assessment.

For each individual, seven behavioral indicators reflecting the neuro-motor progression of pesticide-induced decline (moribund, cramps, apathy, uncoordinated, restless, dorsal, and vertigo) were evaluated. Following the scoring procedure described by Jütte et al.<sup>[12]</sup>, these variables were

treated as numerical features in the analysis. In cases where behavioral scoring captured intensity rather than simple presence/absence, the quantitative grading was retained in the modeling process. This allowed the relationship between behavioral progression and mortality risk to be evaluated in terms of both occurrence and severity.

### Data Preprocessing

Prior to analysis, the dataset was processed to prevent bias during model training. Missing values in the behavioral variables were imputed using a median-based approach that preserves interspecific variance structure. To prevent differences in measurement scales from disproportionately influencing model decisions, all explanatory variables were standardized using z-transformation (mean = 0, standard deviation = 1). To ensure an objective evaluation of model performance, the dataset was partitioned into training (75%) and testing (25%) subsets using a stratified sampling strategy that maintains both class proportions and representation across species. This approach was specifically selected to avoid artificially inflating or diminishing the model's discriminative ability in cases where the high-mortality class contains comparatively fewer observations.

### Machine Learning Algorithms and Hyperparameter Optimization

To evaluate the extent to which behavioral indicators can predict high mortality risk, three ensemble tree-based machine learning algorithms were applied. Random Forest constructs multiple decision trees on bootstrap-resampled subsets of the training data and aggregates their predictions through majority voting, thereby reducing overfitting and improving generalization performance<sup>[13]</sup>. XGBoost employs a gradient boosting framework that iteratively refines decision trees to minimize residual errors, and incorporates L1/L2 regularization and parallel computation, enabling high accuracy and efficiency in large and imbalanced datasets<sup>[14]</sup>. LightGBM further enhances computational efficiency by converting continuous variables into histogram bins and applying a leaf-wise tree growth strategy, which facilitates fast and stable learning in high-dimensional feature spaces<sup>[15]</sup>.

Hyperparameter optimization for each model was conducted using stratified 5-fold cross-validation to maximize predictive performance. To maintain sensitivity, particularly for the high-mortality class, class imbalance was addressed using algorithm-specific weighing strategies: `class_weight = 'balanced'` for Random Forest, `scale_pos_weight = (neg/pos)` for XGBoost, and `class_weight = {0:1, 1:k}` for LightGBM. The key hyperparameters used in the final models are summarized in *Table 1*.

### Model Explainability

To examine the direction and magnitude of each behavioral indicator's contribution to model decisions,

**Table 1.** Machine learning models used in this study and their key hyperparameters

| Model         | Hyperparameters                                                                                                                      |
|---------------|--------------------------------------------------------------------------------------------------------------------------------------|
| Random Forest | <code>n_estimators=500</code> , <code>max_depth=None</code> , <code>class_weight='balanced'</code>                                   |
| XGBoost       | <code>n_estimators=600</code> , <code>learning_rate=0.05</code> , <code>max_depth=4</code> , <code>scale_pos_weight=(neg/pos)</code> |
| LightGBM      | <code>num_leaves=31</code> , <code>learning_rate=0.05</code> , <code>class_weight={0:1, 1:k}</code>                                  |

SHAP analysis was conducted on the XGBoost model. The SHAP beeswarm plot visualized both the sign (positive or negative influence) and the relative effect size of each behavior on mortality risk, thereby revealing a coherent behavioral progression from early to intermediate and terminal stages of collapse in accordance with the model's internal decision structure.

### Performance Evaluation

Multiple evaluation metrics were used to comprehensively assess model performance. While accuracy indicates the overall proportion of correctly classified observations, it is not sufficient on its own when class distributions are imbalanced. Therefore, performance was primarily interpreted through Precision, Recall, and F1-score, which more accurately represent the model's ability to distinguish the high-mortality class. Precision reflects the proportion of individuals predicted as high mortality that were correctly classified, whereas Recall represents the proportion of actual high-mortality individuals that were successfully identified by the model. The F1-score, calculated as the harmonic mean of Precision and Recall, is particularly appropriate for imbalanced datasets. In addition, Support values were reported to indicate the number of instances in each class, allowing these metrics to be contextualized relative to class prevalence. This evaluation strategy prioritizes the reliable early detection of high mortality risk, rather than solely maximizing overall accuracy.

### Computational Environment and Libraries

All analyses were conducted in Python 3.11 using Google Colab. Data processing and management were performed with pandas and NumPy, and visualizations with Matplotlib. The Random Forest model was implemented using scikit-learn, while gradient boosting classifiers were trained with XGBoost and LightGBM. Model explainability was assessed using the SHAP library to identify the contribution of behavioral predictors to mortality risk. All code and analytical steps were executed in a fully reproducible workflow.

## RESULTS

*Table 2* summarizes the performance metrics of the classification models developed to predict high mortality

| Table 2. Early warning classification performance of Random Forest, XGBoost, and LightGBM models based on behavioral predictors |          |                  |               |           |
|---------------------------------------------------------------------------------------------------------------------------------|----------|------------------|---------------|-----------|
| Model                                                                                                                           | Accuracy | Precision (High) | Recall (High) | F1 (High) |
| Random Forest                                                                                                                   | 0.873    | 0.538            | 0.778         | 0.636     |
| XGBoost                                                                                                                         | 0.873    | 0.545            | 0.667         | 0.600     |
| LightGBM                                                                                                                        | 0.762    | 0.333            | 0.667         | 0.444     |

risk based on behavioral response variables. The Random Forest model achieved the highest overall accuracy (87.3%), with a recall of 0.778 for the high-mortality class, indicating strong sensitivity in identifying high-risk cages. XGBoost yielded a similar overall accuracy (87.3%), with a recall of 0.667 and an F1-score of 0.600 for the high-mortality class. In comparison, LightGBM demonstrated lower performance in distinguishing the high-mortality class (F1 = 0.444). These results indicate that sublethal behavioral alterations contain detectable signals associated with increased mortality risk, supporting the potential of behavior-based approaches for early warning assessment.

The confusion matrices shown in Fig 1-A,B,C provide a detailed comparison of the models' ability to detect the high-mortality class ( $y = 1$ ). The Random Forest model correctly classified 77.8% of high-mortality cases (7 out of 9), indicating strong sensitivity in identifying high-risk cages. XGBoost correctly identified 66.7% of high-mortality cases (6 out of 9). In contrast, LightGBM also identified 66.7% of high-mortality cases but produced a higher number of false positives in the low-mortality class. For the low-mortality class ( $y = 0$ ), both Random Forest and XGBoost maintained high classification accuracy (RF: 48/54; XGB: 49/54), whereas LightGBM showed reduced performance (42/54). These results reflect higher true-positive detection in the high-mortality class for Random Forest and XGBoost compared to LightGBM.

SHAP analysis was performed to interpret model outputs and identify the behavioral patterns associated with high mortality risk (Fig 2). The SHAP distribution plots revealed not only the relative importance of each behavioral variable but also the direction of their contribution to mortality classification. Higher values of

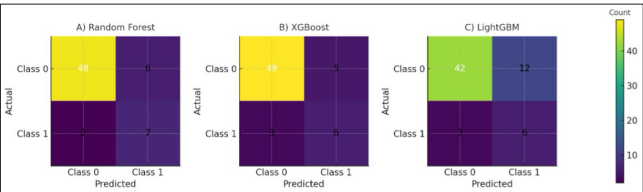


Fig 1. Confusion matrices for the three classification models: A- Random Forest, B- XGBoost, and C- LightGBM, illustrating classification performance for the high-mortality (Class 1) and low-mortality (Class 0) groups

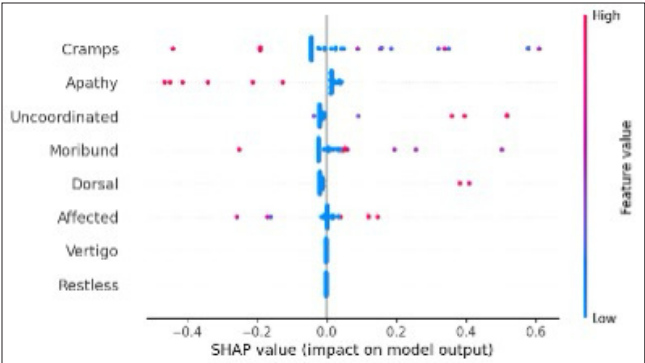


Fig 2. SHAP beeswarm plot illustrating the contributions of behavioral variables to the classification of high-mortality cases

cramps and apathy were predominantly associated with negative SHAP values, indicating that the increase of these behaviors contributed to model predictions of high mortality risk during earlier neuromuscular impairment stages. In contrast, higher values of uncoordinated and dorsal were concentrated in the positive SHAP region, reflecting their stronger association with later-stage loss of motor coordination and postural control. For the moribund variable, SHAP contributions appeared across both positive and negative regions, consistent with its occurrence during rapidly transitioning terminal phases. Taken together, the SHAP patterns indicate a sequential progression of behavioral decline associated with elevated mortality risk, transitioning from cramps → loss of coordination → dorsal posturing → moribund state.

DISCUSSION

Our classification models demonstrated high sensitivity in detecting impending colony-level mortality based on sublethal behavioral alterations. Among them, the Random Forest model achieved the strongest performance, correctly identifying the majority of high-mortality cases (recall = 0.778; accuracy = 87.3%). XGBoost showed comparable overall accuracy (87.3%) with moderately lower sensitivity (recall = 0.667), while LightGBM yielded lower discriminative performance for the high-mortality class (accuracy = 76.2%; F1 = 0.444). Notably, both Random Forest and XGBoost maintained low false-negative rates, minimizing the likelihood of failing to detect cages experiencing severe toxic stress. In the context of early warning systems, prioritizing sensitivity over precision is a strategically appropriate trade-off, as the consequences of overlooking a high-risk colony are substantially greater than issuing a false alert. These results suggest that subtle yet consistent behavioral signals emitted during the early stages of stress can be decoded by machine learning models to provide actionable early risk detection. Accordingly, the behavioral patterns captured in this study may serve as valuable indicators for timely intervention in managed colonies.

The SHAP explainability analysis revealed a clear sequential structure in the behavioral signature associated with lethal pesticide stress. Higher values of cramps and apathy were associated with negative SHAP contributions, indicating their prominence during early stages of neuromuscular impairment. In contrast, elevated uncoordinated and dorsal scores contributed positively to mortality predictions, reflecting later-stage collapse characterized by loss of motor coordination and postural control. The moribund behavior showed mixed SHAP contributions across both positive and negative regions, consistent with its occurrence during rapidly transitioning terminal phases. Together, these patterns indicate a cascading progression of neuromuscular decline: early muscle spasms and reduced activity → loss of coordination → failure to maintain dorsal posture → terminal moribund state. This staged behavioral collapse aligns with known acute neurotoxic responses reported in bees and bumblebees, where pesticide exposure initially disrupts coordination and elevates agitation, followed by reduced mobility and postural failure under sustained stress<sup>[16]</sup>. Our machine-learning-derived behavioral signature therefore reflects established toxicodynamic processes, but presents them within a unified, temporally ordered framework. The identification of this structured progression is a novel contribution, suggesting that monitoring the sequence of behavioral anomalies may provide richer diagnostic insight than evaluating behaviors as isolated symptoms. Future work could validate this cascade across additional species and stressors, yet the present findings already advance our understanding of how pesticide-induced neurotoxicity unfolds behaviorally in pollinator systems.

From an ecological perspective, these findings are particularly significant. Insect pollinators function as keystone species, supporting approximately 75% of global crops and 88% of wild flowering plant species<sup>[17]</sup>. Declines in bee populations therefore pose direct risks to global food security and ecosystem resilience. Recent research has identified contemporary pesticide use -especially neonicotinoids and pyrethroids- as a major driver of these declines<sup>[8]</sup>. For example, Guzman et al. reported that increasing neonicotinoid and pyrethroid use across the United States was associated with substantial reductions in wild bee occupancy, exceeding 40% in some groups<sup>[17]</sup>. Similarly, Dicks et al.<sup>[18]</sup> emphasized that pollinator conservation is essential for ecosystem stability and human well-being, noting that pesticides contribute to pollinator declines not only via acute toxicity but also through indirect sublethal effects. Within this context, our behavioral prediction framework has clear conservation relevance: it enables the detection of lethal pesticide impacts before colony collapse becomes visible. Such early warning capability may provide beekeepers and

ecologists with a practical decision window in which to relocate colonies, adjust pesticide application timing, or implement mitigation measures to preserve pollination services. These results therefore reinforce the growing scientific consensus that sublethal behavioral indicators should be integrated into pollinator health monitoring systems. As Ulrich et al.<sup>[19]</sup> argue, social insects such as bees are keystone species in terrestrial ecosystems, and AI-enabled behavioral monitoring holds considerable potential for ecological protection and restoration. By providing a measurable link between environmental stressors and colony outcomes, our findings contribute to this emerging direction and support proactive pollinator conservation strategies.

From a practical standpoint, these findings highlight pathways for implementing behavior-based monitoring in pollinator health assessment. Ongoing work in Precision Apiculture already leverages non-invasive sensing and machine learning to track colony conditions. For example, acoustic monitoring systems can automatically classify hive states<sup>[20]</sup>, and computer vision platforms have been developed to continuously record behavioral activity over extended periods<sup>[19]</sup>. Likewise, Hossain and Baer introduced an “Electronic Bee Veterinarian” framework that uses temperature sensors and predictive modeling to alert beekeepers to colony stress -whether thermal, pathological, or pesticide-related- several days in advance<sup>[21]</sup>. The behavior scoring approach developed in this study could be integrated into such frameworks. A practical monitoring protocol could combine internal or external hive cameras with computer vision pipelines to quantify key behavioral metrics such as muscle spasms, mobility patterns, and postural stability. These quantified metrics could then be supplied to the machine learning classifier to generate early risk alerts. Such alerts may prompt targeted inspections or mitigation measures, including modifying pesticide application, providing supplemental nutrition, or relocating colonies to safer foraging environments. Importantly, integrating multiple data streams -such as temperature, acoustic activity, and behavioral indicators- will likely yield the most robust early warning systems.

Although this study focused on a pyrethroid insecticide, which is among the most widely used neurotoxic compounds in agricultural systems, the proposed behavior-based early warning framework is not inherently chemical-specific. While behavioral trajectories may vary across pesticides with different modes of action, core manifestations of neurotoxic stress such as impaired coordination, postural instability, and reduced activity are expected to represent convergent functional endpoints, supporting the broader applicability of the approach.

In summary, this study demonstrates that machine learning can transform subtle behavioral disruptions into

interpretable and actionable indicators of lethal stress. This advances pollinator monitoring beyond passive observation and toward a new generation of digital, responsive, and preventative colony health management.

## DECLARATION

**Availability of Data and Materials:** The dataset used in this study is publicly available on OpenAgrar at [https://www.openagrar.de/receive/openagrar\\_mods\\_00092232](https://www.openagrar.de/receive/openagrar_mods_00092232).

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## RESEARCH ARTICLE

# Economic and Veterinary Evaluation of Pet Insurance in Türkiye: Potential, Role in Health, and Impact on Practice

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## Abstract

Pet insurance has emerged as a critical financial tool in developed markets for both animal health management and veterinary practice sustainability, driven by rising global pet ownership, increasing veterinary costs, and extended companion animal lifespans. Insurance mitigates financial barriers for pet owners, improves access to services, and reduces the risk of economic euthanasia. The benefits extend to veterinarians, too, by facilitating treatment decisions and thus strengthening the client–veterinarian relationship. However, despite high adoption rates in countries like Sweden and Germany, the market formation in Türkiye is in its infancy, characterized by limited penetration and public awareness. This study aims to evaluate the potential economic and veterinary outcomes of introducing pet insurance in Türkiye using a scenario-based simulation approach. Three scenarios were developed according to different microchipping and market penetration assumptions. Insurance products currently offered in Türkiye were examined to determine three representative premium levels, which were applied across scenarios to estimate Gross Written Premium, expected claims, and technical profitability. Analyses indicate that even a 5% penetration rate could generate an annual Gross Written Premium volume exceeding TRY 400 million. The findings highlight the insurance's potential to support the financing of the veterinary sector, expand preventive care, and enhance owner affordability. From a welfare perspective, insurance adoption can improve early diagnosis and reduce treatment abandonment, thereby elevating animal welfare. The research underscores the dual economic and ethical role of insurance as both a market instrument and a public good in animal health management. Ultimately, the study points to the need for awareness campaigns and supportive policy frameworks for the future development of the Turkish market.

**Keywords:** Animal welfare, Companion animals, Healthcare access, Pet insurance, Veterinary medicine

## INTRODUCTION

The global increase in pet ownership, driven by urbanization and rising animal welfare awareness, has led to a significant increase in demand for veterinary health services and a resulting economic burden on owners. Pet owners increasingly view their animals not merely as property but as family members <sup>[1-5]</sup>. This trend is particularly evident among younger generations, where prioritizing pet health is common <sup>[5]</sup>. Globally, pet populations exceed 1 billion, with more than half of the world's population owning at least one pet <sup>[6]</sup>. Regions like the EU, China, and the United States hold the largest pet populations <sup>[6]</sup>.

In this context, pet insurance has emerged as a crucial financial tool to manage substantial veterinary costs, from regular check-ups to chronic disease treatments. Pet insurance functions similarly to human health insurance,

with owners paying a premium to cover a portion or the entirety of veterinary expenses, though policy specifics (e.g., pre-existing conditions) vary <sup>[7]</sup>. This insurance type has achieved high prevalence in some European countries, such as Sweden (where over 40% of pets are insured), though overall adoption remains low in many developing and large markets, including the United States <sup>[8]</sup>.

From a veterinary perspective, pet insurance is vital for animal welfare and the sustainability of clinical practice. Insured pets benefit from better access to regular care and early diagnosis, improving overall quality of life <sup>[9]</sup>. Studies confirm that insurance facilitates the recording and treatment of common and complex diseases at a higher rate <sup>[10,11]</sup>, allowing veterinarians to practice evidence-based medicine <sup>[12]</sup>. The availability of insurance provides a necessary safeguard against the rapidly rising costs of veterinary services, especially given the increasing prevalence of chronic diseases and longer lifespans in



pets <sup>[13-15]</sup>. Research indicates that insured households are more likely to pursue recommended treatments <sup>[10,11]</sup> and report lower levels of “economic euthanasia”, where pets are euthanized due to unaffordable treatment costs <sup>[16]</sup>. Despite these benefits, challenges remain, including owners’ limited understanding of policy coverage and exclusions, which can lead to dissatisfaction <sup>[7,17]</sup>. Lack of owner awareness is cited as a major barrier to wider adoption <sup>[18,19]</sup>.

In Türkiye, the insurance market generally remains underdeveloped compared to OECD averages, with an overall insurance penetration rate below 2.5% <sup>[20]</sup>. Nevertheless, the market exhibits remarkably high nominal growth rates across several non-life insurance classes, suggesting significant potential for emerging segments <sup>[20]</sup>. Pet insurance is a nascent field in Türkiye, gaining wider recognition around 2015. Current products typically focus on accident and emergency medical expenses rather than comprehensive health coverage, covering issues like traffic accidents, fractures, and accidental injuries <sup>[21]</sup>. Currently, there are no official sector-wide statistics on pet insurance penetration rates in Türkiye. However, the high growth potential is supported by key factors: the significant companion animal population (approximately \$4.7 million domestic cats and \$1.4 million domestic dogs) <sup>[22]</sup>, and mandatory microchipping regulations which have established a foundational database of over \$2 million registered companion animals, a key precondition for many insurance policies <sup>[23]</sup>.

Given the documented global benefits of pet insurance on animal welfare <sup>[10,11,16]</sup> and the high growth dynamics of the Turkish insurance sector <sup>[22]</sup>, a critical gap exists in understanding the potential market scale and financial viability of pet insurance in Türkiye. This study aims to quantify and evaluate the current situation and potential development areas for pet insurance in Türkiye through a scenario-based simulation. Specifically, this study investigates the potential impacts of introducing pet insurance in Türkiye by modeling key financial indicators (premium and claims flow) under varying market penetration and registration assumptions. This research offers new insights for both the insurance industry and veterinary professionals by combining economic and animal welfare perspectives.

## MATERIAL AND METHODS

### Ethical Statement

This study did not involve any procedures requiring ethical approval.

### Compilation of Supplementary Data and Visuals

Based on the information obtained from the publications

cited in the introduction, a map showing pet ownership rates across the world was created to provide a global context for the study. The multifaceted advantages of pet insurance, including persistent challenges and limitations, were summarized and structured into a table format by synthesizing the information gathered from the literature review. This synthesized framework provides an overview of the benefits for different stakeholders, including pet owners, veterinarians, and companion animals.

### Data Sources and Scope

The analysis relies on a simulation model built upon publicly available market data and primary market research. The key data sources used to define the scope and parameters of the simulation are as follows:

- *Companion Animal Population:* The total companion animal population in Türkiye (approximately 4.7 million cats and 1.4 million dogs) was estimated using figures derived from the European Pet Food Industry Federation (FEDIAF) <sup>[22]</sup>.
- *Microchipping Data:* Data concerning the baseline number of microchipped and registered companion animals (cats, dogs, and ferrets) in Türkiye (2.031.112 total) were sourced from the Ministry of Agriculture and Forestry as of January 1, 2021 <sup>[23]</sup>.
- *Market Parameters and Product Analysis:* The range for the annual premium (TRY 3.500 - TRY 5.000) and the typical coverage details used in the simulation were derived from a detailed primary review and analysis of pet insurance products offered by the main insurers operating in the Turkish market <sup>[21]</sup>. This review specifically encompassed the offerings from:
  - Türkiye Sigorta - “Sempatı” pet insurance (standard and comprehensive packages).
  - Allianz Sigorta - “Sevimli Dostum” pet insurance (various coverage limits).
  - AXA Sigorta - pet insurance (five package options).
  - Anadolu Sigorta - “Patim Güvende” pet insurance (Basic and Plus packages).
  - Zurich Sigorta - “Neşeli Patiler” pet insurance (Standard and Premium packages).
  - Magdeburger Sigorta - “Can Dostum” pet insurance (single package).
  - Fiba Sigorta - “Fi-Pati” pet insurance (SOS, Mini, and Maxi packages; no upper age limit for Mini and SOS options).
  - AK Sigorta - “Pati” pet insurance (Basic, Plus, and Plus-VetAmerikan packages).

The analysis focused on standard or ‘mini’ packages to establish the baseline market pricing and coverage assumptions utilized in the simulation. An examination of pet insurance products available in the Turkish insurance market reveals that standard or ‘mini’ packages typically offer treatment coverage (including emergency, outpatient, or inpatient care) ranging from 25.000 TRY to 35.000 TRY, and third-party liability coverage between 10.000 TRY and 20.000 TRY. It was observed that the annual premium for these basic policies is generally between 3.500 TRY and 5.000 TRY. As policy coverage is expanded, annual premiums can increase significantly, reaching up to 25.000 TRY.

### Simulation Model and Calculation Methodology

To assess the potential size and financial performance of the pet insurance market in Türkiye, a quantitative simulation model was developed using Python (Jupyter notebook).

### Scenario Definition

Three distinct simulation scenarios were defined based on varying assumptions regarding the penetration rate of microchipping among the total companion animal population (cats and dogs). Scenario 1 represents the current market, with approximately 2.03 million microchipped cats and dogs. Scenario 2 assumes that the microchipping rate increases to 60% of the total pet population (around 3.66 million animals), while Scenario 3 assumes an 80% rate (about 4.88 million animals), reflecting a possible outcome of wider regulatory enforcement. Within each scenario, different market penetration rates (1%, 5%, and 10%) were considered to estimate the number of potential policies sold. The annual premium was assumed to range between TRY 3.500 and TRY 5.000, corresponding to typical “standard” or “mini” packages offered by insurers in Türkiye.

### Financial Indicators and Formulas

For each combination of scenario and market penetration rate, three key financial indicators were calculated:

- **Gross Written Premium (GWP):** the total amount of premiums collected, representing the nominal size of the market. The calculation of GWP utilized three distinct premium levels for the premium per policy: 3.500 TRY (Low), 4.250 TRY (Medium), and 5.000 TRY (High).
- **Expected Claims (EC):** the estimated total value of claims to be paid by insurers, derived from an assumed **loss ratio** of 60-70%, meaning that insurers are expected to pay out 60-70% of collected premiums as claims.  $EC = GWP \times \text{Loss Ratio}$
- **Technical Profit (TP):** the residual amount after

deducting both claims and operational expenses (assumed at 20% of GWP) from total premiums, representing the insurer’s underwriting margin.  $\text{Technical Profit} = GWP \times (1 - \text{Loss Ratio} - \text{Expense Ratio})$

While these ratios are benchmarked against mature markets, their application to the Turkish context is justified by two specific market dynamics. First, the 60-70% loss ratio aligns with the target technical profitability thresholds required for long-term sustainability in the Turkish non-life insurance sector, ensuring that the product remains valuable to the consumer without eroding the insurer’s capital base. Second, the 20% operating expense ratio is predicated on a ‘digital-first’ distribution strategy. Given Türkiye’s high mobile internet penetration and the growing trend of direct-to-consumer sales in niche insurance products, it is assumed that reduced intermediary commissions and automated underwriting will keep operational costs significantly lower than traditional agency-based models.

## RESULTS

The global distribution of pet ownership rates, compiled from various international publications, is presented in [Fig. 1](#) to provide a contextual overview of the market landscape.

[Table 1](#) summarizes the multifaceted advantages of pet insurance for different stakeholders, including pet owners, veterinarians, and companion animals. In addition, it highlights persistent challenges and limitations that may hinder broader adoption and effective utilization of such policies.

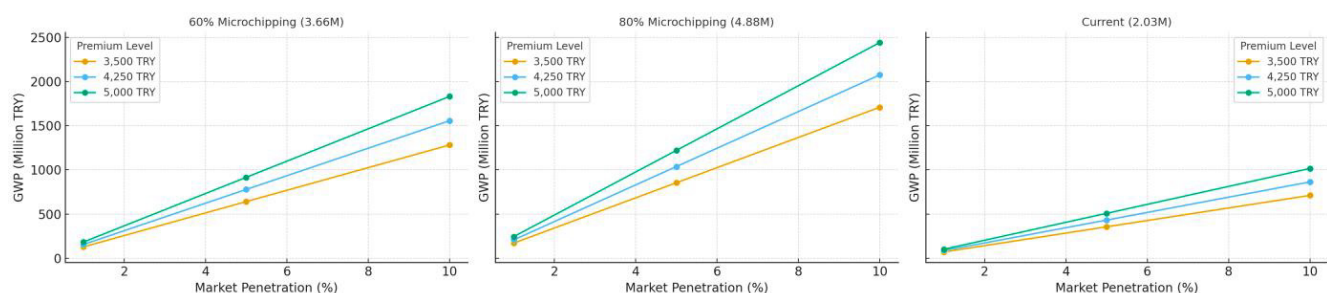
The simulation results reveal that the potential size of the pet insurance market in Türkiye is highly sensitive to both the level of market penetration and the spread of microchipping among companion animals. [Fig. 2](#) illustrates the impact on Gross Written Premium (GWP) across three different premium levels for the current microchipping rate of 2.03 million and hypothetical



**Fig 1.** Pet Ownership Rates Worldwide

**Table 1.** Comparative overview of pet insurance: advantages for pet owners, veterinarians, and pets, alongside key challenges and limitations

| Advantages for Pet Owners                                                                                                                                                                                                                                                                                                                             | Advantages for Veterinarians                                                                                                                                                                         | Advantages for Pets                                                                                                                                                    |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Reduces out-of-pocket veterinary expenses<br>Increases access to advanced treatments<br>Provides financial security in emergencies<br>Enhances pet welfare and life expectancy                                                                                                                                                                        | Reduces financial conflicts with clients<br>Increases treatment acceptance rates<br>Enables focus on best medical options, not just affordability<br>Strengthens trust and communication with owners | Decreases euthanasia rates<br>Access to better care opportunities<br>Regular veterinary check-ups<br>Longer lifespan<br>Access to advanced technology-based treatments |
| Challenges and Limitations                                                                                                                                                                                                                                                                                                                            |                                                                                                                                                                                                      |                                                                                                                                                                        |
| Lack of awareness and understanding among owners<br>Insufficient promotion and education about policies<br>Affordability issues: premiums + veterinary bills still costly for some owners<br>Variability in policy coverage across companies<br>Lack of a widespread insurance culture<br>Limited marketing and promotional efforts for pet insurance |                                                                                                                                                                                                      |                                                                                                                                                                        |

**Fig 2.** Simulated Gross Written Premium vs Market Penetration across microchipping scenarios

microchipping rates of 60% and 80, respectively, as market penetration increases. It is evident that GWP is substantially and linearly affected by the increases in both the chipping rate and penetration.

**Table 2** demonstrates that at 5% market penetration within the current microchipped population, total GWP increases proportionally with higher premium levels, while the Expected Claims (EC) calculated as a fixed proportion of GWP according to the assumed loss ratio (60-70%) follow the same upward pattern. The **Technical Profit** narrows significantly as the loss ratio increases from 60% to 70%, highlighting the critical importance of accurate risk pricing and claims management in sustaining profitability.

**Table 3** compares alternative scenarios based on higher microchipping rates (60% and 80% of the total pet population). Under constant pricing and penetration assumptions (TRY 4.250 annual premium, 5% market penetration, 70% loss ratio), the overall premium volume rises from TRY 432 million in the current situation to TRY 1.04 billion in the 80% microchipping scenario-an increase of more than 140%. This illustrates the direct link between regulatory enforcement of pet registration and the expansion potential of the insurance market.

A comparative evaluation of the simulation outcomes demonstrates clear quantitative differences across scenarios.

For the current microchipped population, increasing the annual premium from TRY 3.500 to TRY 5.000 results in a proportional increase in Gross Written Premium of approximately 43%, while Technical Profit increases by a similar magnitude under a constant loss ratio. However, changes in the assumed loss ratio exert a markedly stronger effect on profitability: increasing the loss ratio from 60% to 70% leads to an average reduction of nearly 50% in Technical Profit across all premium levels. This highlights that underwriting performance and claims management play a more critical role in financial sustainability than premium pricing alone.

In contrast, scenario comparisons based on microchipping coverage reveal that structural market expansion has the greatest impact on overall market size. Holding premium level, penetration rate, and loss ratio constant, increasing microchipping coverage from the current level to 80% of the pet population results in more than a twofold increase in Gross Written Premium. These comparative findings indicate that regulatory enforcement of pet registration and identification may generate substantially larger market gains than isolated adjustments in pricing or penetration strategies.

From a policy and strategic perspective, these results suggest that further institutional encouragement of pet registration and awareness programs could substantially accelerate the development of the pet insurance sector

**Table 2.** Financial outcomes for current microchipped pets

| Annual Premium (TRY) | Loss Ratio (%) | GWP (TRY)   | EC (TRY)    | TP (TRY)    |
|----------------------|----------------|-------------|-------------|-------------|
| 3.500                | 60             | 355.446.000 | 213.267.600 | 71.089.200  |
| 3.500                | 70             | 355.446.000 | 248.812.200 | 35.544.600  |
| 4.250                | 60             | 431.873.000 | 259.123.800 | 86.374.600  |
| 4.250                | 70             | 431.873.000 | 302.311.100 | 43.187.300  |
| 5.000                | 60             | 507.778.000 | 304.666.800 | 101.555.600 |
| 5.000                | 70             | 507.778.000 | 355.444.600 | 50.777.800  |

GWP: Gross Written Premium; EC: Expected Claims; TP: Technical Profit

**Table 3.** Comparison of chip scenarios

| Chip Scenario        | Microchipped Count | GWP (TRY)   | EC (TRY)    | TP (TRY)   |
|----------------------|--------------------|-------------|-------------|------------|
| Current Microchipped | 2.031.112          | 431.613.000 | 302.129.100 | 43.161.300 |
| Chip Rate 60%        | 3.660.000          | 777.750.000 | 544.425.000 | 77.775.000 |

GWP: Gross Written Premium; EC: Expected Claims; TP: Technical Profit

in Türkiye. Even at conservative penetration levels, the segment has the potential to contribute meaningfully to the non-life insurance portfolio, while also promoting responsible pet ownership and financial resilience among households.

## DISCUSSION

Pet insurance systems have been shown internationally to support improved access to veterinary care, reduce financial barriers for pet owners, and enhance continuity of treatment [6,24]. It is supported by recent studies that, particularly in nations with a high prevalence of pet insurance, the insurance elevates veterinary expenditure among pet owners and enhances access to advanced diagnostic and therapeutic modalities. Furthermore, evidence suggests that pet insurance facilitates client compliance with complex treatment plans, which veterinarians note can reduce cost pressure during clinical decision-making [25,26]. Consequently, this development may indirectly contribute to the enhanced predictability of veterinary clinics' revenue streams [25]. Similarly, North American industry data indicate that insured pets receive more diagnostic and preventive services, demonstrating the role of insurance in promoting better clinical outcomes and reducing cost-driven treatment delays [8]. These global experiences suggest that structured insurance adoption may serve as a catalyst for strengthening veterinary healthcare systems in emerging markets, including Türkiye. Building upon these findings, the present study contributes by providing a macroeconomic and simulation-based evaluation of how expanding pet insurance coverage could affect the financial stability of

veterinary practices in an emerging market context such as Türkiye.

The scenario-based simulation not only demonstrates the potential financial outcomes for insurers but also provides valuable insights into the implications for veterinary professionals, pet owners, and overall animal welfare.

Scenario 1 (Current microchipped population) reflects the current status of the Turkish pet market, in which approximately two million registered animals could generate around TRY 430 million in premium volume at a moderate adoption rate of 5%. Although this scenario indicates an emerging market, the relatively limited insurance coverage implies that the majority of veterinary expenses are still paid out-of-pocket. Consequently, veterinarians continue to face situations where pet owners may delay or avoid necessary treatments due to cost constraints, leading to suboptimal health outcomes.

Scenario 2 (60% microchipping penetration) represents a more proactive regulatory environment, with approximately 3.6 million registered pets. Under this assumption, the total premium volume could exceed TRY 770 million, providing a significantly larger financial buffer for both pet owners and veterinary practices. In this context, pet insurance can improve treatment accessibility, allowing veterinarians to offer more comprehensive diagnostic and therapeutic procedures. The stabilization of payment streams may also enable clinics to invest in advanced medical equipment and staff training, indirectly enhancing the quality of care.

Scenario 3 (80% microchipping penetration) reflects a mature and well-regulated market. The potential premium volume surpassing TRY 1 billion implies widespread adoption of financial protection for pet health. This would fundamentally shift the dynamics of small animal practice in Türkiye. Routine and preventive care such as vaccinations, dental treatments, and chronic disease management would likely become more standardized, as insurance coverage reduces economic hesitation among pet owners. For veterinarians, this evolution could result in more predictable caseloads, better client compliance, and reduced ethical stress associated with cost-driven treatment refusals.

Across all scenarios, the introduction and expansion of pet insurance can generate a positive feedback loop between financial protection, early intervention, and overall animal welfare. As the insured population grows, veterinary clinics could experience improved liquidity and planning capacity, which in turn supports sustained investments in public health and zoonotic disease prevention.

This study demonstrates that the expansion of pet insurance in Türkiye could have substantial financial and societal

benefits, extending beyond the insurance sector itself. The scenario analyses indicate that even moderate increases in market penetration driven by broader microchipping compliance could generate hundreds of millions of Turkish Lira in premium volume, creating a more resilient and accessible ecosystem for veterinary healthcare. From a veterinary perspective, the availability of pet insurance directly translates into improved treatment accessibility, earlier disease detection, and enhanced continuity of care. The mitigation of financial constraints reduces the frequency with which veterinarians encounter ethical dilemmas, lowers the necessity to modify treatment protocols due to cost, and even decreases the incidence of cost-driven euthanasia decisions <sup>[27]</sup>.

International experience from developed pet insurance markets demonstrates that insurance penetration is often supported by complementary public policies and institutional arrangements <sup>[28]</sup>. However, the transferability of these models to emerging markets requires careful adaptation to local regulatory frameworks. In developed markets like Sweden and the United Kingdom, strong animal registration systems and data integration have enhanced market transparency, facilitating more accurate risk pooling for pet insurance schemes <sup>[28,29]</sup>. Academic and policy discourses increasingly advocate for the establishment of subsidized veterinary care programs or safety nets to assist low-income households with preventive and catastrophic expenses <sup>[30]</sup>. Furthermore, the integration of pet insurance into employee benefit packages has emerged as a significant mechanism to encourage voluntary participation and expand coverage, particularly in the North American market <sup>[8]</sup>. Collaborative models involving direct billing systems and streamlined claims processing have significantly reduced financial barriers for owners and administrative burdens for clinics, thereby improving overall service accessibility <sup>[31]</sup>. Consequently, these mechanisms not only ensure financial sustainability but also foster a culture of responsible pet ownership.

Several limitations of the present study should be acknowledged. First, the scenario-based simulation relies on assumed loss ratios and operating expense levels derived from international literature, which may not fully capture the heterogeneity of the Turkish insurance and veterinary markets. Second, behavioral responses of pet owners and veterinarians to insurance adoption were not directly modeled and may influence real-world outcomes. Third, the study does not explicitly account for the impact of high inflation on claim severity, which represents a significant external risk factor for pricing stability in the current economic climate.

Despite these limitations, the findings offer meaningful guidance for policy and practice. In the Turkish context, targeted education and awareness campaigns aimed at

pet owners and veterinary professionals could improve insurance literacy and trust. Specifically, veterinary faculties should consider integrating insurance literacy modules into their curricula to prepare future practitioners for client financial counseling. Policy measures such as incentives for microchipping, pilot state-supported preventive care funds, and frameworks for structured collaboration through shared digital health data standards between insurers and veterinary clinics may further accelerate market development. Collectively, these strategies could enhance financial predictability for veterinary practices, improve access to care, and strengthen animal welfare outcomes.

At the policy level, integrating pet insurance within a broader framework of animal registration and welfare legislation could yield significant public health gains. Encouraging microchipping and promoting insurance literacy among pet owners should therefore be considered complementary strategies. For veterinary practitioners, collaborating with insurers to design transparent, needs-based coverage options could reduce the ethical and financial dilemmas frequently encountered in small animal practice. Ultimately, a more structured and financially inclusive pet health system would strengthen not only the economic sustainability of veterinary services but also the overall wellbeing of companion animals in Türkiye.

## DECLARATIONS

**Availability of Data and Materials:** All data supporting the findings of this study are included within the manuscript. Additional materials or simulation codes used in the analysis are available from the corresponding author upon reasonable request.

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Kafkas Universitesi Veteriner Fakultesi Dergisi do not accept advertising and sponsorships that are believed to create a potential conflict of interest. If the article sent to Kafkas Universitesi Veteriner Fakultesi Dergisi is for the promotion of a commercial product and/or the work carried out is directly supported by a company, it is rejected without consideration.

**OPEN ACCESS STATEMENT**

Kafkas Universitesi Veteriner Fakultesi Dergisi is an open access publication. The journal's publication model is based on Budapest Open Access Initiative (BOAI) declaration. Articles published in Kafkas Universitesi Veteriner Fakultesi Dergisi are available online, free of charge at <https://vetdergikafkas.org/archive.php>.

Except for commercial purposes, users are allowed to read, download, copy, print, search, or link to the full texts of the articles in this journal without asking prior permission from the publisher or the author. The open access articles in the journal are licensed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International (CC BY-NC 4.0) licence.

**ARTICLE EVALUATION AND PUBLICATION PROCESS****• Initial Evaluation Process**

Articles submitted to Kafkas Universitesi Veteriner Fakultesi Dergisi are primarily evaluated by the editors and associate editors. At this stage, articles not having suitable scope and aims, with low original research value, containing scientific and ethically important errors, having low potential to contribute to science and the journal, and having poor language and narration are rejected by the editor without peer-review process. Initial evaluation process takes up to most 2 weeks.

**• Preliminary Evaluation Process**

Articles that are deemed appropriate for editorial evaluation are sent to the subject editor related to the category of articles to be examined in terms of scientific competence and to the statistics editor for evaluation in terms of statistical methods. The subject editors examine the article in all aspects and report their decisions (rejection, revision or peer-review) to the chief editor. This stage takes about 1 month.

**• Peer-review Process**

Double-blind peer-review is applied to the articles that have completed preliminary evaluation process. Suggestions of subject editors are primarily considered in referee assignment. In addition, reviews can be requested from the referees registered in the journal's referee pool. At least 2 referees are assigned for peer-review. Opinion of more referees can be required depending on the evaluation process. At this stage, referees send their decision (reject, revision or accept) about the article to the editor-in-chief. If the rejection decision given by a referee reflects sufficient examination and evidence-based negativities or ethical problems about the scientific content and accuracy of the article, this decision is checked by the editor-in-chief and associate editors and submitted to the authors regardless of the other referees' decisions. The time given to referees to evaluate an article is ~4 weeks.

**• Publication Process of an Article**

Total evaluation period of an article, which is completed in the peer-review phase after completing the initial and preliminary evaluation process, takes 4-6 months. The articles that have completed the subject editorial and peer-review evaluation stages and accepted by the editorial are sent to the corresponding author for final checks and necessary final additions. After the acceptance, the article designed in the publication format of the journal is given an DOI number and published immediately on the Article in Press page. When it is time to publish the periodic edition of the journal, a selection is made from the articles kept on the Article in Press page, taking into account the submission date. The time it takes for the article to be published by taking the page number is 6-12 months.

**NO PUBLICATION FEE**

Processing and publication are free of charge with the journal. There is no article processing charges, submission fees or any other fees for any submitted or accepted articles.

**RESPONSIBILITIES OF THE PUBLISHER, EDITORS AND ASSOCIATE EDITORS**

The publisher (Dean of the Faculty of Veterinary Medicine of Kafkas University) contributes to the execution of the journal's routine processes such as printing, archiving, and mailing, in line with requests from the editor.

The publisher undertakes to carry out an independent and fair decision-making mechanism for its editors and assistants in the article evaluation process and decisions.

The publisher undertakes to carry out an independent and fair decision-making mechanism for its editors and associate editors in the article evaluation process and decisions.

Editor-in-chief/editors/associate editors of Kafkas Universitesi Veteriner Fakultesi Dergisi evaluate the articles submitted to the journal regardless of their race, gender, religious belief, ethnicity, citizenship or political views. In addition, it undertakes not to give any information about the article except for the authors, subject editors and referees.

Kafkas Universitesi Veteriner Fakultesi Dergisi follows internationally accepted principles and criteria and takes the necessary decisions to apply in the journal.

Editor-in-chief/editors/associate editors conduct the evaluation and decision process in the journal in coordination within the principles of confidentiality and have independent decision-making authority and responsibility without being affected by any internal or external factors.

Editor-in-chief/editors/associate editors make and implement all kinds of planning for the development of the journal and its international recognition. They also follow national and international meetings or events on the development of journals and article evaluation, and ensures that the journal is represented on these platforms.

The editor-in-chief/editors/associate editors make every effort to ensure that the journal's subject editors and referee pool have international qualifications. Likewise, it makes the necessary attempts to strengthen the author's profile.

Editor-in-chief/editors/associate editors make plans to improve the quality of the articles published in the journal and carry out the necessary process.

Editor-in-chief/editors/associate editors regularly conduct and control the initial evaluation, preliminary evaluation, peer review and acceptance-rejection decisions of articles submitted to the journal. While carrying out these procedures, features such as the suitability of the study for the aims and scope of the journal, its originality, the up-to-date and reliability of the scientific methods used, and the potential it will contribute to the development of the journal as well as its benefit to science/practice are taken into consideration.

Editor-in-chief/editors/associate editors systematically review, inspect and make decisions about the articles submitted to the journal in terms of features such as author rights, conflict of interest, observance and protection of animal rights, and compliance with research and publication ethics.

The editor-in-chief conducts the evaluation/revision process between the authors and subject editors and referees, and ensures that it is completed within the prescribed time.

**ARCHIVE POLICY**

The editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi and the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) keep all the articles (electronic and printed) published in the journal in their archives. All articles and their attachment files sent to the journal are kept securely in the archive. In light of the technological developments, the editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi regularly performs electronic processes for the development and updating of materials in digital environment and presents them to its readers on condition of keeping in safe the original documents and information regarding the articles.

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 Üniversitesi Veteriner Fakültesi 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 Üniversitesi Veteriner Fakültesi 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 Üniversitesi Veteriner Fakültesi 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 Üniversitesi Veteriner Fakültesi Dergisi. It is more appropriate to submit them with acceptable time intervals for the journal's policy.

## 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: McIlwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4<sup>th</sup> 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 checked 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

