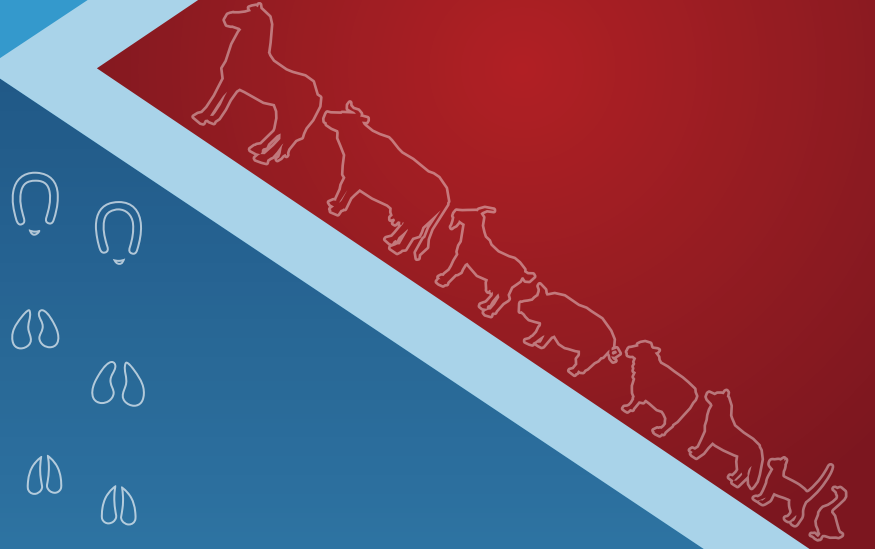


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REVIEW ARTICLE

Host Genetic Determinants of Resistance to Bovine Babesiosis and Its Tick Vector: Implications for Sustainable Disease Control

Mehroz LATIF ^{1†} , Abdullah AZEEM ^{1†} , Muhammad Kasib KHAN ^{1(*)} , Muhammad Shahid MAHMOOD ² , Sultan ALI ² , Muhammad Umer ASHRAF ² , Sundas AFRESHAM ¹ , Muhammad Adnan Sabir MUGHAL ³ , Zaheer ABBAS ¹ , Muhammad Asif RAZZAQ ¹ 

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Abstract

Bovine babesiosis, caused primarily by the tick-borne apicomplexans *Babesia bovis* and *Babesia bigemina*, remains one of the most economically important haemoparasitic diseases affecting cattle in tropical and subtropical regions. Conventional control strategies, including acaricides, chemotherapy, and live blood-derived vaccines, are limited by resistance, chemical residues, cold-chain requirements, and incomplete strain protection, prompting interest in host genetic resistance as a sustainable complement. Indicine (*Bos indicus*) cattle, and some taurine breeds, exhibit heritable variation in resistance to both *Babesia* infection and its *Rhipicephalus* tick vector. This review integrates current knowledge of the immunological basis of resistance with advances in quantitative and functional genomics and their applications in breeding. We examine disease pathobiology, innate and adaptive immune mechanisms, breed-associated and within-breed variation, and the genetic architecture of resistance revealed through heritability estimates, bovine leukocyte antigen and immune-related genes, quantitative trait loci, genome-wide association studies, and selection signatures. Throughout, we distinguish direct evidence (*Babesia* infection measured) from indirect evidence (tick burden, general immune response, or tropical adaptation). We conclude that resistance is polygenic and environmentally influenced. Direct genomic evidence for *Babesia* resistance remains limited; most mapped loci derive from tick-count phenotypes.

Keywords: Bovine babesiosis, *Babesia bovis*, Genomic selection, Host genetic resistance, *Rhipicephalus microplus*, Sustainable disease control

INTRODUCTION

Bovine babesiosis is a tick-borne haemoprotozoan disease of cattle caused principally by the intraerythrocytic apicomplexan parasites *Babesia bovis* and *Babesia bigemina*, with *Babesia divergens* important in parts of Europe ^[1]. Transmission depends on ixodid ticks, mainly *Rhipicephalus* (*Boophilus*) species, which acquire the parasite during feeding and pass it transovarially to the next generation ^[2]. Several agents of the disease, and *B. divergens* in particular, also infect humans, which gives the problem a zoonotic dimension ^[3].

The scale of the burden is large. Roughly 80% of the world's cattle are raised in regions at risk of tick-borne disease ^[4], and a systematic meta-analysis placed the pooled global

prevalence of bovine babesiosis at 29%, highest in South America and lowest in Asia, with wide regional variation ^[5]. In endemic countries the disease causes mortality, abortion, lost milk and weight gain, and the recurring cost of treatment and tick control, with smallholders absorbing a disproportionate share of these losses ^[6]. Regional surveys, including work across Punjab in Pakistan and molecular characterization of piroplasms in cattle from Azad Kashmir, continue to record high *Babesia*/piroplasm prevalence in cattle and to expose gaps in diagnosis and control in South Asian systems ^[7-9].

Current control rests on three pillars, each now under strain: acaricides, chemotherapy, and live blood-derived vaccines. Host genetic resistance has therefore emerged as a promising complement to these conventional control measures.



The central argument of this review is that heritable host resistance to *Babesia* and to its tick vector is real, measurable, and potentially exploitable, and that it should be treated as a complementary pillar of control rather than a curiosity. Here, tick resistance means a lower tick burden, measured as tick count, and *Babesia* resistance means a lower *Babesia* infection level, usually measured by quantitative polymerase chain reaction (qPCR); these two terms are used throughout. We distinguish resistance, which reduces tick or pathogen burden, from tolerance, which reduces disease impact at a given burden; studies scored on clinical outcome may capture both. Indicine (*Bos indicus*) cattle are markedly more resistant than taurine (*Bos taurus*) animals under controlled challenge ^[10], and variation persists within breeds, which is what makes selection feasible. What this review adds beyond existing accounts is an explicit integration of three literatures usually read separately: the immunology that defines the biological substrate of resistance, the quantitative and functional genomics that map and explain it, and the breeding and One Health strategies that translate it into the field.

We therefore summarize the disease and the limits of current control; describe the immune pathways on which resistance loci act; review the empirical evidence for breed-associated and within-breed resistance; synthesize the genetic architecture from heritability through genome-wide association and selection signatures; examine functional-genomic mechanisms and parasite and vector genetic variation; and assess how this knowledge can be deployed sustainably, before setting out the main knowledge gaps.

LITERATURE SEARCH STRATEGY

This article is a narrative review, and the literature was assembled through a structured but non-exhaustive search. PubMed, Web of Science, Scopus and Google Scholar were searched from database inception until the final search on 5 June 2026. Search strings combined terms for the disease and its agents (“bovine babesiosis”, “*Babesia bovis*”, “*Babesia bigemina*”, “tick-borne disease”, “*Rhipicephalus microplus*”) with terms for host genetics and control (“host genetic resistance”, “breed resistance”, “*Bos indicus*”, “heritability”, “quantitative trait loci”, “genome-wide association”, “selection signature”, “genomic selection”, “BoLA” (bovine leukocyte antigen), “transcriptome”, “vaccine”), using Boolean operators and truncation where the database permitted. The reference lists of retrieved reviews and primary papers were screened by hand, and a small number of further records were identified in this way. In total, approximately 1500 records were retrieved and 600 were screened by title and abstract.

Titles and abstracts were screened against three questions: whether host genotype influences *Babesia* infection or

tick burden in cattle, which genomic regions or immune pathways underlie that variation, and how the resulting knowledge can be applied in breeding and integrated control. Full texts were retained when they reported original phenotypic, genetic, genomic or functional data in cattle, or when they offered an authoritative synthesis of such data. Records were excluded when they concerned babesiosis in non-bovine hosts without comparative relevance, when they reported diagnosis or prevalence with no bearing on host variation or control, or when they duplicated data reported more fully elsewhere. Work published in the last ten years was prioritized, although older studies were kept where they remain the primary source for a finding, as with the controlled breed-challenge experiments of the 1990s and the earliest quantitative trait locus scans. Because the body of direct *Babesia*-resistance genomics is small, every study of that type that was identified has been retained, whereas the much larger tick-resistance literature was sampled for representative and methodologically strong reports. Sixty-six sources are cited in the final review.

BOVINE BABESIOSIS: ETIOLOGY, BURDEN, AND THE LIMITS OF CURRENT CONTROL

Causative Agents, Vectors, and Transmission

B. bovis and *B. bigemina* differ in biology and virulence. *B. bovis* is the more pathogenic, producing infected erythrocytes that sequester in the microvasculature and cause cerebral and respiratory disease, whereas *B. bigemina* typically causes a more acute but less neurovirulent haemolytic syndrome ^[1]. Both are transmitted by *Rhipicephalus* ticks with transovarial passage, a feature that sustains parasite populations independently of continuous cattle-to-cattle spread ^[2]. Vector competence is specific: the invasive Asian longhorned tick *Haemaphysalis longicornis*, despite acquiring *B. bovis* deoxyribonucleic acid (DNA), failed to transmit the parasite transstadially or transovarially, which constrains predictions of geographic spread ^[11]. The diversity of co-circulating haemoparasites is often underestimated, and molecular surveys in endemic regions frequently detect mixed piroplasm and *Anaplasma* communities that complicate diagnosis and control ^[12,13]. Recent South Asian work identifying cattle piroplasms in Azad Kashmir and cytochrome-b based *Theileria annulata* lineages in Pakistani bovines reinforces that *Babesia* control often occurs within a broader tick-borne-pathogen landscape ^[9,14].

Epidemiology, Economic Impact, and Endemic Stability

Whether babesiosis causes heavy losses in a given herd depends less on the presence of the parasite than on

the balance between infection pressure and the age structure of susceptibility, summarized in the concept of endemic stability. Where transmission is high and calves are infected while still protected by innate age-related resistance, clinical disease is rare; where transmission is unstable, naive adults are exposed and outbreaks follow [2,6]. This conditions every control decision, since measures that reduce tick contact can destabilize a previously stable system. The burden itself is unevenly distributed: pooled prevalence varies widely by region and host species, exceeding one-tenth of animals in parts of East Asia and reaching high levels in water buffaloes, which act as additional reservoirs [15,16], while Indonesian work on *Babesia naoakii* shows that ruminant babesiosis outside the classical *B. bovis* and *B. bigemina* framework can be clinically and epidemiologically important in Asian production systems [17,18]. Climate change adds further uncertainty, with distribution models projecting expansion of *R. microplus* into higher latitudes and altitudes under warming scenarios [19].

Current Control Tools and Their Limitations

The mechanisms, strengths, limitations, and sustainability profiles of the main control tools are compared in [Table 1](#). Two points deserve emphasis here. First, acaricide resistance in *R. microplus* is now widespread and multi-class, arising through target-site mutations and metabolic

detoxification, and molecular markers that reliably predict field resistance remain elusive, since several historically reported resistance mutations occur in both resistant and susceptible isolates [20,21]. Second, vaccine development has been difficult. Live attenuated blood vaccines protect but carry contamination and cold-chain risks [22], while rationally designed subunit and viral-vectored candidates have induced strong T helper 1 responses yet failed to elicit the neutralizing antibodies needed for protection on challenge [23]. Anti-tick vaccines that target parasite-independent tick antigens offer a complementary route but have shown variable, often incomplete, efficacy [24]. Plant-derived acaricides remain a secondary but active line of research, with recent studies/reviews testing essential oils and *Moringa oleifera* extracts against livestock ticks, including *R. microplus* [25,26]. Taken together, these limits, rather than any single failure, make a complementary host-encoded contribution attractive ([Fig. 1](#)).

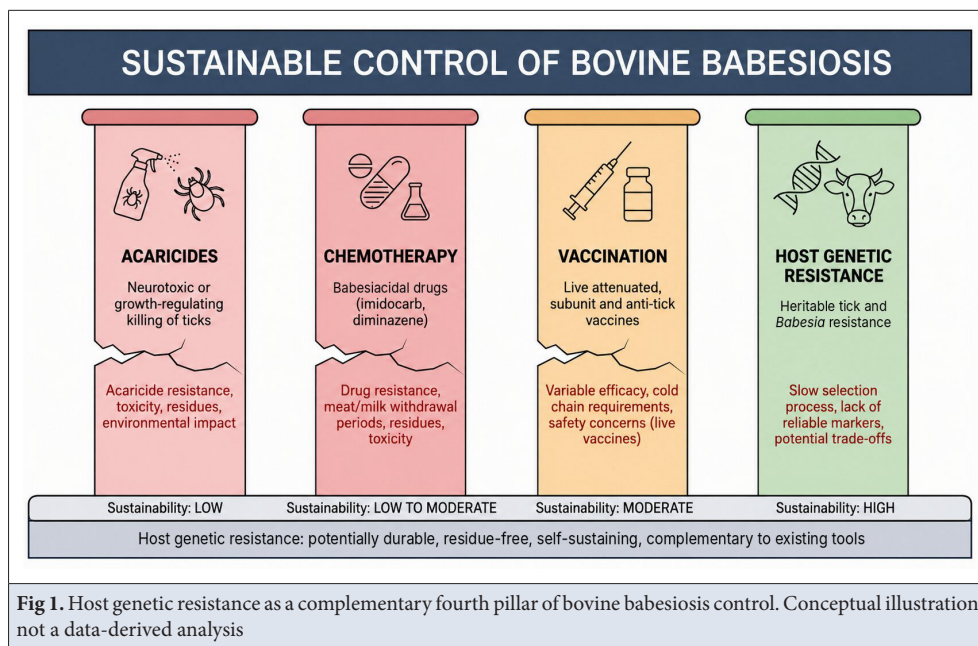
Zoonotic Dimensions and the One Health frame

Beyond the direct losses noted above, economic weight accumulates through the chronic carrier state, which sustains transmission while depressing performance below the threshold at which owners typically seek treatment [2]. The disease also constrains the use of high-producing taurine genetics in the tropics, because the productivity gain of taurine breeds is offset by their

Table 1. Principal tools for controlling bovine babesiosis and its tick vector, compared by mechanism, strengths, limitations, and sustainability

Control Approach	Mechanism/Basis	Key Strengths	Principal Limitations	Sustainability	References
Chemical acaricides	Neurotoxic or growth-regulating killing of ticks	Rapid knockdown; cheap; widely available	Multi-class resistance; milk and meat residues; environmental cost; can destabilize endemic stability	Low	[6,20,21]
Chemotherapy (imidocarb, diminazene)	Babesiocidal drugs	Effective acute treatment; can clear carrier state	Narrow arsenal; residue and withdrawal limits; emerging insensitivity; no new classes	Low to moderate	[27]
Live attenuated/blood-derived vaccines	Pre-immunization with attenuated parasites	Strong, durable protection; field-proven	Cold-chain dependence; contamination and reversion risk; batch variability	Moderate	[22,27]
Subunit/viral-vectored vaccines	Recombinant parasite antigens (e.g., MSA-2c, RAP-1)	Defined; safe; scalable	Incomplete strain coverage; weak neutralizing antibody; no licensed product	Moderate (developmental)	[23,28]
Anti-tick vaccines	Immunization against tick antigens (Bm86, serpins, FER2/TPM)	Reduce tick fitness; benign; slow acaricide resistance	Variable, often incomplete efficacy; antigen and strain dependence; multi-dose	Moderate to high	[24,29,30]
Botanical/biological control	Plant extracts; entomopathogens	Low residue; locally sourced	Early stage; variable potency; limited field validation	Moderate	[25,26,31]
Host genetic resistance (this review)	Heritable tick resistance and <i>Babesia</i> resistance	Potentially durable; self-sustaining; no residues; additive to other tools	Slow to deploy; phenotyping-limited; possible productivity trade-offs	High	[32,33]

Sustainability ratings are qualitative author judgments based on the cited evidence, considering durability of effect, risk of resistance, residues, environmental impact and need for repeated inputs or a cold chain. MSA-2c, merozoite surface antigen 2c; RAP-1, rhoptry-associated protein 1; FER2, ferritin 2; TPM, tropomyosin



susceptibility to tick fever, which is the central tension that genetic improvement of resistance sets out to resolve [34]. A zoonotic dimension compounds the animal-health burden, since several *Babesia* species infect humans, splenectomized and immunocompromised people are at particular risk of severe disease, and the absence of a licensed human vaccine leaves control dependent on managing infection in animal reservoirs and limiting tick contact [3]. These features place bovine babesiosis within a One Health frame, in which animal genetics, human health, and tick ecology cannot be addressed in isolation.

PATHOBIOLOGY AND THE HOST IMMUNE RESPONSE AS THE SUBSTRATE FOR GENETIC VARIATION

Resistance loci do not act in a vacuum; they exert their effects through defined immune and physiological pathways, so the biology on which they operate is the necessary starting point.

Infection Biology and Pathogenesis

After tick inoculation, *Babesia* merozoites invade erythrocytes, replicate, and lyse the host cell, producing the haemolytic anaemia, haemoglobinuria, and fever that define acute disease [1]. The distinctive virulence of *B. bovis* follows from cytoadhesion: parasite proteins exported to the erythrocyte surface mediate binding to vascular endothelium, sequestering infected cells in the brain and other organs and producing the neurological and respiratory signs of cerebral babesiosis [35]. The variant

erythrocyte surface antigen 1 family, encoded by the *ves* multigene family, drives both this sequestration and antigenic variation, and recent transcriptomic work identifies *ves1α* expression as the principal determinant of cytoadhesion strength [35]. The accompanying inflammatory response is double-edged, controlling parasite replication while also contributing to the pathology of severe disease [36].

Innate Immunity

Control of acute infection is heavily spleen-dependent, with splenic macrophages activated to produce nitric oxide, reactive oxygen species, and inflammatory cytokines that limit parasitaemia [1]. The well-documented age-related resistance of calves, which underlies endemic stability, is largely innate and spleen-associated rather than antibody-mediated [2]. Differences between resistant and susceptible animals are detectable at these innate nodes: in cattle of differing indicine ancestry, animals with a *Bos indicus* maternal lineage mounted an earlier and more connected innate response to tick infestation, with greater monocyte and natural killer (NK) cell involvement, than animals of taurine lineage [37].

Adaptive Immunity

Protective adaptive immunity is dominated by CD4+ T helper 1 responses, in which interferon gamma (IFN-γ) primes macrophages, supported by antibodies that opsonize parasites and parasite antigens displayed on the erythrocyte surface [27]. Persistently infected animals develop concomitant immunity that limits disease on re-exposure, and antigen presentation by major histocompatibility complex (MHC) molecules restricts which T-cell epitopes are recognized, linking the adaptive

response directly to host genotype^[38]. The failure of a multi-antigen subunit vaccine to protect despite strong T helper 1 induction indicates that cellular responses alone are insufficient and that antibody quality matters^[23].

The relative contributions of the innate and adaptive arms are not fixed across the course of infection. Survival of the acute phase depends on rapid innate control before adaptive immunity matures, which is why the spleen and macrophage activation are decisive in naive animals, whereas durable protection in recovered animals rests on memory T cells and antibody^[1,27]. This temporal structure has a practical corollary that recurs later in this review: interventions that accelerate or broaden innate responsiveness, such as trained-immunity strategies, act on the same early window in which genetically resistant animals already hold an advantage^[39]. It also explains why animals of different genetic background diverge most clearly during early infection, when innate differences are expressed before the adaptive response converges^[37]. For genetic dissection the implication is concrete: a resistance phenotype measured at a single time point can misrepresent an animal's true standing, so the timing of sampling matters as much as the trait that is scored.

From Immune Phenotype to Genetic Variation

The nodes above, that is, MHC-restricted antigen presentation, cytokine signaling, and macrophage effector function, vary between animals and between breeds, and that variation is heritable. Expression of chemokine and Toll-like receptor genes during natural infection differs with genetic group and tracks with *Babesia* infection level^[36]. This provides the mechanistic rationale for the genetic dissection that follows, on the working assumption that at least some of the loci mapped in later sections correspond to heritable variation in these immune

pathways. The assumption is reasonable but largely untested, since few mapped loci have yet been connected experimentally to a specific immune function. The major pathobiological and immunogenetic control points linking infection biology to host resistance are shown in Fig. 2.

THE PHENOMENON OF BREED-ASSOCIATED RESISTANCE

The clearest evidence that host genetics shapes outcome comes from breed comparisons.

Bos indicus versus *Bos taurus*

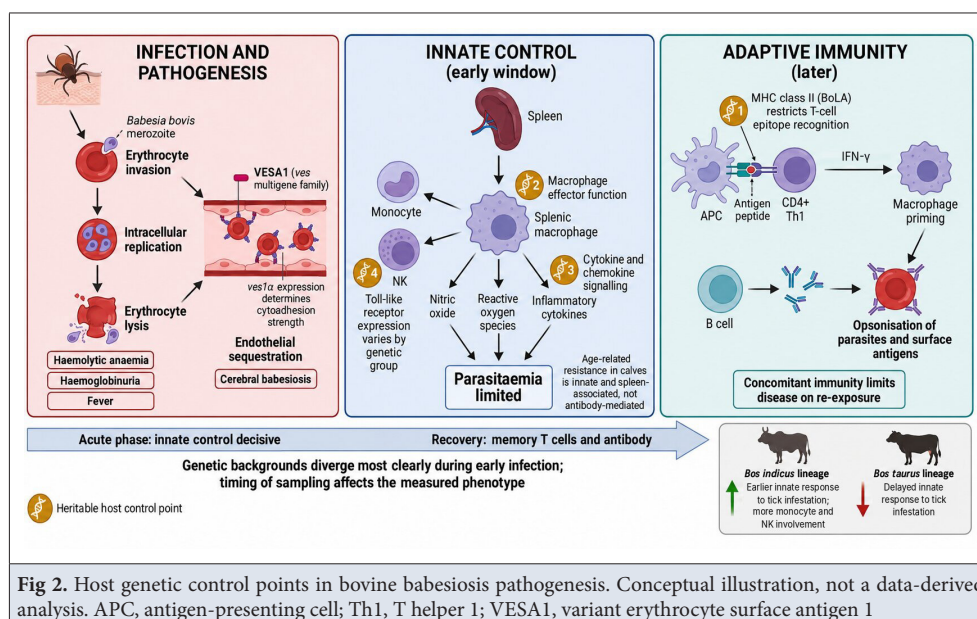
Under controlled challenge with virulent *B. bovis*, pure *Bos indicus* cattle are markedly more resistant than *Bos taurus* cattle, with crossbreds intermediate^[10]. Natural-infection studies in dairy and beef systems also show wide variation in *Babesia* infection levels among taurine and high-taurine animals^[36,40].

The Crossbreeding Gradient: How Much Zebu is Enough?

The protection conferred by indicine ancestry is dose-dependent, and recent evidence sharpens an important caveat: a small proportion of zebu background in crossbred calves was not sufficient to measurably improve resistance to natural *Babesia* infection, with high-taurine composites carrying higher parasite DNA loads than purebred taurine animals^[34]. This argues against assuming that any indicine admixture yields useful protection and has direct consequences for crossbreeding design.

Within-breed Phenotypic Variation

Resistance is not purely a between-breed phenomenon. Resistant, intermediate, and susceptible phenotypes



segregate within taurine and within zebu populations, and individual animals differ consistently over time in both tick burden and *Babesia* infection level, which is precisely the variation that selection can exploit ^[41,42]. In a tropically adapted taurine breed, resistance also tracked with coat and hair characteristics and varied with age, indicating that several heritable traits contribute to the resistant phenotype ^[42].

Disentangling Tick Resistance from *Babesia* Resistance

A point that is often blurred deserves emphasis: tick resistance and *Babesia* resistance are correlated but genetically distinct traits. Repeatability analyses show that an animal's tick count and its *Babesia* infection level are only weakly to moderately correlated, so an animal resistant to ticks is not automatically resistant to the parasite ^[41]. This distinction is consequential for the genetics that follows, because most mapping studies have measured tick count as a proxy and comparatively few have phenotyped *Babesia* infection directly.

This distinction also determines how the evidence in the sections that follow should be weighted, and it is worth setting out the grading applied from this point onward. Direct evidence comes from studies in which *Babesia* infection or clinical babesiosis was itself phenotyped and related to host breed or genotype. This category is small, and most of what exists has already been introduced: controlled challenge of indicine and taurine cattle with virulent *B. bovis* ^[10], comparison of natural infection across crossbred and purebred animals ^[34], differences in immune-gene expression and *Babesia* infection level between genetic groups under natural challenge ^[36], and repeatability estimates for infection level in dairy and beef herds ^[40,41]. The sections that follow add only a small number of quantitative-genetic and genomic studies that phenotyped *Babesia* infection directly.

Indirect evidence through the vector comes from the much larger body of work in which tick count or tick resistance was the measured phenotype ^[41,42], and it bears on *Babesia* only insofar as reducing tick burden reduces exposure, which the weak correlation between tick count and *Babesia* infection level shows to be a partial relationship ^[41]. Almost all of the mapping, association and prediction studies reviewed in the next section fall into this category.

Functional support comes from transcriptomic and immunological studies that separate resistant from susceptible animals but did not phenotype *Babesia* infection ^[4,37]. General adaptation evidence comes from selection scans in tropically adapted breeds, reviewed below, in which immune-related sweeps are recovered alongside heat tolerance and other adaptive traits and

cannot be assigned to babesiosis specifically. Where the sections that follow rest on the second, third or fourth of these categories, this is stated rather than left implied.

HOST GENETIC ARCHITECTURE OF SUSCEPTIBILITY AND RESISTANCE

The heart of the question is which genes and loci underlie resistance, how much of the variation they explain, and how repeatable the signals are across populations.

Heritability and Genetic Parameters

Resistance traits are heritable but only modestly so, which sets realistic expectations for selection. The pattern is informative rather than incidental. Heritability for tick count is low to moderate and highly variable across breeds and seasons, whereas heritability for *Babesia* infection level is consistently lower still, and the genetic correlation between the two traits is weak ^[33,43]. Two consequences follow. Direct selection on *Babesia* resistance will be slower than on tick resistance, and gains in one cannot be assumed to carry over to the other. Encouragingly, genetic correlations between resistance and production traits are generally weak rather than strongly antagonistic, which keeps combined improvement feasible ^[44].

Candidate Genes and Immunogenetics

The bovine MHC, the BoLA region, recurs as a candidate across mapping studies, which is consistent with its role in antigen presentation and T-cell epitope restriction. This recurrence comes largely from tick-resistance mapping, however, and the MHC is a gene-dense, highly polymorphic region that is recovered frequently in genome scans for many traits, so its repeated appearance is suggestive rather than confirmatory. Specific *BoLA-DRB3* alleles associate with susceptibility or resistance to other bovine pathogens, such as bovine leukemia virus ^[45], and, more directly for this review, particular *BoLA-DRB3* alleles were associated with reduced risk of *B. bigemina* infection while others were linked to increased susceptibility to *B. bovis* in Colombian Creole cattle ^[46]. Beyond the MHC, candidate windows from genome-wide studies repeatedly implicate immune and inflammatory genes, including cytokine and effector loci, which is broadly consistent with the immune pathways described earlier. The mapped windows are wide and gene-rich, so the genes named within them remain candidates awaiting functional confirmation rather than established effectors ^[33,47]. The Colombian Creole study is one of very few published associations between a defined host locus and *Babesia* infection status in cattle. It was observational and based on small samples from three breeds, so the alleles it identifies are best treated as leads for replication rather than as validated markers. The wider *BoLA-DRB3* literature concerns other pathogens

and supports the general expectation that the region is relevant to infectious-disease outcome, not the specific claim that particular alleles govern babesiosis ^[45].

Quantitative Trait Loci Mapping

Linkage mapping in indicine by taurine crosses established that resistance is polygenic. A genome-wide scan in a *Bos taurus* by *Bos indicus* population identified several quantitative trait loci (QTL) for tick resistance, including a strong signal on the chromosome that carries the MHC, with other loci detected only in one season ^[48]. That seasonal pattern is not noise: reaction-norm analyses formally demonstrate single nucleotide polymorphism (SNP) by environment interaction, such that the genetic control of tick resistance depends on the level of tick challenge, with different loci relevant at different infestation pressures ^[47]. This environment dependence helps explain why signals are not always reproducible across studies conducted under different exposure.

Genome-wide Association Studies and Genomic Regions

Higher-resolution association studies have refined these signals. For tick resistance, genome-wide association studies (GWAS) in indigenous and composite breeds have identified significant regions and candidate genes and have supported low-density tag-SNP panels for prediction ^[49,50]. For *Babesia* infection level specifically, a dedicated genomic study in Hereford and Braford cattle identified top SNPs distributed across several chromosomes that overlapped immune-function candidate genes, and reported that infection level and tick count, though related, mapped partly to different regions, which reinforces the genetic distinction between *Babesia* resistance and tick resistance ^[33].

Selection Signatures in Indigenous and Zebu Breeds

Natural selection has left detectable footprints on resistance-related loci in tropically adapted cattle, providing an independent line of evidence. Genome scans in indicine and African breeds repeatedly recover selective sweeps spanning immune-response and heat-tolerance genes, including, in Indian and Chinese zebu, loci with direct roles in tick resistance and immune function, and, in African dryland zebu, gene-rich immune sweeps shared across breeds ^[51-54].

That immunity and adaptation genes are recurrent targets of selection is consistent with the resistance phenotypes of these breeds, but the inference is indirect in a way that matters. These scans were run on breed samples rather than on animals phenotyped for *Babesia* infection, and the swept regions recovered usually span heat tolerance, tick resistance and general immune function together, so they cannot be attributed to babesiosis resistance specifically.

Selection signatures are therefore best read as evidence that tropical adaptation has a genomic basis which includes immunity, and as a source of candidate regions for testing, rather than as evidence of *Babesia* resistance.

Non-additive Variation and the Limits of Additive Models

Most heritability estimates and genomic predictions for resistance assume an additive model, in which marker effects sum independently. This is a useful approximation but an incomplete one. Dominance and epistasis are plausible for immune traits, whose products act within regulatory networks rather than in isolation, and kernel-based machine-learning methods that explicitly compare additive, dominance, and epistatic models can both improve prediction and indicate which inheritance mode best fits a given trait ^[55]. The stakes are practical: if non-additive variance is substantial, additive breeding values will systematically misrank the resistance of specific crosses, and the error will be largest precisely where it matters most, in the composite and crossbred animals that dominate tropical production ^[32]. The recurrent appearance of regulatory rather than protein-coding candidates, including a regulatory locus prioritized from Braford skin, is consistent with an architecture in which network context modifies the effect of individual variants ^[4]. How much of the resistance variance is non-additive is therefore not a statistical nicety but a direct determinant of how accurately a breeding program can rank candidate animals.

Synthesis of the Architecture

The genetic-parameter estimates discussed above are collated in [Table 2](#), and the mapped genomic regions, candidate genes and selection signatures, classified by category of evidence, are summarized in [Table 3](#). Three conclusions follow. First, resistance is polygenic, built from many loci of small effect rather than a few major genes, which is why marker panels rather than single markers are needed for prediction. Second, tick and *Babesia* resistance share part of their architecture but are not identical, and the evidence base is lopsided, because the great majority of mapped loci come from tick-count phenotypes while direct *Babesia*-infection genomics remains thin ^[33,43]. The asymmetry is made explicit in the evidence column of [Table 3](#), where a single entry of nine rests on a phenotype in which *Babesia* infection itself was measured.

Third, the MHC and core immune pathways recur as themes across linkage, association, and selection-signature studies. This recurrence is biologically plausible, but the regions concerned are gene-dense and highly polymorphic and are frequently recovered in scans for unrelated traits, and the studies differ in breed, phenotype and design. The pattern is best treated as a convergence

Table 2. Representative heritability and genetic-parameter estimates for tick resistance and *Babesia*/hemoparasite infection in cattle

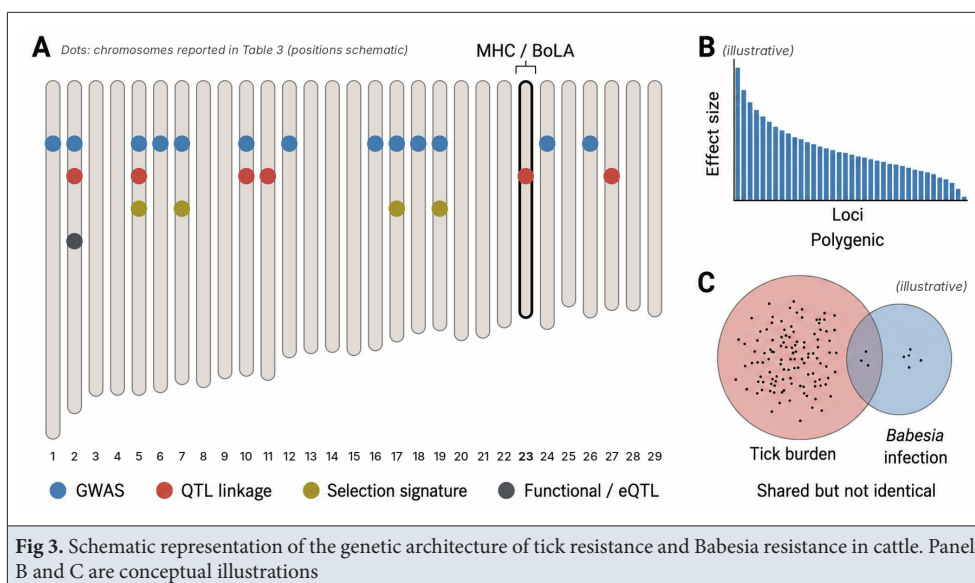
Trait	Breed/Population	Estimate	Method	Reference
Tick count (<i>R. microplus</i>)	Nellore (<i>B. indicus</i>), Brazil	$h^2 = 0.06-0.30$ (age-specific)	Bayesian random regression	[44]
Tick count (multiple ixodid species)	Nguni (indigenous), South Africa	$h^2 = 0.02-0.17$	GWAS; animal and sire models	[49]
Tick count (monthly, by location)	Nguni (indigenous), South Africa	$h^2 = 0.00-0.89$ (month/site-specific)	Bivariate sire models	[56]
Tick count (<i>R. microplus</i>)	Angus (<i>B. taurus</i>), Brazil	$h^2 = 0.16$; repeatability 0.21	Bayesian multi-trait	[43]
<i>B. bovis</i> infection level	Angus (<i>B. taurus</i>), Brazil	$h^2 = 0.03$; repeatability 0.07	Bayesian multi-trait	[43]
<i>B. bovis</i> infection level	Hereford and Braford, Brazil	$h^2 = 0.10$; genetic corr. with tick count 0.15	Single-step GBLUP, bivariate	[33]
Tick resistance (multi-breed)	7 breeds (Brazil, Australia, South Africa)	$h^2 = 0.05-0.42$; genetic corr. -0.01 to 0.87	Multi-breed genomic prediction	[32]
Tick count and <i>Babesia</i> infection level (repeatability)	Holstein and Canchim, Brazil	Low to moderate; weak tick- <i>Babesia</i> correlation	qPCR; mixed models	[40,41]

Ranges are not single population estimates; they span estimates that vary with age, month, anatomical location, tick species, breed or environment within the cited study. h^2 , heritability; GBLUP, genomic best linear unbiased prediction; qPCR, quantitative polymerase chain reaction

Table 3. Mapped genomic regions, candidate genes, and selection signatures for tick resistance and *Babesia* resistance in cattle, classified by category of evidence. Evidence categories: Direct, *Babesia* infection measured; Indirect, tick count measured; Functional support, expression data only; General adaptation, selection scans not specific to babesiosis

Population/Breed	Trait Phenotyped	Evidence Category	Chromosome/Region	Candidate Genes or Pathways (Not Functionally Validated)	Method	Reference
Hereford and Braford, Brazil	<i>B. bovis</i> infection level and tick count	Direct (infection level); indirect (tick count)	Top SNPs on BTA 1, 2, 5, 6, 12, 16, 17, 18, 24, 26	Immune-pathway candidate genes	Single-step GBLUP GWAS	[33]
Gyr x Holstein F2 (<i>B. indicus</i> x <i>B. taurus</i>)	Tick count	Indirect, through tick resistance	6 QTL; season-specific (BTA 2, 10 dry; BTA 5, 11, 27 rainy); BTA 23 in both seasons	MHC region (BTA 23)	Genome-wide microsatellite linkage scan	[48]
Nguni (indigenous), South Africa	Tick count (mainly <i>Amblyomma hebraeum</i>)	Indirect, through tick resistance	Genome-wide significant on BTA 7, 10, 19	Immune-region candidates	GWAS and regional heritability mapping	[49]
Hereford and Braford, Brazil	Tick count, across challenge levels	Indirect, through tick resistance	Multiple chromosomes; SNP by environment	Immune, inflammation and tissue-repair genes	GWAS with reaction norms	[47]
Hereford and Braford, Brazil	Tick count (prediction)	Indirect, through tick resistance	58 tag SNPs (genome-wide)	Low-density predictive panel	Bayesian tag-SNP selection	[50]
Braford, Brazil	Tick count, with skin transcriptome	Functional support	cis-eQTL; <i>PADI4</i> locus	WNT/ β -catenin, NF- κ B, JAK/STAT; <i>PADI4</i>	GWAS, RNA-seq and eQTL integration	[4,57]
Sahiwal (<i>B. indicus</i>), India	Tropical adaptation (no infection or tick phenotype)	General adaptation	BTA 17 (<i>IL2</i>) among 146 swept regions	<i>IL2</i> , <i>IFNL3</i> , <i>IRF8</i> , <i>BLK</i>	Selection signatures (ddRAD)	[51]
Dehong humped (<i>B. indicus</i>), China	Heat tolerance and disease resistance (no infection or tick phenotype)	General adaptation	Multiple selective sweeps	<i>LIPH</i> , <i>TP63</i> , <i>CBFA2T3</i> (immunity)	F_{ST} , XP-EHH, XP-CLR	[52]
African / Sudanese zebu	Dryland adaptation (no infection or tick phenotype)	General adaptation	Gene-rich sweeps on BTA 5, 7, 19	Immune-response genes	F_{ST} and haplotype sweep scans	[53]

Evidence categories follow the grading defined in the subsection "Disentangling tick resistance from *Babesia* resistance". Direct: *Babesia* infection itself measured and related to host genotype. Indirect: tick count was the measured phenotype. Functional support: expression data without a *Babesia* phenotype. General adaptation: selection scans in tropically adapted breeds, not attributable to babesiosis specifically. One of nine entries rests on a direct *Babesia* phenotype. BTA, *Bos taurus* autosome; ddRAD, double-digest restriction site-associated DNA sequencing; F_{ST} , fixation index; GBLUP, genomic best linear unbiased prediction; XP-CLR, cross-population composite likelihood ratio; XP-EHH, cross-population extended haplotype homozygosity



worth pursuing rather than as independent confirmation. The weakest part of the evidence is reproducibility across populations and environments, a problem that SNP by environment interaction shows is partly real biology rather than statistical noise [47]. The combined evidence from QTL mapping, GWAS, selection signatures and functional genomics is summarized in Fig. 3. The distinction between tick resistance and *Babesia* resistance that runs through this evidence is illustrated in Fig. 4.

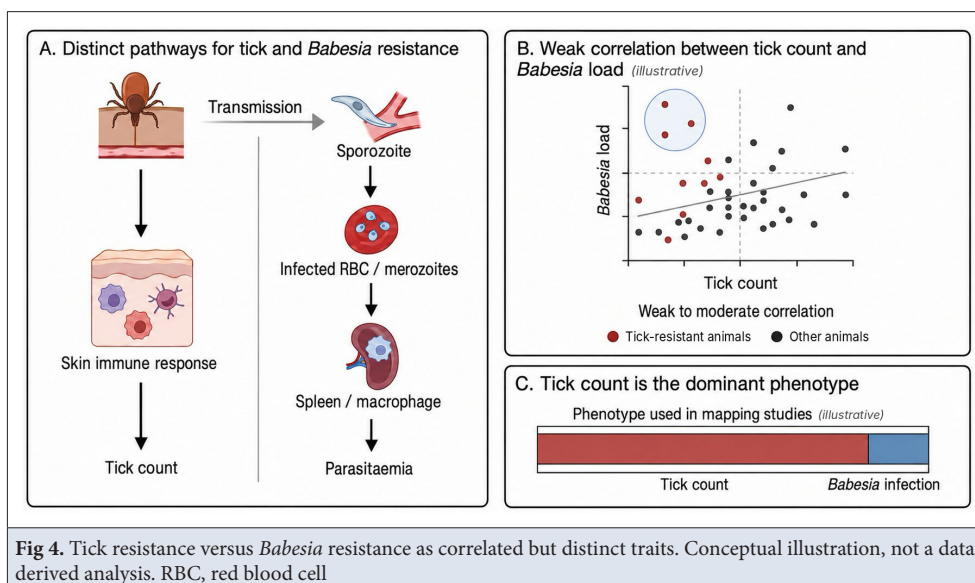
FUNCTIONAL GENOMICS AND MOLECULAR MECHANISMS

Association identifies where in the genome resistance lives; functional genomics asks what actually differs between resistant and susceptible animals. One qualification governs this entire section. The transcriptomic and

systems-biology work available in cattle was carried out on animals classified by tick burden rather than by *Babesia* infection, so the pathways described below are established for tick resistance and remain candidates, not demonstrated mechanisms, for *Babesia* resistance.

Host Transcriptomics

Transcriptomic profiling can distinguish resistant from susceptible animals, although the evidence in cattle rests on a small number of studies in a narrow range of breeds. In Braford cattle, ribonucleic acid sequencing (RNA-seq) of skin from artificially infested resistant and susceptible hosts identified differentially expressed genes consistent with specific anti-tick mechanisms, with resistant animals showing distinctive skin-remodeling and leukocyte-recruitment signatures [57]. The phenotype in that study was tick burden, and the contrasting groups came from



a single composite breed, so the signatures describe anti-tick responses in that population and have not been tested against *Babesia* infection.

Implicated Pathways

A recurring set of pathways is reported, though the independence of these reports is limited. Resistance-associated transcriptional programs have implicated the Wingless/integrated (WNT)/ β -catenin pathway, nuclear factor kappa B (NF- κ B), and Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling, together with proteolysis and tissue-repair processes, with WNT supported both by expression data and by a parallel association signal^[57]. A systems-biology synthesis of Braford resistance reported compatible modules and prioritized peptidyl arginine deiminase type IV (*PADI4*) as a candidate at a regulatory locus^[4]. Two qualifications follow. First, both analyses concern Braford cattle phenotyped for tick burden, so the agreement between them reflects a shared population and phenotype rather than independent replication across breeds or against *Babesia* infection. Second, WNT, NF- κ B and JAK/STAT are pleiotropic pathways recovered across a wide range of inflammatory and tissue-remodeling contexts, so their appearance here is compatible with a role in resistance without establishing one. These pathways are best regarded as prioritized hypotheses for functional testing.

Integrative and Systems Biology

The current frontier is integrating association with functional data to move from correlation toward causation. Combining GWAS with expression data to identify expression quantitative trait loci (eQTL) can prioritize causal candidates, and machine-learning approaches are increasingly used to annotate regulatory variants, including cross-species prediction of enhancers in cattle from better-annotated genomes^[58]. These methods are promising but still limited by small, tissue-restricted datasets, and few causal variants for resistance have been confirmed.

Parasite and Vector Genetics as Effect Modifiers

Resistance is one corner of a three-way interaction. Parasite and tick genetics modify it. *B. bovis* populations are genetically diverse and geographically sub-structured, which shapes immunity and the portability of antigen-based markers and vaccines across regions^[59]. Comparative genomics of *Babesia* species shows extensive variation in the multigene families that encode surface and virulence proteins, the same families that drive antigenic variation and cytoadhesion^[60]. On the vector side, *B. bovis* infection itself alters gene expression in *Rhipicephalus microplus* larvae, and reference transcriptomes spanning

the parasite's vertebrate and invertebrate stages are now available to dissect this host-vector-parasite interaction^[61,62]. The practical implication is that a marker or antigen validated against one parasite or tick population may not transfer to another, a caveat that applies equally to host-side markers selected in one environment.

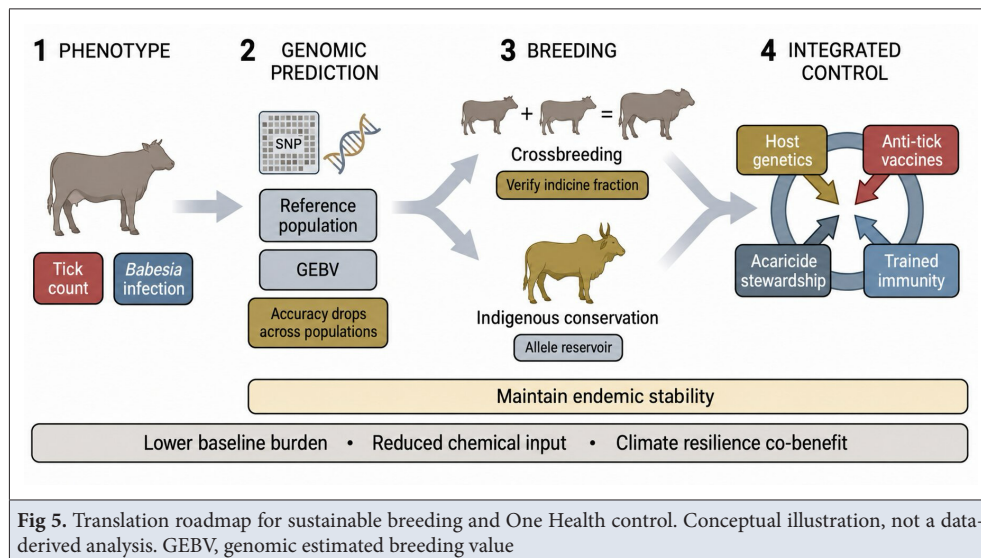
Two operational lessons follow from treating resistance as one vertex of a host-parasite-vector triad. First, the genetic diversity and geographic sub-structuring of *B. bovis* mean that immunity selected or acquired against one parasite population may be only partial against another, so resistance phenotypes and any antigen-based companion tools should be validated in the target epidemiological setting rather than assumed to transfer^[59,60]. Second, because the parasite reprograms gene expression in the tick and depends on tick physiology for transmission, vector competence is itself a variable that interacts with host resistance, and the stage-resolved reference data now available make it feasible to ask which host responses actually disrupt transmission rather than merely lowering parasitaemia^[61,62]. Separating resistance that reduces an individual animal's burden from resistance that reduces onward transmission is an underexplored distinction, and it bears directly on whether genetic selection can shift herd-level infection pressure and therefore on how selection interacts with endemic stability.

TRANSLATING HOST GENETICS INTO SUSTAINABLE DISEASE CONTROL

The implications half of the title concerns how this knowledge becomes durable, field-deployable control. A practical roadmap for translating resistance phenotypes and genomic data into breeding and integrated One Health control is shown in *Fig. 5*.

Marker-Assisted and Genomic Selection

Genomic estimated breeding values for tick resistance can be computed with useful accuracy, and tag-SNP panels selected by Bayesian methods predict tick resistance in Hereford and Braford cattle at densities low enough to be economical^[50]. The same genomic framework extends to *Babesia* infection level, where infection and tick-count traits can be evaluated jointly^[33]. A central practical question is portability: multi-breed and multi-country genomic prediction of tick resistance is feasible, but accuracy depends strongly on the size and composition of the reference population and degrades when training and target populations differ in linkage-disequilibrium phase^[32]. The clearest near-term gains are therefore within-population, with cross-population prediction still requiring larger and more diverse reference cohorts.



Breeding Strategy and Indigenous-Breed Conservation

Two complementary strategies follow. Crossbreeding can capture indicine resistance while retaining taurine productivity, but the evidence that a small zebu fraction is insufficient^[34] means crossbreeding programs must target and verify a meaningful indicine contribution rather than assume it. In parallel, locally adapted indigenous breeds are reservoirs of resistance alleles shaped by generations of natural selection, and characterizing and conserving them is a genetic-resource priority, as selection-signature studies in Indian, Chinese, and African zebu suggest, although these signatures reflect general adaptation, not confirmed *Babesia* resistance^[51-53]. Conservation here is not only cultural but functional, protecting alleles that breeding programs may later need.

Integration within One Health and Integrated Pest Management

Host genetics is a complement to, not a replacement for, other tools, and its value is greatest inside an integrated framework. Trained-immunity approaches, including bacillus Calmette-Guérin (BCG)-based strategies, are being explored to broaden protection against babesiosis^[39], and next-generation anti-tick vaccines, such as multi-antigen cocktails that reduce tick fitness, can be layered with genetic selection and rational acaricide stewardship^[29]. For the reason set out above in the discussion of endemic stability, these elements must be coordinated rather than applied piecemeal^[6]. Genetic resistance is attractive within this framework because it is potentially durable and leaves no residues, addressing the failure modes of acaricides and drugs set out in *Table 1*.

Trade-offs and Sustainability

Selecting for resistance is not cost-free, and honest accounting matters. Resistance must be balanced against

growth, milk, and fertility, although the generally weak genetic correlations between resistance and production traits suggest the trade-offs are manageable rather than prohibitive^[44]. Resistance traits also intersect with climate resilience, since many of the same indigenous breeds combine disease resistance with heat tolerance in selection-signature scans, which reflect general adaptation, not confirmed *Babesia* resistance^[54].

For smallholder and tropical producers, who bear the heaviest burden and have the least access to acaricides and cold-chain vaccines, a self-sustaining genetic contribution is especially valuable, and regional framing for South Asian and Pakistani systems should weigh this equity dimension explicitly^[7,8,63].

The genomic resources needed to act on this are also expanding. Whole-genome analyses of indigenous African cattle recover candidate regions putatively linked to adaptation to ticks and vector-borne infection^[64], and comparable characterization of indigenous and composite breeds in southern Africa shows that locally adapted populations retain distinctive diversity that crossbreeding can erode if it is not documented first^[65]. Both point to the same practical conclusion for tropical smallholder systems, which is that the raw genetic material for a durable contribution may already exist in the breeds these producers keep, and that the priority is to characterize and use it rather than to displace it. The realistic position is that genetics lowers the baseline burden and the intensity of chemical control needed, rather than eliminating the need for other tools.

CHALLENGES, KNOWLEDGE GAPS, AND FUTURE DIRECTIONS

An honest appraisal of the field identifies several bottlenecks and a concrete agenda. The first is phenotyping. Resistance

is defined inconsistently across studies as clinical outcome, *Babesia* infection level, or tick count, and these are not interchangeable. Tick counts are laborious and noisy, and infection levels require quantitative molecular assays, so scalable, objective, standardized phenotypes are the rate-limiting step for everything downstream.

Progress here is partly a matter of technology. Quantitative molecular assays already allow *Babesia* infection level to be measured objectively rather than scored by eye, and high-throughput sequencing can resolve the full community of co-infecting haemoparasites in a single sample, which would let resistance be phenotyped against a defined parasite background rather than a presumed one.

Pairing such assays with routine regional surveillance, including in under-sampled South Asian systems, would generate the large, consistently phenotyped cohorts that genomic prediction requires while simultaneously informing control. Molecular screening of ticks also continues to reveal pathogens not previously reported in particular vectors and regions ^[66].

The second is population structure. Reference populations are small and region-specific, and markers transfer poorly across breeds and environments because linkage-disequilibrium phase differs and because, as SNP by environment analyses show, the underlying genetic control is itself environment-conditional. Larger multi-breed, multi-environment cohorts are needed before prediction can be portable.

The third, and most specific to this review, is the scarcity of direct *Babesia*-resistance genomics. Most inference is borrowed from tick-resistance studies, yet tick and *Babesia* resistance are genetically distinct, and dedicated cohorts phenotyped for *Babesia* infection remain few. Closing this gap is the single most valuable empirical step the field could take.

The fourth is functional validation. Candidate genes are abundant but confirmed causal variants are almost absent. Gene-editing tools now make validation tractable: clustered regularly interspaced short palindromic repeats (CRISPR)-based editing has been used to interrogate immune-gene function in bovine cells, and Cas9-expressing cattle have been generated, providing a route from candidate gene to experimental test in the target species. The limiting factors are efficiency, cost, and the regulatory and welfare questions that editing raises.

Emerging analytical tools may help but should be appraised soberly. Machine-learning methods for genomic prediction can model non-additive effects and generate hypotheses about inheritance mechanisms, while regulatory-variant annotation, single-cell immunogenomics, long-read reference genomes, and multi-omics integration each

address part of the causation problem. None is a shortcut around the need for large, well-phenotyped cohorts.

CONCLUSION

The host genome is a promising complementary pillar of bovine babesiosis control. Indicine cattle resist *Babesia* better than taurine cattle under controlled challenge, and potentially exploitable variation persists within breeds. Resistance is polygenic, but direct *Babesia* genomic evidence remains limited, and selection signatures reflect general tropical adaptation, not confirmed *Babesia*-resistance loci. Candidate pathways such as WNT, NF- κ B and JAK/STAT, identified in tick-resistance studies, await functional validation.

Genetics is complementary to vaccines and vector management, not a substitute for them, and its place is inside a coordinated One Health framework that also manages endemic stability. Three conditions will determine whether this potential is realized: scalable and standardized phenotyping, larger multi-breed and multi-environment reference cohorts, and experimental confirmation of causal variants. If these are met, genetics-informed breeding could help lower the babesiosis burden and the intensity of chemical control required, with the greatest benefit for the resource-limited tropical producers who currently bear the heaviest cost.

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REVIEW ARTICLE

Prebiotics in Prevention of *Salmonella* Shedding in Layer HensAhmed I. ALAJAJI¹ , Abdulaziz M. ALMUZAINI¹ ¹ Department of Veterinary Preventive Medicine, College of Veterinary Medicine, Qassim University, Buraydah 51452, SAUDI ARABIA

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Abstract

Salmonella infection in laying hens is one of the biggest challenges to poultry production and food safety because of its role in contaminating eggs and being transmitted to humans as a zoonotic infection. The use of antibiotics in control strategies was well established until there has been growing interest in searching alternative control strategies due to the growing concerns with antimicrobial resistance. Prebiotics which are defined as non-digestible feeds components which selectively stimulates desirable intestinal microbiota have been found promising as functional feed additives in the diet of poultry. Fructo-oligosaccharides, mannan-oligosaccharides, inulin and β -glucans have the ability to influence the gut microbiosis, improve mucosal immunity and decrease intestinal colonization of pathogenic microorganisms such as *Salmonella*. Prebiotics aid competitive exclusion and the production of short-chain fatty acids and an improved intestine barrier through the stimulation of the growth of beneficial bacteria like *Lactobacillus* and *Bifidobacterium*. All these mechanisms are associated with the reduction of cecal colonization and fecal shedding of *Salmonella* in layer hens. This review is a summary of the epidemiology of *Salmonella* in poultry, the biological importance of major prebiotics, their mechanisms of action, and effect on poultry performance, which clearly point at them as a sustainable instrument in the enhancement of poultry health and food safety.

Keywords: *Salmonella*, prebiotics, poultry nutrition, salmonellosis, control, gut microbiota, zoonotic bacteria

INTRODUCTION

Poultry-related food borne pathogens are a major public health issue in the world today ^[1]. Of these pathogens, *Salmonella* spp. still are a significant part of the most significant zoonotic agents associated with poultry and egg-related human infections ^[2]. Poultry products represent significant reservoirs of a number of *Salmonella* serovars especially *Salmonella* Enteritidis and *Salmonella* Typhimurium capable of colonizing the intestinal tract of birds without causing serious clinical effects but can contaminate eggs, meat and the surrounding environment ^[3]. This is an asymptomatic carrier that complicates the management of *Salmonella* especially in large poultry enterprises ^[4]. The poultry business in the world has been growing very fast over the past few decades to satisfy the growing need of animal protein at low cost ^[5]. As the growth has occurred, management of foodborne pathogens is now of high priority among producers, regulating agencies and government agencies that deal with health ^[6]. *Salmonella* infection in laying hens can cause horizontal infection by feces contamination and vertical infection by an infected reproductive organ, both

of which can cause egg contamination before its shell develops ^[7]. It is therefore possible that infected layer flocks provide the consumer with a possible source of infection ^[8]. Historically, antibiotics were commonly applied in the poultry production to prevent diseases, treat them, and enhance the growth ^[9]. Nevertheless, the high usage of antimicrobial agents in animal production has led to the development and spread of antimicrobial resistant bacteria ^[10]. The growing cases of antibiotic resistance have brought serious concerns about safety and sustainability of antibiotics use on livestock ^[11]. To counter this, the use of antibiotic growth promoters in the production of poultry in many countries is limited or banned. Such regulatory developments have triggered a lot of research on alternative measures that can keep poultry healthy and productive with minimum dependence on antibiotics. Multiple alternative control strategies are in use to control different bacterial, viral, fungal, and parasitic diseases of poultry and other animals ^[12,13]. Among all alternative control strategies, probiotics are considered as most effective because of their diverse medicinal and biological activities ^[14,15].

The use of effective feed additives to improve gut health



and boost the natural resistance of birds to pathogens is one of the promising directions ^[16]. However, prebiotics have attracted significant amount of attention among these additives because of their capacity of regulating the intestinal microbiota and enhancing the beneficial microbial populations which results in lowering the antimicrobial resistance ^[17]. Prebiotics are generally non-digestible carbohydrates that cannot be digested in the upper tract and get to the lower intestine where they are fermented by benevolent bacteria ^[18]. The most typical ones are fructo-oligosaccharides, mannan-oligosaccharides, galacto-oligosaccharides and inulin ^[19]. The intestinal microbiota is at the centre of the gut health and pathogen colonization prevention ^[20]. A healthy microbial community is able to inhibit pathogenic bacteria by the help of competition exclusion, antimicrobial metabolites production and the activation of host immune systems ^[21]. Prebiotics serve the selective proliferation of positive microorganisms that incline to thrive in the poultry food when added to chicken diets (*Lactobacillus* and *Bifidobacterium*) ^[22]. During fermentation, these microorganisms synthesize short chain fatty acids (SCFAs) which include acetate, propionate and butyrate out of prebiotic substrates ^[23]. The benefits of SCFAs include decreasing the intestinal PH, inhibiting the growth of pathogens, and enhancing the intestinal epithelial integrity ^[24]. Some studies have shown that dietary supplementation of prebiotics has the power of decreasing colonization and shedding of *Salmonella* in poultry ^[25]. Indicatively, fructo-oligosaccharides have been found to reduce cecal loads of *Salmonella* Enteritidis in laying hens and fecal shedding concomitantly with increased mucosal immune responses ^[26]. It has been linked to these effects, including the increased number of helpful bacteria and increased intestinal immunoglobulin A (IgA), which is a significant part of mucosal immunity ^[27].

Besides microbiota modulation, prebiotics also have the ability to regulate the host immune functions ^[28]. Most prebiotic compounds stimulate innate and adaptive immune responses by promoting the activation of immune cells, such as macrophages, dendritic cells, and lymphocytes ^[29]. Certain prebiotics have also been shown to enhance the structure of the intestinal epithelium, with respect to the height of villus and nutrient uptake ^[30]. Better gut morphology helps in the enhancement of nutrient usage and the general performance of poultry ^[31]. Prebiotics have one more benefit since they do not interfere with other means of controlling diseases ^[32]. Probiotics may be administered as synbiotics together with prebiotics to increase the gut microbial homeostasis and resistance to pathogens ^[33]. These combined strategies are being seen more and more as powerful parts of overall systems of poultry health management ^[34]. Full knowledge

of the complexes of how various prebiotics can affect gut microbiota and pathogen settlement is needed to maximize them in poultry systems of production ^[35].

There are some extensive reviews of the effectiveness of prebiotics in colonizing reduction of *Salmonella* in poultry, but most of them include a strong emphasis on broiler chickens. The physiology and production of laying hens is fundamentally different. They have much longer production cycles, high calcium requirements for eggshell development, have special physiological stresses like moult and pose a special public health risk through the vertical transmission of *Salmonella* through eggs. Thus, an important knowledge need is the mechanism of action of prebiotics in the special physiological conditions of the layer hen. This article will focus on laying hens and is not a broad review as in previous articles. Critically reviews current literature on prebiotic interventions (fructo-oligosaccharides, mannan-oligosaccharides, inulin, and β -glucans), as well as their mechanisms of action with regard to the stressors present during different stages of egg production and their effectiveness in reducing intestinal colonization and fecal shedding of *Salmonella* to prevent egg contamination.

LITERATURE SEARCH STRATEGY

A structured search strategy was used to identify texts adequately and objectively for evaluation. Peer-reviewed articles published in the English language between 2020 to 2026 were searched in the databases PubMed, Scopus and Google Scholar. The main search string used was a Boolean combination of words: ("prebiotic" OR "fructo-oligosaccharide" OR "mannan-oligosaccharide" OR "inulin" OR "beta-glucan") AND ("*Salmonella*") AND ("layer hen" OR "laying hen" OR "pullet"). Studies that were only performed on broiler chickens and studies that have been conducted on the non-*Salmonella* pathogens were excluded from this review unless specifically needed to compare physiological mechanisms between the two types of chicken. For prebiotic mechanisms, *Salmonella* colonization, fecal shedding, mucosal immunity, and layer hen performance, articles were chosen on the basis of their relevance to the topics. Also existing systematic reviews were consulted in order to avoid duplication of scientific questions in this narrative review.

EPIDEMIOLOGY OF *SALMONELLA* IN LAYER HENS

The *Salmonella* infections are still considered as the most prominent issues in the production of poultry as they are widely distributed and may lead to foodborne illness in humans ^[2]. Many *Salmonella* serovars have been identified in poultry since *Salmonella* Enteritidis and *Salmonella*

Typhimurium are the most commonly identified with egg-borne and poultry outbreaks [36]. They can colonize the intestinal tract of chickens and stay in the ceca over long periods without overt clinical disease [37]. *Salmonella* epidemiology of layer hens is multidimensional and multi-source of transmission [38]. Horizontal transmission is by consuming infected feed, water, littering or fecal matter of infected birds [39]. After being introduced to a flock, the pathogen may propagate quickly because of the high stocking density and contamination of the environment [40]. Cecum is a major reservoir where *Salmonella* may proliferate and survive giving rise to unremitting fecal shedding [41]. Vertical transmission is also a very important factor in the epidemiology of *Salmonella* in laying hens [42]. Contaminated hens can develop bacteria in their reproductive tract specifically the ovary and oviduct. This may cause the eggs to be contaminated during formation in the reproductive tract [43]. Internally contaminated eggs have a high food safety risk as the bacteria might withstand the normal provision of food and therefore cause an infection to the consumers [4].

The persistence of the environment also makes the task of control harder [44]. *Salmonella* is able to endure long periods in poultry houses, litter, dust and equipment. Rats, insects and wild birds might also act as reservoirs and vectors of the pathogen and enable the introduction of the pathogen into the poultry birds [45]. Moreover, infected feed materials are found to be the possible causes of infection [46]. The intestinal microbiota of chicken is very essential in determining the susceptibility to *Salmonella* colonization [47]. Laying hens have a more delicate 'ecological niche' due to the regular extreme physiological changes that occur during the onset of lay and induced molting, increasing the susceptibility of the bird for colonization at this time compared to broilers. Stable intestinal microbiota can also be considered a natural defence mechanism against these diseases in healthy birds that are naturally balanced [48]. This can be accomplished by the presence of favourable microorganisms in ecological niches in the gut and compete with pathogens over nutrients and attaching sites [21]. Such a disturbance of this microbial balance is referred to as dysbiosis that may predispose to infection and colonization by pathogens [49]. There are various management aspects that determine the prevalence of *Salmonella* in layer flocks [50]. The introduction and spread of the pathogen may be caused by poor biosecurity, insufficient sanitation, and poor handling of feed [51]. Moulting, transportation, high stocking density and other stress conditions also have the potential to increase an individual to infection due to the weakening of the immune system and changes in gut microbial composition [52].

The poultry industry has also practiced several control strategies such as vaccination, better biosecurity exercises,

and surveillance programs within the industry over the last few years to curb the prevalence of *Salmonella* [53]. Nonetheless, these measures are not likely to eradicate the pathogen in the poultry production systems fully [9]. Therefore, nutritional treatment, i.e., the prebiotic and probiotic use, have increasingly become the focus of interest as an adjunctive approach to counteract intestinal colonization and fecal shedding of *Salmonella* [54].

PREBIOTICS AS FUNCTIONAL FEED ADDITIVES

Prebiotics are considered to be non-digestible food substances which selectively induce growth or action of the healthy microorganisms in the gastrointestinal tract [55]. These substances are usually complex carbohydrates like oligosaccharides which are not fermented in the upper tract of the digestive system and are fermented by the intestinal microbes of the lower tract (Fig. 1) [56]. Prebiotics increase intestinal microbial balance through fermentation that supports the growth of positive bacteria such as *Lactobacillus* and *Bifidobacterium* [57]. Prebiotics are also being considered as an important functional feed additive because of their gut health benefits, nutrient absorption, and their ability to decrease pathogen colonization in poultry nutrition [58]. Prebiotics may inhibit the growth of pathogenic microorganisms by promoting the growth of the beneficial microorganisms by competitive exclusion, generation of antimicrobial metabolites, and lowering of the pH of the intestinal environment [33]. All these effects lead to a better intestinal barrier integrity and increased immune responses in chickens [59]. Some forms of prebiotics have been studied in poultry production and these include fructo-oligosaccharides, mannan-oligosaccharides, inulin, and β -glucans [60]. The individual unique structural properties and modes of action of each of these compounds affect the gut microbiota and host physiology (Table 1) [61]. It has been found that these prebiotics can greatly decrease the colonization of such pathogens as *Salmonella* through enhancing the mucosal immunity and improving the microbial balance [25].

Fructo-oligosaccharides

Fructo-oligosaccharides (FOS) are short fructose polymers that are obtained as a result of chicory, onions, garlic, and bananas [62]. The compounds cannot be digested by the host enzymes in the upper gastrointestinal tract and pass on to the large intestine where they can be fermented by the beneficial bacteria [63]. In chickens, supplementation with FOS has been identified to have a considerable effect on the intestinal microflora and the gut condition [64]. Selective stimulation of beneficial bacterial populations is one of the main methods according to which FOS work [65]. The fermentation of FOS by *Lactobacillus* and

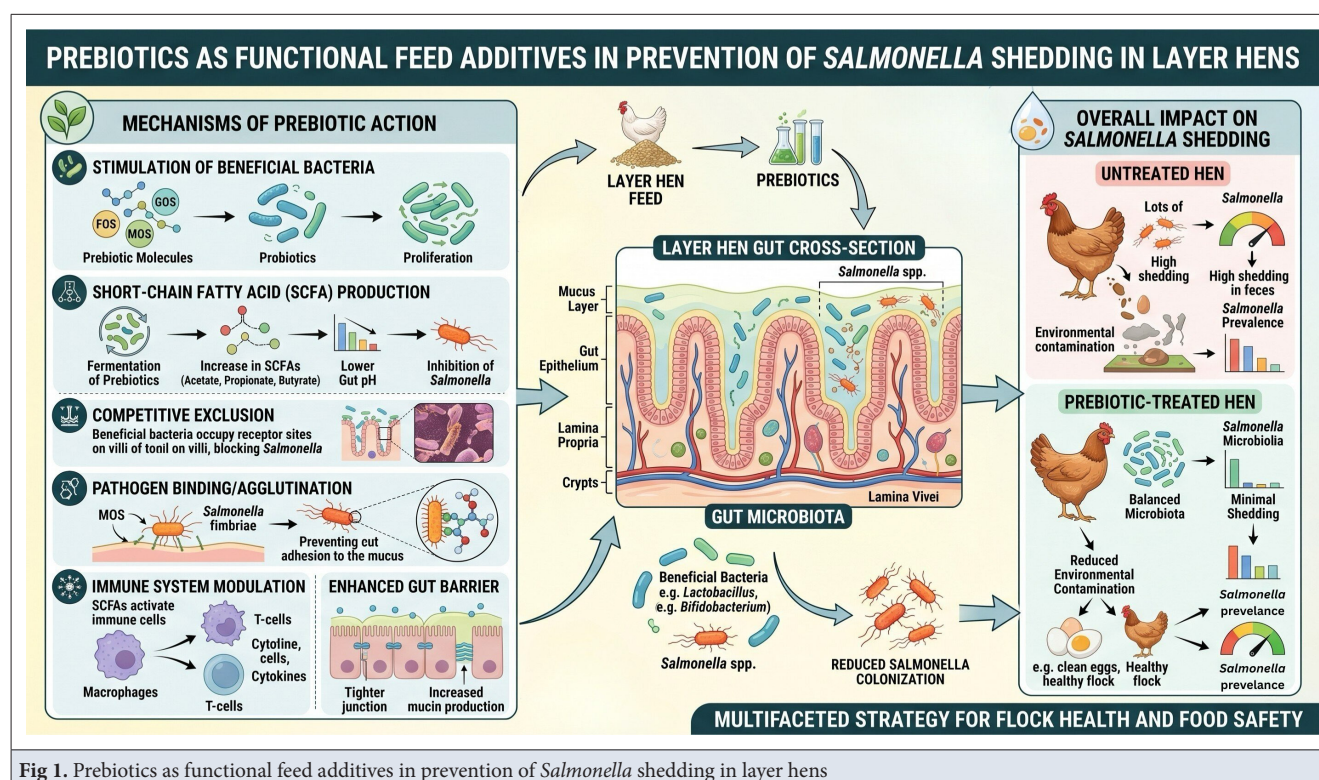


Fig 1. Prebiotics as functional feed additives in prevention of *Salmonella* shedding in layer hens

Table 1. Effects of dietary prebiotics on intestinal microbiota, immune responses, and *Salmonella* colonization or shedding in layer hens

Sr. No.	Prebiotic Type	Source	<i>Salmonella</i> Serovar Studied	Inclusion Level in Diet	Experimental Design	Results	Effects on Performance	Ref.
1	Fructo-oligosaccharides	Chicory root derived oligosaccharide	<i>Salmonella</i> Enteritidis	0.375-0.75% of diet	<i>In vivo</i> layer challenge (Molt-induction)	Reduced cecal colonization and liver/ovary infection in multiple trials	Maintained post-molt production performance	[132]
2	Fructo-oligosaccharides	Plant-derived oligosaccharide	<i>Salmonella</i> Enteritidis	0.5-1% dietary supplementation	<i>In vivo</i> challenge	Significant reduction in fecal shedding and cecal load	No negative effect on growth or egg production	[133]
3	Fructo-oligosaccharides	Plant fructans	<i>Salmonella</i> Enteritidis	Variable (synbiotic formulation)	<i>In vivo</i> synbiotic layer challenge	Reduced cecal <i>Salmonella</i> load during infection	Maintained egg production	[134]
4	Mannan-oligosaccharides	Yeast cell wall (<i>Saccharomyces cerevisiae</i>)	<i>Salmonella</i> Enteritidis	0.1-0.5%	<i>In vivo</i> layer field trial	Reduced intestinal pathogen colonization	Improved feed conversion and egg production	[135]
5	Mannan-oligosaccharides	Yeast-derived	<i>Salmonella</i> Enteritidis	Commercial feed additive	<i>In vivo</i> layer challenge	Reduced intestinal and ovarian infection	Improved intestinal health	[136]
6	MOS + <i>Bacillus</i> probiotic (Synbiotic)	Yeast MOS + <i>Bacillus</i> spp.	<i>Salmonella</i> Enteritidis	Commercial synbiotic formulation	<i>In vivo</i> synbiotic layer challenge	Reduced intestinal colonization and egg contamination risk	Maintained production	[137]
7	Inulin	Chicory root fructan	<i>Salmonella</i> spp.	0.5-1% dietary inclusion	<i>In vivo</i> layer field trial	Reduced intestinal pathogen populations	Improved gut morphology	[133]
8	Inulin + Postbiotic	Chicory fructan + LAB metabolites	<i>Salmonella enterica</i>	Experimental formulation	<i>In vitro</i> and <i>in vivo</i> layer evaluation	Antimicrobial activity against <i>Salmonella</i>	Improved intestinal mucosal architecture	[133]
9	β -Glucans	Yeast or fungal polysaccharides	<i>Salmonella</i> Typhimurium	0.05-0.1%	<i>In vivo</i> layer field trial	Reduced pathogen colonization	Improved disease resistance	[138]
10	Mixed Prebiotics	Various oligosaccharides	<i>Salmonella</i> spp.	Variable	Meta-analysis of multiple studies	Overall reduction in fecal and cloacal shedding	No negative effects on productivity	[54]

Bifidobacterium species also increases the short-chain fatty acid generation such as acetate, propionate and butyrate [23]. These organic acids reduce the pH in the intestine and provide an unfavourable habitat to the pathogenic bacteria including *Salmonella* [66]. Moreover, SCFAs are an energy source of intestinal epithelial cells and help to increase intestinal integrity [24]. The laying hen experimental studies have shown that food supplementation of FOS can greatly reduce cecal colonization and shedding of *Salmonella* Enteritidis of the feces [67]. However, the strength of this evidence varies significantly depending on the age of the flock and the challenge dose. While FOS is highly effective in early-lay hens, its efficacy often diminishes during late-stage production or forced molting, suggesting that age-related dysbiosis may overpower prebiotic interventions if dosages are not properly adjusted. Due to FOS supplemented diets, infected birds had lower pathogen loads and improved mucosal immune responses such as increased IgA-producing cells in the intestinal lamina propria [68].

FOS also have an effect on host immune response by stimulating the innate and adaptive immune response [69]. The compounds have the potential to alter the expression of immune related genes including Toll-like receptors and cytokines, which are vital in the pathogen recognition and immune signalling [70]. There are boosted immunity and resistance to intestinal pathogens [71]. The other mechanism is competitive exclusion [72]. The beneficial bacteria, which are stimulated by FOS, occupy the sites of attachment in the intestinal epithelium and prevent infection by the pathogenic microorganisms [73]. Such microbial rivalry prevents the possibility of *Salmonella* to be attached to intestinal cells and become an infection [74]. Their capacity to regulate the intestinal microorganisms, augment immune responses, and prevent the proliferation of pathogens make them useful feed supplements towards the advancement of the wellbeing of chickens and the decrease of *Salmonella* shedding in layer hens [75].

Mannan-oligosaccharides

Mannan-oligosaccharides (MOS) [18] are a group of products, mainly derived by the cell wall of the yeast *Saccharomyces cerevisiae*, and are also used as prebiotic feed supplements in poultry feed [76]. MOS are polysaccharide mannose polymer with exceptional biological characteristics being in a position to regulate intestinal microbiota and enhance immunity [77]. Inhibition of bacterial adhesion to epithelial cells of the intestine is amongst the most significant mechanisms through which MOS inhibit pathogen colonization [78]. *Salmonella* has type-1 fimbriae which are used to attach to mannose residues in host cells, and this is common in many pathogenic bacteria [79]. MOS serve as molecular decoys as they present alternative binding sites to such

fimbriae [80]. Consequently, the pathogens bind to MOS molecules instead of to the intestinal epithelial cell, which prevents the colonization process and allows the pathogen to be eliminated in the gastrointestinal tract [78]. However, a major restriction of the existing MOS literature is that the data is often extrapolated from a model developed for broilers. Many of the studies do not include the high calcium diets and long gut transit times common to laying hens which could potentially affect the binding affinity of MOS and efficacy in laying hens.

MOS, likewise, enhance the development of the beneficial bacteria like *Lactobacillus* and *Bifidobacterium*, which lead to the competition of the pathogenic microorganisms [81]. These useful bacteria break down foods to generate short chain fatty acids which reduce the pH of the intestines and prevent the growth of pathogens [82]. The increased microbial balance leads to increased intestinal health and lower pathogen burden. Besides their microbiota-modulating properties, MOS have been revealed to induce immunity in poultry [83]. MOS supplementation enhances the production of antibodies as well as the growth of lymphoid organs which are vital constituents of avian immune system [84]. The benefits of having improved immune responses is that birds have a better chance of withstanding infections as well as being healthy [85].

The MOS supplementation has been linked to better absorption of nutrients and enhanced digestion [86]. MOS help to enhance the morphology of the intestines and nutrient absorption through the promotion of the beneficial microbial populations and the reduction of the number of the pathogenic bacteria [87]. This eventually enhances the performance and productivity of poultry [88].

Inulin

Inulin is a naturally occurring fructan polysaccharide that is present in most plants such as chicory root, Jerusalem artichoke, garlic, and also onions [89]. Similar to other prebiotic substances, inulin is indigestible in the proximal gastrointestinal tract and gets into the lower intestine where it is fermented by useful microorganisms [90]. The inulin fermentation by intestinal bacteria leads to the generation of short chain fatty acids that are very essential in the intestinal health [82]. SCFAs reduce the level of intestinal pH, prevent the proliferation of pathogenic bacteria, and supply energy to the epithelial cells [91]. This increases the integrity of barriers of the intestine and decreases the chances of invasion of pathogens [92]. Inulin supplementation in chickens has also been reported to have a large effect on gut microbial composition by raising the count of profitable microbes including *Lactobacillus* and *Bifidobacterium* [93]. These bacteria can cause competitive exclusion by holding ecological niche in the gut and compete with pathogens in the nutrients

and attachment sites ^[94]. Consequently, the intestinal tube becomes less colonized by the pathogenic bacteria like *Salmonella* ^[95].

Inulin is also significant in the regulation of immune system ^[96]. It has been established that the inulin supplementation may be used to boost the synthesis of immunoglobulins and the activation of immune cells contributing to the defence against the pathogens ^[97]. Better immune responses are also a contributing factor to high resistance to intestinal infections ^[98]. Also, inulin enhances the intestinal morphology by raising the length of the villus and the surface area in the small intestine ^[99]. These structural improvements increase the absorption of nutrients and digestion ^[100]. Enhanced nutrient use leads to enhanced growth performance and aggregate productivity in poultry ^[101]. Inulin is also an effective prebiotic ingredient that can be used to reduce the colonization of pathogens and improve the health of the gut in poultry through its individual effect on gut microbiota, immune system and intestinal morphology ^[22].

β-Glucans

β-Glucans are a group of polysaccharides that are typically obtained in yeast, fungi, cereal and some of the algae ^[102]. These are compounds that are made of glucose molecules, which are connected through β-glycosidic bonds and are commonly known to possess immunomodulatory effects ^[103]. The application of β-glucans as functional feed additives in the poultry nutrition is becoming more common to boost the immune response and disease resistance ^[104]. Stimulation of the innate immune system is one of the main mechanisms of β-glucans actions because they are perceived by particular receptors on the macrophages, dendritic cells, and heterophils ^[105]. Interaction of β-glucans to the receptors triggers signalling pathways that increase phagocytosis, cytokine generation, and clearance of pathogens ^[106]. Dietary supplementation of poultry with β-glucans has been demonstrated to increase both cellular and humoral immune response ^[107]. The increased production of antibodies and the augmented functioning of immune cells lead to heightened resistance against bacterial infections and β-glucans can also regulate the inflammatory reactions, which may avoid over-protection of tissue damage during the stage of infection ^[108]. β-glucans also lead to the modification of gut microbiota composition ^[109]. Beneficial bacteria can ferment these polysaccharides and thus grow and be active ^[110]. Higher population of good microorganisms has been associated with competitive elimination of pathogenic bacteria as well as better microbial balance in the intestines ^[111].

Moreover, β-glucans enhance the intestinal barrier activity through the increase of epithelial integrity and the mucosal immunity ^[112]. Enhanced gut barrier operation

decreases the capacity of the pathogen to invade the intestinal lining and initiate infection by *Salmonella* ^[47]. Studies have established that β-glucans supplementation has the potential of enhancing growth performance, immune functions, and environmental stress resistance in short-term poultry trails ^[113]. However, it is important to remember that the strongest of these immune responses has been seen in fast growing broilers. There is limited evidence to demonstrate the efficacy of β-glucan throughout an entire commercial laying cycle, and it is not conclusive. This represents a significant research gap.

IMPACT OF PREBIOTICS ON POULTRY PERFORMANCE

Besides having antimicrobial and immunomodulatory qualities, prebiotics have great impact on poultry productivity and performance ^[114]. Healthy gut and superior microbial balance will lead to higher levels of digestion, nutrient absorption and immunity that will ultimately lead to high growth and egg development in laying hens ^[115]. The regulation of intestinal microbiota is one of the main ways in which prebiotics can affect the performance of poultry ^[25]. Prebiotics induce the growth of desirable microorganisms (*Lactobacillus* and *Bifidobacterium*) in the gastrointestinal system, which form a stable ecosystem ^[116]. The beneficial bacteria outcompete the pathogenic microorganisms on nutrients and attached space, which minimizes the colonization of the pathogen and intestinal inflammation ^[117]. The short-chain fatty acids are also produced by prebiotic fermentation and are used to provide energy to the intestinal epithelial cells ^[118]. These metabolites regulate the intestinal health by improving the integrity of the epithelia and inducing cell growth in the intestinal mucosa ^[119]. A better morphology of the intestine with a higher height and surface area of the villus height will result in more efficient nutrient absorption ^[120]. Increased nutrient absorption results in increased feed efficiency and growth performance of poultry ^[121]. Multiple researches have also indicated that prebiotic oligosaccharides supplementation has the potential to increase feed ratio, egg production, and egg mass in laying hens ^[122]. The difficulty of interpreting these performance metrics, however, cannot be ignored. Many of the trials have short supplementation periods (e.g. 4-8 weeks) and small numbers of birds and do not adequately reflect the long duration of a commercial laying cycle under the rigorous physiological and metabolic demands of the commercial layer. As an illustration, MOS supplementation has been found to enhance egg production and feed efficiency via the regulation of gut microbiota and a better provision of nutrients ^[86]. The prebiotics too affect the parameters of egg quality ^[123]. Certain investigations have indicated the increase of the eggshell thickness, albumen

quality and yolk composition after supplementation with some oligosaccharides ^[124]. Eggshell quality is also of great value in diminishing microbial contamination and enhancing egg safety ^[125]. The other notable advantage of prebiotics is that they boost flagella and lessen death rates in chicken flocks ^[126]. Prebiotic supplementation of birds is also associated with better immune parameters with increased antibody and improved resistance to infectious diseases ^[127]. Enhanced immune response lowers disease occurrence and leads to a high flock productivity ^[128]. Besides enhancing the health and productivity of the birds, prebiotics can also assist the birds to survive stressful environmental conditions like heat stress ^[129]. It has been shown that prebiotic supplementation is able to stabilize gut microbiota, enhance immune reaction when facing a stressor situation, thus ensuring performance stability and diminishing the adverse physiological outcomes ^[130]. Prebiotic addition to the poultry feed is a promising policy of optimizing performance, disease resistance and ensuring sustainable production of poultry ^[131].

FUTURE PROSPECTS

The growing market interest in nutritional solutions to improve gut health and disease resistance has been driven by the growing demand of producing poultry free of antibiotics. Prebiotics are the potential category of feed supplements that can be used to enhance the intestinal microbial status and the decrease of colonization with pathogens in poultry. The future studies will be directed towards the discovery of new prebiotic compounds that have improved functional properties. Recent developments in microbiome studies and molecular biology are also allowing researchers to gain a clearer insight into the interplay between the various elements of diet, intestinal microbiota, and immune responses by the host. These findings can be used to develop specific prebiotic preparations that can be used selectively to stimulate the growth of favourable microbial communities that will outcompete pathogens like *Salmonella*. The other research opportunity is the development of synbiotic formulations which are a combination of pre and probiotics. These combinations have the ability to offer synergistic effects which at once introduces useful microorganisms as well as offers substrates that are conducive to the growth of these microorganisms. Precision nutrition strategies also could be very helpful in the optimization of prebiotic supplementation. The effect of prebiotics is strongly dependent on the age of the birds, genetic strain and environmental factors, making it important to make layer trials over the full production cycle, rather than using extrapolated broiler data. Adapting existing prebiotic formulations to the specific physiological needs and longevity of commercial layer systems to maximize

effectiveness and predictability of prebiotics in these systems will be essential for future prebiotic formulations.

CONCLUSION

Controlling *Salmonella* infection in poultry is an important issue to the poultry industry and health in general. Since the issues of antimicrobial resistance are still evolving, the need to find efficient alternatives to antibiotics has become the focus of higher significance as well. Prebiotics have become promising functional feed supplements that could be used to enhance the gut health and decrease pathogen colonization in poultry. Fructo-oligosaccharides, mannan-oligosaccharides, inulin, and β -glucans possess beneficial effects which occur in a variety of ways. They are modulation of intestinal microbiota, stimulation of the beneficial bacterial population, the production of short-chain fatty acids, mucosal immune enhancement, and pathogen adhesion inhibition in the intestinal epithelial cells. These processes make prebiotics play a role in less colonization and fecal shedding of *Salmonella* in hens in the layer. Prebiotics can also be used to improve poultry performance by improving nutrient digestibility, feed conversion and egg production besides increasing disease resistance. The fact that they can enhance immune response and stabilize gut microbiota also helps them to be more resilient to environmental stress and infectious diseases. Prebiotics are a viable and long-term solution to enhancing the health of poultry and preventing the transmission of foodborne pathogens. The future study and the refined use of prebiotics in the nutrition of poultry will be important in aiding safe and efficient poultry production systems.

DECLARATIONS

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REVIEW ARTICLE

Fermented Chinese Herbal Medicines Improve Intestinal Health in Livestock and Poultry via Microbial Transformation of Bioactive Compounds and Anti-Inflammatory Mechanisms

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Abstract

Intestinal inflammation, underpinned by gut dysbiosis, epithelial barrier dysfunction and dysregulated TLR4/NF- κ B-mediated innate immunity, represents a major constraint on livestock production efficiency and animal welfare. Traditional Chinese herbal medicines (TCHMs) possess multi-component, multi-target anti-inflammatory profiles and favourable safety margins; however, their therapeutic efficacy is limited by poor oral bioavailability, extensive first-pass metabolism and substantial pharmacokinetic variability. Microbial fermentation can remodel herbal constituents through polysaccharide depolymerisation, flavonoid glycoside hydrolysis, phenolic acid liberation and alkaloid/tannin modification, potentially yielding metabolites with improved bioaccessibility and intestinal anti-inflammatory activity. This narrative review synthesises current evidence on fermentation-mediated phytochemical transformation of Chinese medicinal herbs and the intestinal health effects of the resulting preparations in production animals. Fermentation microorganisms, metabolic profiles and condition-dependent phytochemical transformations are critically examined and integrated with tissue-level protective mechanisms encompassing gut microbiota remodelling, short-chain fatty acid metabolism, epithelial tight-junction reinforcement, suppression of aberrant TLR4/MyD88/NF- κ B signalling and pathological NLRP3 inflammasome activation, as well as Nrf2-dependent antioxidant defence. Evidence obtained directly from fermented herbal preparations in production animals is explicitly delineated from that extrapolated from general probiotic or short-chain fatty acid (SCFA) literature. Importantly, much of the mechanistic evidence remains preclinical, and few studies have validated efficacy, optimal dosing or safety under commercial production conditions. Finally, a "strain-ingredient-metabolite-microbiota-host" mechanistic framework is proposed to guide the standardisation of fermentation protocols, quality control and the veterinary application of fermented TCHMs.

Keywords: Antioxidant defence, Chinese medicinal herbs, Gut microbiota, Microbial fermentation, Short-chain fatty acid

INTRODUCTION

Intestinal inflammation encompasses a spectrum of disorders hallmarked by mucosal barrier impairment, gut dysbiosis, and dysregulated innate immunity. In production animals, post-weaning diarrhoea (PWD) in piglets, frequently triggered by enterotoxigenic *Escherichia coli* (ETEC) infection and weaning stress, represents a leading cause of morbidity, growth retardation and economic loss in the swine industry ^[1,2]. Pathologically, ETEC-derived lipopolysaccharides and enterotoxins activate TLR4/MyD88/NF- κ B signalling, driving TNF- α ,

IL-1 β and IL-6 production whilst concurrently disrupting tight junction localisation and increasing epithelial permeability ^[3,4]. These convergent pathologies illustrate that intestinal inflammation emerges not from isolated cytokine dysregulation, but from the integrated collapse of microbiota homeostasis, barrier integrity and inflammatory control.

Traditional Chinese herbal medicines (TCHMs) offer multi-component, multi-target anti-inflammatory potential; however, their bioactive constituents -polysaccharides, flavonoid glycosides, saponins and alkaloids- are constrained



by poor oral bioavailability. High molecular weight and extensive hydrogen bonding limit their bioaccessibility, i.e., release from the herbal matrix during gastrointestinal digestion, whilst limited membrane permeability, extensive first-pass metabolism and microbiota-dependent metabolic variability further restrict the fraction reaching systemic circulation in an active form (i.e., bioavailability), resulting in negligible systemic exposure and highly inconsistent therapeutic responses [5-7]. For instance, oral ginsenosides exhibit absolute bioavailability below 5% [8], whilst most flavonoid glycosides require microbial deglycosylation prior to absorption [9]. Overcoming these bottlenecks of poor absorption, poor targeting and high metabolic variability is essential for realising the intestinal anti-inflammatory efficacy of TCHMs.

Microbial fermentation addresses these constraints through targeted bioconversion [10]. In production animals, fermented herbal preparations have demonstrated direct efficacy: a probiotic-fermented complex of *Radix isatidis*, *Radix scutellariae* and *Radix glycyrrhizae* reduces diarrhoea incidence and improves growth performance in weaned piglets [11]; fermented soybean meal attenuates ETEC K88-induced PWD by modulating inflammatory cytokines and caecal microbiota [12]; and fermented herbal feed additives enrich beneficial *Lactobacillus* whilst improving feed conversion in piglets [13]. Mechanistically, fermentation depolymerises polysaccharides into bioactive oligosaccharides, hydrolyses glycosides into membrane-permeable aglycones, and modifies alkaloids and tannins to attenuate toxicity whilst preserving bioactivity [14]. Murine dextran sulphate sodium (DSS)-colitis models further indicate that fermented *Astragalus* suppresses colonic TNF- α , IL-1 β and IL-6, upregulates IL-10, and restores tight junction protein expression [15,16]. However, such murine findings serve solely as mechanistic explorations; their extrapolation to porcine PWD should be regarded as tentative given species-dependent differences in immune architecture, microbiota composition and disease pathogenesis-specifically, DSS induces chemical epithelial injury and Th1/Th17-mediated transmural inflammation, whereas porcine PWD is primarily driven by ETEC enterotoxins, fimbrial adhesion and weaning-induced barrier collapse [17].

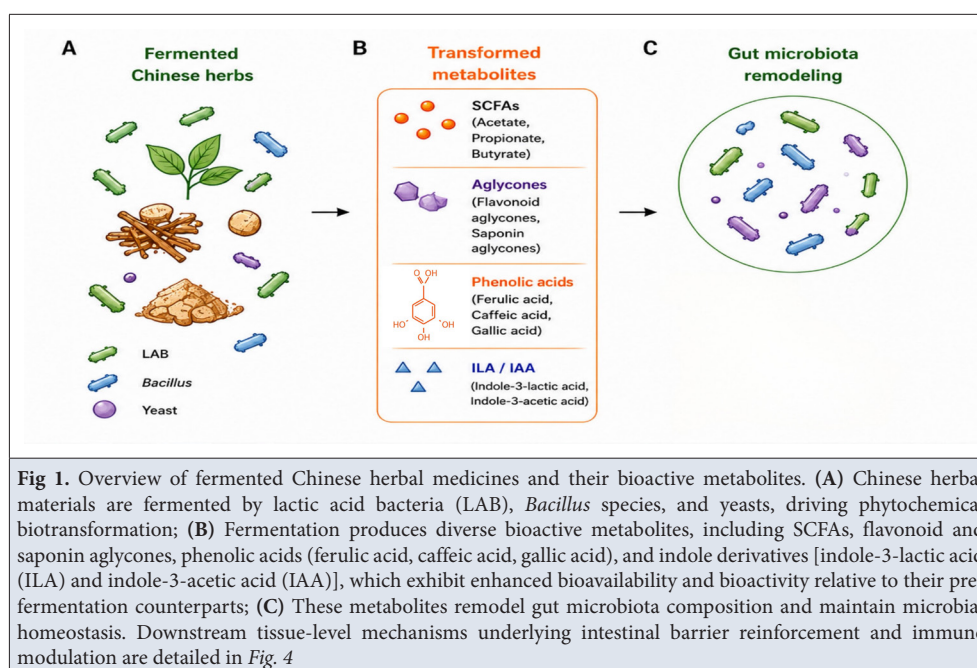
Despite this growing body of work, the specific knowledge gap addressed by this review remains insufficiently served by the existing literature. Prior reviews of fermented TCHMs have been written primarily from phytochemical, pharmacological or human clinical perspectives, cataloguing component-content changes and *in vitro* bioactivities; the few reviews touching on animal production have largely listed growth-promoting effects without critically appraising the strength or origin

of the underlying evidence [18-20]. Three unresolved gaps persist. First, evidence obtained directly from fermented herbal preparations in livestock and poultry has not been systematically separated from mechanisms extrapolated from rodent colitis models, cell-culture systems or the general probiotic and SCFA literature, leaving the actual strength of support for proposed intestinal mechanisms unclear. Second, fermentation variables (microbial strain, substrate, temperature, pH, moisture, oxygen availability and duration) have rarely been linked to the resulting metabolite profiles and intestinal outcomes, which hampers standardisation and reproducibility. Third, safety and quality-consistency concerns specific to veterinary use (including microbial contamination, mycotoxins, toxic metabolites and batch-to-batch variation) remain largely unaddressed.

To address these gaps, the present narrative review makes two distinctive contributions. First, it centres specifically on the production-animal intestinal anti-inflammation dimension, explicitly distinguishing evidence derived directly from Chinese fermented herbal preparations in livestock from mechanisms extrapolated from general probiotic, SCFA or murine colitis literature. Second, it advances a unified “strain-ingredient-metabolite-microbiota-host” mechanistic framework that integrates fermentation biotransformation with gut microbiota remodelling and host tissue-level pathways, providing a conceptual foundation for standardising fermentation protocols and advancing targeted microbial fermentation strategies in veterinary practice. Fig. 1 provides an overview of this process: herbal materials are biotransformed by probiotic microbes into bioavailable metabolites that remodel gut microbiota, with downstream tissue level protective mechanisms presented in Fig. 4. The following sections examine fermentation microorganisms and metabolic profiles, condition-dependent phytochemical transformations, and gut anti-inflammatory mechanisms, before evaluating therapeutic efficacy in livestock production.

LITERATURE SEARCH AND REVIEW METHODOLOGY

This article is a narrative review; no systematic protocol was registered and the PRISMA framework was not applied. Literature was retrieved from PubMed, Web of Science, Scopus and CNKI up to June 2026, using combinations of terms covering fermentation (“fermentation”, “biotransformation”, “probiotic fermentation”), subject (“traditional Chinese medicine”, “Chinese herbal medicine”) and outcomes (“intestinal inflammation”, “gut microbiota”, “intestinal barrier”, “weaned piglet”, “broiler”, “livestock”); reference lists of retrieved articles were



screened for additional sources. Peer-reviewed English or Chinese publications examining microbial fermentation of Chinese medicinal herbs and reporting phytochemical transformations, fermentation conditions, intestinal or anti-inflammatory outcomes, or production performance were included; conference abstracts, dissertations and non-fermentation studies were excluded. No formal risk-of-bias assessment was performed; instead, evidence is appraised qualitatively throughout, distinguishing data obtained directly in livestock and poultry from rodent, cell-culture and general probiotic/SCFA evidence, and demonstrated effects from proposed mechanisms.

FUNDAMENTALS OF MICROBIAL FERMENTATION AND BIOTRANSFORMATION OF HERBAL ACTIVE COMPOUNDS

Microbial Strains and Metabolic Profiles in Chinese Herbal Fermentation

Chinese herbal fermentation typically employs *Lactobacillus*, *Bacillus* species, yeasts, and mixed microbial consortia. *Lactobacillus* produce lactic acid, acetic acid, exopolysaccharides, and glycoside hydrolases, thereby acidifying the fermentation substrate and facilitating glycoside biotransformation [21]. *Bacillus* species demonstrate robust stress tolerance and secrete proteases, amylases, cellulases, and pectinases, rendering them well suited for the fermentation of herbal residues and fibrous feedstocks. Chang et al. [22] developed a synbiotic formulation comprising probiotic-fermented herbal extracts and biogenic selenium nanoparticles, illustrating

the field's transition from monostrain fermentation to polymicrobial synbiotic systems. Strain selection dictates the trajectory of biotransformation. Co-fermentation with *Lactobacillus acidophilus* and *Bacillus subtilis* enhances the bioactivity of honeysuckle-Cassia seed extracts [23]. Liao et al. [24] optimised *B. subtilis*-mediated fermentation of the 'Four Yellow' formula and assessed its anti-*Escherichia coli* efficacy and growth-promoting activity. These findings suggest that future Chinese herbal fermentation should move beyond conventional probiotics towards strain screening based on defined biotransformation capacity and targeted anti-inflammatory functionality.

Effects of Fermentation Conditions on Active Compound Biotransformation

Fermentation conditions critically govern enzyme activity and biotransformation efficiency, thereby determining product quality. Temperature and pH fluctuations directly modulate enzyme secretion profiles and catalytic stability. During solid-state fermentation, temperature gradually increases during spore germination, then rises sharply owing to metabolic heat generation during rapid growth. Concomitantly, pH initially increases due to protein deamination and NH_3 release, followed by a sharp decline as organic acids accumulate and NH_3 is consumed. This pH-temperature coupling governs the temporal dynamics of enzyme production: pectinases and invertases peak during rapid pH decline, whereas xylanase and α -galactosidase synthesis is markedly delayed [25]. pH directly modulates enzyme activity. For instance, recombinant AnGlu04478 exhibits optimal activity at pH 5.0 and retains substantial residual activity following 2 h incubation across pH 3.0-8.0. Its thermal optimum is

40°C, with robust stability between 25 and 50°C. Metal ions differentially modulate β -glucosidase activity: Na^+ , K^+ , Ca^{2+} and Mg^{2+} serve as activators or inhibitors, whereas SDS and EDTA consistently suppress enzyme function. Substrate induction also critically regulates enzyme secretion. For example, ginsenoside supplementation in *Aspergillus niger* fermentation markedly upregulates β -glucosidase gene expression, thereby enhancing rare ginsenoside biotransformation [26].

Moisture, oxygen availability and fermentation duration likewise shape biotransformation outcomes. Moisture governs substrate swelling, nutrient diffusion and enzyme secretion, and its optimum is strain-dependent: filamentous fungi tolerate low water activity, whereas bacteria and yeasts require 40-70% moisture [27,28]. Oxygen regime directs the transformation route-aerobic fungal processes favour oxidative bioconversion, whereas anaerobic lactic fermentation redirects metabolism towards organic acid accumulation [29]. Duration determines whether conversion halts at intermediates or reaches terminal metabolites, and over-incubation may degrade products through non-specific proteolysis [30]. These parameters act interactively, and their optimal windows are strongly strain-dependent (Table 1).

Organic acid accumulation indirectly promotes polysaccharide hydrolysis by lowering environmental pH. Lactic acid, acetic acid, and other fermentation-derived organic acids alter polysaccharide molecular weight and functional properties through pH-dependent mechanisms. Distinct microbial communities generate unique acid profiles, resulting in divergent polysaccharide structural and functional modifications. Concomitantly, fermentation-generated free radicals attack glycosidic bonds and side-chain moieties, inducing polysaccharide cleavage and structural remodeling. Such modifications

may unmask cryptic active sites, thereby enhancing antioxidant and immunomodulatory capacity [35].

Fermentation conditions also contribute substantially to poor inter-study reproducibility [36]. Variations in microbial strains, fermentation duration, or drying protocols applied to identical herbal materials yield divergent polysaccharide molecular weights, organic acid contents, and low-molecular-weight phenolic acid profiles. Fan et al. [37] compared the pro-digestive effects of *Massa Medicata Fermentata* (MMF) before and after processing, integrating metabolomic and microbiome analyses to demonstrate that processing methods concurrently modulate chemical composition and gut microbiota structure. Critically, most published studies report only a subset of relevant parameters; strain identity, moisture content and oxygen regime are the most frequently omitted, which not only accounts for much of the apparent inconsistency between studies but also precludes quantitative correlation of process parameters with metabolite outcomes. Claims that a given condition “enhances” bioactive transformation should accordingly be interpreted with caution unless the full parameter set is disclosed. Consequently, evaluating fermented Chinese herbal medicines requires a continuous evidence chain linking process parameters, metabolite profiles, and biological effects. Accordingly, Fig. 2 delineates the interrelationships among microbial strains, fermentation conditions, and metabolites.

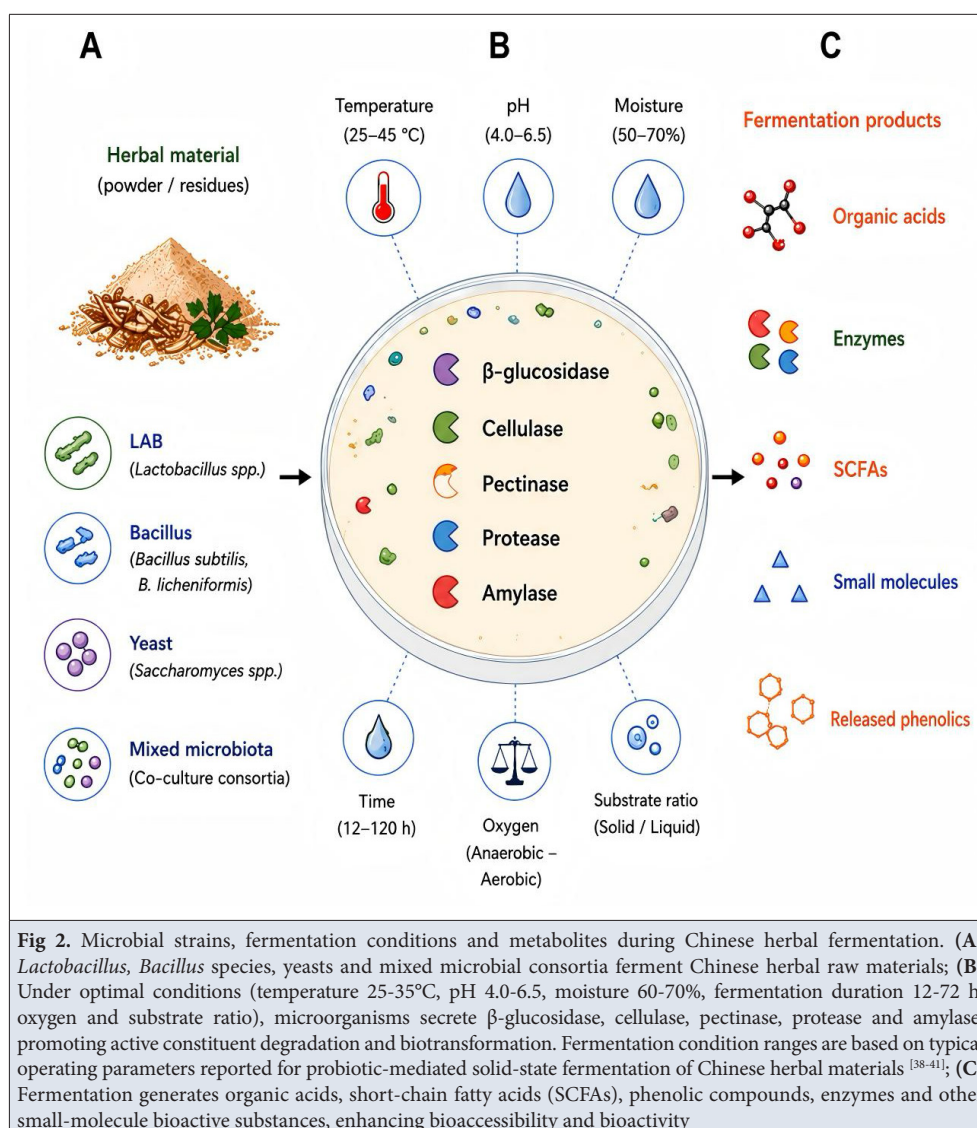
EFFECTS OF FERMENTATION ON BIOACTIVE COMPOUNDS IN CHINESE HERBAL MEDICINES

Biotransformation of Polysaccharides and Glycosides

Polysaccharides constitute a critical material basis for the intestinal anti-inflammatory activity of Chinese

Table 1. Comparison of the principal microbial groups used in herbal fermentation and their condition-dependent biotransformation profiles

Microbial Group	Representative Genera	Key Enzymes	Typical Moisture/O ₂ Regime	Major Biotransformations	Ref
Lactic acid bacteria	<i>Lactiplantibacillus</i> , <i>Lactobacillus</i> , <i>Pediococcus</i>	β -glucosidases, esterases, proteases	Higher moisture (40-70% in SSF); anaerobic/microaerophilic	Glycoside $\rightarrow$ aglycone hydrolysis; organic acid accumulation; phenolic liberation	[31]
<i>Bacillus</i> spp.	<i>B. subtilis</i> , <i>B. licheniformis</i>	Proteases, cellulases, amylases	Moderate-high moisture; aerobic	Cell-wall disruption and release of bound phenolics; glycoside hydrolysis; peptide generation	[32]
Yeasts	<i>Saccharomyces</i> , <i>Candida</i>	β -glucosidases, invertase	Moderate moisture; facultative anaerobic	Glycoside hydrolysis; flavour-active metabolite formation; ethanol/ester production	[31]
Filamentous fungi	<i>Aspergillus</i> , <i>Rhizopus</i> , <i>Monascus</i>	Cellulases, xylanases, pectinases, tannase, β -glucosidases	Low water activity tolerated; strictly aerobic, gas-exchange-dependent	Lignocellulose degradation; extensive glycoside $\rightarrow$ aglycone conversion; alkaloid modification	[26,33]
Mixed/co-culture consortia	LAB + yeast + <i>Bacillus</i> combinations	Complementary enzyme repertoires	Stepwise or simultaneous; regime depends on dominant member	Sequential transformations unattainable by single strains	[34]



herbal medicines. Fermentation deploys polysaccharide hydrolases, cellulases, and pectinases to reduce polysaccharide molecular weight and increase soluble oligosaccharide content, thereby enhancing microbial utilisation. Yang et al.^[38] demonstrated the prebiotic potential of fermented *Astragalus* polysaccharides, which modulate gut microbiota composition *in vitro*. Laoxianghuang polysaccharides also promote IL-10 production through gut microbiota-dependent linoleic acid metabolism^[39]. These findings indicate that polysaccharide-mediated anti-inflammation relies on a sequential cascade from polysaccharide structure through microbial fermentation and metabolite generation to immune modulation. Glycoside biotransformation represents another critical mechanism for fermentation-enhanced bioavailability. Many flavonoids, saponins, and anthraquinones occur naturally as glycosides, which are highly polar and poorly absorbed^[40]. Fermentation-derived β-glucosidases liberate aglycones, a transformation

experimentally demonstrated by direct compositional analyses^[41–44]; by contrast, the subsequent epithelial absorption and inflammatory target engagement of the liberated aglycones remain largely inferred from studies of isolated, non-fermented compounds and have rarely been demonstrated for fermented preparations themselves. Ma et al.^[41] screened flavonoid glycoside-hydrolysing strains for the bioconversion of Shuanghuanglian medicinal residue, enabling its valorisation. This study underscores that targeted screening of biotransformation-capable strains is pivotal for elucidating fermentation-enhanced efficacy.

Biotransformation of Flavonoids, Phenolic Acids, and Alkaloids

Flavonoids and phenolic acids represent the predominant bioactive classes underpinning the anti-inflammatory efficacy of fermented Chinese herbal medicines^[42]. Fermentation releases conjugated phenolic acids, remodels

flavonoid glycoside-to-aglycone ratios, and generates novel phenolic metabolites [43]. Metabolomic analyses have documented marked phenolic remodelling during MMF fermentation, with differential metabolites primarily categorised into lipids, amino acids, phenolic acids, organic acids, flavonoids and alkaloids; structural equation modelling further identified *Bacillus velezensis* and fungal species as key drivers of flavonoid and phenolic acid accumulation [44]. Zhang et al. [45] demonstrated that chebulagic acid ameliorates DSS-induced colitis while restoring gut microbiota homeostasis and attenuating oxidative stress. It should be emphasised that chebulagic acid evidence derives from the DSS colitis model; its direct applicability to porcine ETEC-PWD remains speculative and requires species-specific validation.

Alkaloid biotransformation is highly dependent on both the herbal matrix and the microbial strain employed. Certain alkaloids undergo deglycosylation, demethylation, redox reactions, and conjugation, thereby modulating their polarity, toxicity, and anti-inflammatory potential. Investigations into intestinal absorption and component interactions in Forsythia-Honeysuckle formulae reveal potential absorptive and metabolic interplays among constituents [46]. Consequently, evaluating alkaloid-containing fermented formulations necessitates assessment beyond total alkaloid content to encompass constituent ratios, metabolite profiles, and safety.

Effects of Fermentation on Active Compound Physicochemical Properties

Fermentation modulates the molecular weight, solubility, acid-base properties, stability, and bioaccessibility of herbal bioactive compounds. Polysaccharide degradation enhances solubility and microbial fermentability; glycoside hydrolysis remodels polarity and membrane permeability; organic acid generation acidifies the microenvironment and suppresses pathogenic proliferation. Bai et al. [47] demonstrated that MMF modulates gut microbiota composition in rats with functional dyspepsia; as detailed above, Fan et al. [37] and Zheng et al. [44] concurrently established that MMF fermentation remodels phenolic metabolite profiles and microbial community structure. Fermentation also potentiates the anti-inflammatory activity of ox bile through NLRP3 inflammasome suppression [48]. Collectively, these findings indicate that physicochemical remodelling ultimately translates into microecological and immunological outcomes. Fig. 3 delineates the biotransformation pathways and functional enhancement mechanisms of key bioactive compounds during fermentation. Overall, polysaccharide depolymerisation and glycoside-to-aglycone hydrolysis are supported by direct compositional evidence, whereas most downstream functional attributions remain proposed mechanisms that await targeted experimental validation.

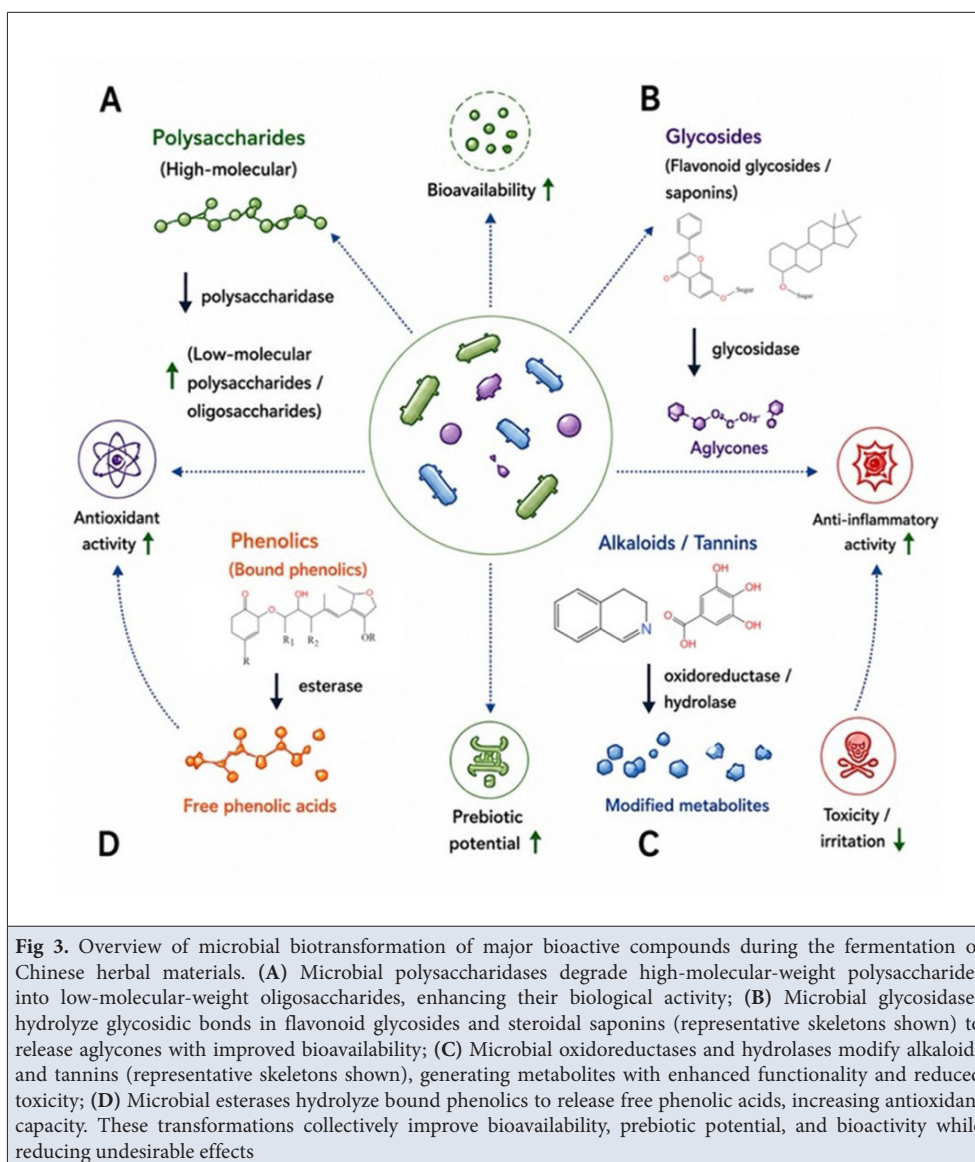
Research Progress on the Enhancement of the Biological Activity of Traditional Chinese Medicinal Herbs Through Fermentation

In terms of biological activity, fermented Chinese herbal medicines primarily exhibit anti-inflammatory, antioxidant, gut microbiota-regulating and gut barrier-enhancing effects. Existing research has shown that fermented herbal preparations can exert protective effects both *in vivo* and *in vitro* under LPS stimulation [49]; fermented Pueraria root extract can alleviate DSS-induced colitis by reducing pro-inflammatory cytokine levels [50] fermented *Astragalus* and its metabolites modulate inflammatory states, improve gut microbiota structure and promote intestinal barrier repair in DSS colitis mice [51]. These murine mechanistic insights, while informative, should not be equated with fermented-herb efficacy in porcine PWD, given the divergent aetiologies of chemical colitis versus bacterial enterotoxigenic diarrhoea. These results indicate that the synergistic effects of fermentation are not only reflected in the transformation and enhancement of bioactive components, but are also further demonstrated by improvements in the host's inflammatory response and intestinal function.

MECHANISMS OF ACTION OF FERMENTED CHINESE HERBS IN INTESTINAL ANTI-INFLAMMATION

Regulation of Gut Microbiota Composition and Metabolic Function

The gut microbiota is a key determinant of host health. A healthy gut microbiota typically exhibits high diversity, high gene richness, and stable metabolic function, all of which are crucial for maintaining intestinal homeostasis [52]. Disruption of the gut microbiota following weaning is a major cause of diarrhoea in piglets. Using 16S rRNA sequencing, Konstantinov et al. [53] and Lu et al. [54] analysed gut microbiota composition and metabolic shifts in piglets during the neonatal and weaning periods. The results indicated that the gut microbiota of neonatal piglets (2-12 days) was dominated by *E. coli* and various *Lactobacillus* species, with *Lactobacillus* predominating during the pre-weaning stage. Lactic acid and acetic acid produced by these bacteria maintain a low gut pH, thereby inhibiting pathogen colonisation. However, following weaning, *Lactobacillus* abundance declined significantly, whilst levels of potential pathogens, represented by *Clostridium* and *Escherichia*, gradually increased. This indicates that weaning significantly alters gut microbiota structure and metabolic activity, potentially increasing the risk of dysbiosis and diarrhoea. Such changes weaken lactic acid- and bacteriocin-mediated antibacterial effects, thereby reducing colonisation resistance against pathogenic bacteria.



In terms of gut microbiota regulation, fermented Chinese herbal medicines selectively enrich beneficial commensals that produce short-chain fatty acids [55]. Fermented bamboo shoots increase the abundance of *Akkermansia*, *Anaerovorax* and *Bacteroides* in the gut of DSS-induced colitis mice, while reducing the disease activity index [56]. Extrapolation of such murine DSS-colitis findings to porcine post-weaning diarrhoea should be regarded as tentative, given species- and model-dependent differences in pathogenesis, immune architecture and microbiota composition [57]. *Lactobacillus helveticus* H3 restores *Lactobacillus* and *Bifidobacterium* populations suppressed by pathogenic *E. coli*, while reducing *Escherichia-Shigella* colonization [58]. Fermentation-derived polyphenolic metabolites also serve as selective growth factors for *Lactobacillus* and *Bifidobacterium*, while inhibiting pathogens such as *Clostridioides difficile* and *E. coli* [59]. *Akkermansia muciniphila*, a mucin-degrading bacterium,

produces acetate, butyrate and propionate; its abundance negatively correlates with disease activity index and pro-inflammatory factor levels [60]. It must be stressed that these microbiota shifts are largely correlational: compositional changes demonstrated by 16S rRNA profiling do not, by themselves, establish a causal contribution to improved intestinal health. Direct mechanistic evidence for fermented herbal interventions -such as faecal microbiota transplantation or germ-free validation- remains scarce; a notable exception is the transplantation-validated tryptophan-metabolite pathway discussed below [61].

At the metabolite level, SCFAs, including acetate, propionate and butyrate, are key anti-inflammatory molecules derived from dietary fibre fermentation by intestinal beneficial bacteria [61]. As the primary energy source for colonic epithelial cells, butyrate exerts anti-inflammatory effects principally through GPR109A activation and histone deacetylase (HDAC)

inhibition, while its induction of Treg differentiation and IL-10 production is largely independent of NLRP3 inflammasome activation [62,63]. Functionally, butyrate strengthens intestinal epithelial barrier integrity by upregulating tight junction proteins (claudin-1, ZO-1 and occludin) via AMPK activation and alleviates cytokine-induced barrier injury by downregulating claudin-2 [64-66]. These protective effects have been validated in weaned piglets, where supplemental butyrate improved intestinal tight junction structure and reduced the incidence of post-weaning diarrhoea [67]. Notably, direct evidence supporting the barrier-protective and anti-inflammatory functions of herbal fermentation-derived butyrate against porcine PWD remains insufficient [68]. Unlike butyrate, acetate and propionate act mainly through GPR43 to trigger physiological NLRP3 inflammasome activation and subsequent IL-18 maturation, which sustains epithelial homeostasis and differs from pathological NLRP3 activation in intestinal inflammation [69]. Additionally, butyrate facilitates inflammation resolution by inducing Foxp3 Treg cells and IL-10 secretion via GPR43/GPR109A signalling and HDAC inhibition [70]. Moreover, propionate relieves intestinal inflammation by suppressing IL-8 and VCAM-1 expression through HDAC inhibition, whereas acetate primarily mediates epithelial protection via GPR43 signalling [62].

Beyond SCFAs, tryptophan metabolites such as indole-3-lactic acid (ILA) and indole-3-acetic acid (IAA) constitute another vital mechanistic axis underlying the beneficial effects of fermented Chinese herbal medicines. Solid-state fermented *Lactocaseibacillus paracasei* cultures enrich ILA and IAA, activate aryl hydrocarbon receptor (AhR) signalling and strengthen the mucosal barrier-effects abolished by antibiotic-induced microbiota depletion yet restored by faecal microbiota transplantation or metabolite supplementation [71,72]. These tryptophan metabolites stimulate the expression of IL-22, mucins and antimicrobial peptides via AhR activation, facilitating intestinal barrier repair and homeostasis [72,73].

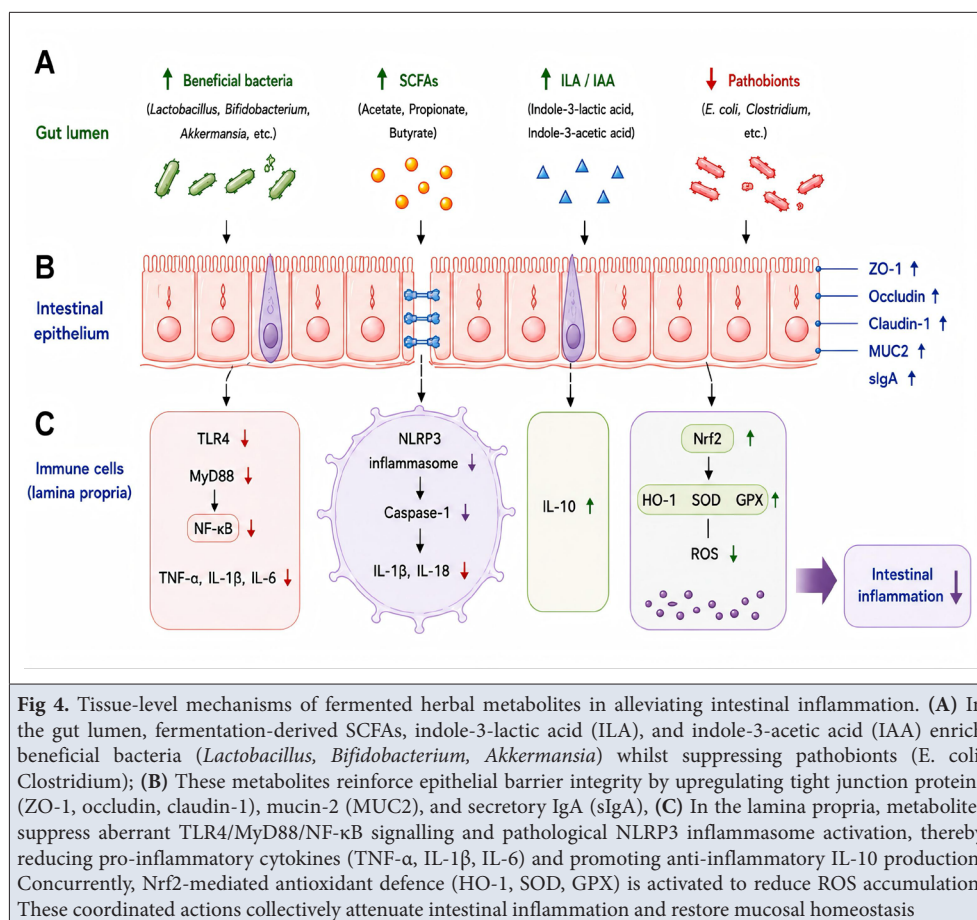
Intestinal Barrier Function Improvement

Intestinal barrier damage is a key step in the progression from microbial dysbiosis to clinical diarrhoea. The intestinal barrier comprises mechanical, chemical, immune and biological barriers, with the mechanical barrier serving as the first line of defence against pathogen and harmful substance invasion [74]. Normal barrier integrity is maintained by the mucus layer, epithelial cells, tight junctions and mucosal immunity. Weaning stress reduces energy intake and epithelial renewal capacity in piglets, leading to decreased villus height and crypt hyperplasia, ultimately compromising intestinal barrier integrity. Liu et al. [75] and López-Colom et al. [76] demonstrated that enterotoxins, lipopolysaccharides and

pro-inflammatory factors produced by enterotoxigenic *E. coli* (ETEC) damage the intestinal mucosal layer and tight junction structures, increasing permeability and ultimately triggering intestinal inflammation.

Fermented Chinese herbal medicines repair the intestinal mucosal barrier through multiple mechanisms, as illustrated in Fig. 4, including upregulating tight junction protein expression, suppressing aberrant immune activation and enhancing mucosal immunity. Tight junction proteins are key molecules maintaining intestinal epithelial physical barrier integrity; fermentation products promote tight junction protein expression by regulating Hippo/YAP and PI3K/Akt/mTOR signalling pathways [77]. For example, in a direct production-animal study, a fermented herbal formulation (FHF) composed of *Radix isatidis*, *Folium isatidis*, *Radix scutellariae*, *Fructus forsythiae* and *Radix glycyrrhizae* -fermented with *Enterococcus faecium* and *Saccharomyces cerevisiae*-upregulated Claudin-1, Occludin and ZO-1 expression at both mRNA and protein levels in the jejunum of weaned piglets, concomitantly reducing serum diamine oxidase (DAO) and TNF- α levels [4]. Fermented plant extract (FPE) significantly reduces intracellular ROS levels in IPEC-J2 intestinal epithelial cells, restoring barrier integrity and cell migration capacity, indicating direct barrier-protective effects of fermented Chinese herbal medicines [78]. Importantly, increased expression of barrier-related proteins alone does not necessarily demonstrate improved intestinal permeability; functional evidence is available for only a subset of preparations, and most studies lack direct permeability measurements such as FITC-dextran flux, transepithelial electrical resistance or serum D-lactate.

Concurrently, as depicted in Fig. 4-C, fermentation-derived metabolites suppress aberrant TLR4/MyD88/NF- κ B signalling and pathological NLRP3 inflammasome activation in lamina propria immune cells, thereby reducing pro-inflammatory cytokines (TNF- α , IL-1 β and IL-6) and promoting anti-inflammatory IL-10 production [4]. Notably, while these signalling pathways are well characterised in general intestinal immunology, their direct inhibition by fermented herbal metabolites has been specifically demonstrated in LPS-stimulated IPEC-J2 cells and weaned piglets for FHF, and in THP-1 macrophages for fermented plant extracts [78]; extrapolation to other fermented preparations or species should remain cautious. Moreover, activation of the Nrf2 antioxidant pathway enhances HO-1, SOD and GPX expression, further reducing ROS accumulation and attenuating intestinal inflammation (Fig. 4) [79]. At the mucosal immunity level, enrichment of beneficial bacteria such as *A. muciniphila* increases mucus layer thickness, protects intestinal mucosal integrity and maintains immune homeostasis [60]. Furthermore, fermentation products enhance intestinal



chemical barrier function by promoting sIgA secretion, and maintain lamina propria immune cell homeostasis by regulating dendritic cell maturation and Treg/Th17 balance, thereby synergistically repairing the intestinal mucosa at both structural and immune barrier levels [69]. Collectively, these coordinated actions at the epithelial and immune levels synergistically restore intestinal mucosal homeostasis (Fig. 4). Nevertheless, it must be acknowledged that strong correlation with weak causality remains a limitation: microbiota changes may be either the cause or consequence of inflammation, and increased SCFA levels may directly protect the barrier or may simply accompany beneficial bacterial enrichment without proven causality in the context of fermented herbal interventions.

Modulating Inflammatory Response and Oxidative Stress

The immune-inflammatory response is also a key factor in post-weaning diarrhoea development in piglets. LPS is recognised by TLR4 and activates MyD88, IKK and NF-κB signalling, leading to TLR4 and NF-κB upregulation and subsequent induction of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6. NF-κB activation not only promotes inflammatory factor release but also affects tight junction protein localisation and epithelial

cell apoptosis [80]. Fermentation-derived metabolites suppress aberrant TLR4/MyD88/NF-κB signalling and pathological NLRP3 inflammasome activation, thereby reducing TNF-α, IL-1β and IL-6 production whilst promoting anti-inflammatory IL-10 secretion and Nrf2-mediated antioxidant responses (Fig. 4). Thus, TLR4/NF-κB signalling modulation alleviates inflammation and serves as a key pathway linking pathogen expansion, exacerbated inflammation and barrier disruption. The JAK-STAT pathway is also involved in mucosal immune regulation following weaning stress in piglets [81].

Fermented Chinese herbal medicines block the intestinal inflammatory cascade by inhibiting pro-inflammatory signalling pathways, activating antioxidant defence systems and restoring the balance between pro- and anti-inflammatory factors [69]. It should be noted that many of these anti-inflammatory mechanisms are derived from general SCFA and polyphenol literature; where effects are demonstrated specifically with fermented herbal preparations, this is indicated explicitly. Regarding pro-inflammatory pathway inhibition, fermentation products act on mitogen-activated protein kinase and nuclear factor κB signalling. Extracts from fermented and aged mountain-cultivated ginseng shoots inhibit phosphorylation of extracellular signal-regulated kinase,

p38 and c-Jun N-terminal kinase in lipopolysaccharide-stimulated RAW 264.7 macrophages, and prevent NF- κ B p65 nuclear translocation, thereby reducing production of pro-inflammatory mediators including TNF- α , IL-1 β , IL-6 and inducible nitric oxide synthase [82]. In THP-1 macrophages, fermented plant extracts inhibit nuclear factor κ B activation via a Toll-like receptor 4-independent pathway, downregulating pro-inflammatory factors including tumour necrosis factor- α and monocyte chemotactic protein, while upregulating anti-inflammatory IL-10 [78]. *Lactobacillus* fermented ginseng sprout extract also inhibits lipopolysaccharide-induced nitric oxide and prostaglandin E2 production, and downregulates cyclooxygenase-2 and pro-inflammatory cytokine expression.

Regarding antioxidant stress, fermentation products activate the nuclear factor E2-related factor 2 (Nrf2)/antioxidant response element (ARE) signalling pathway. Under oxidative stress, Kelch-like ECH-associated protein 1 (Keap1) undergoes conformational changes, enabling Nrf2 dissociation, stabilisation and nuclear translocation. Following heterodimerisation with small Maf proteins, Nrf2 binds to ARE, thereby initiating transcription of downstream antioxidant enzymes including haem oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1, superoxide dismutase and glutathione peroxidase [83]. Direct *in vivo* evidence for fermented herbal preparations in production animals demonstrates that one fermented herbal formulation upregulates Nrf2 and downstream antioxidant enzymes while decreasing MDA concentrations in weaned piglets [84]. Nevertheless, much of the canonical Nrf2/ARE signalling framework described above originates from general oxidative stress research and investigations using non-fermented herbal compounds. Traditional herbal formulations such as Gegen Qinlian Decoction counteract oxidative stress by activating the Nrf2/ARE pathway, with puerarin, berberine and glycyrrhizin as the primary active drivers [85]. A distinction is warranted between direct antioxidant effects -radical scavenging by fermentation-liberated phenolics, demonstrated largely in cell-free or cell-culture assays- and activation of endogenous antioxidant defence via the Nrf2/HO-1 axis, for which *in vivo* production-animal evidence remains limited to a small number of trials [84]. The former cannot be assumed to translate into *in vivo* redox modulation.

Notably, Nrf2 and NF- κ B pathways exhibit reciprocal cross-regulation [86]. The NF- κ B p65 subunit inhibits the Nrf2 pathway by competitively binding the transcriptional co-activator cyclic adenosine monophosphate response element-binding protein (CREB), or by recruiting histone deacetylase 3 (HDAC3) to induce local deacetylation [87]. Conversely, Nrf2 activation also suppresses NF- κ B

transcriptional activity. By simultaneously regulating these two pathways, fermented Chinese herbal medicines reduce pro-inflammatory factor production (TNF- α , IL-1 β , IL-6), while promoting anti-inflammatory interleukin-10 expression, thereby restoring the dynamic balance between pro- and anti-inflammatory factors. Based on microbiota, barrier, inflammatory and oxidative stress mechanisms, Fig. 4 summarises the key mechanisms by which fermentation-derived metabolites alleviate intestinal inflammation.

APPLICATION OF FERMENTED CHINESE HERBAL MEDICINES IN INTESTINAL INFLAMMATION

Applications in Livestock and Poultry Production

Available evidence from production-animal studies indicates that specific fermented herbal preparations exert species- and formulation-dependent effects on intestinal health and production performance, though the breadth of evidence varies considerably across livestock classes (Table 2).

In pigs, fermentation of Chinese herbal mixtures and composite preparations has been associated with improved growth performance and reduced diarrhoea incidence in weaned piglets challenged by weaning stress or enterotoxigenic *E. coli* [93,94]. A recent study demonstrated that a fermented herbal formulation (FHF) containing *Radix isatidis*, *Folium isatidis*, *Radix scutellariae*, *Fructus forsythiae* and *Radix glycyrrhizae* -fermented with *Enterococcus faecium* and *Saccharomyces cerevisiae*- improved average daily gain, reduced diarrhoea rate, enhanced jejunal tight junction protein expression (Claudin-1, Occludin, ZO-1), elevated acetic and butyric acids, and enriched *Limosilactobacillus* and *Lactobacillus* in weaned piglets [85]. These effects appear to be mediated through concurrent modulation of gut microbiota composition, enhancement of tight junction protein expression and attenuation of systemic inflammatory markers such as TNF- α [84,94]. Fermented herbal extracts have also been reported to improve nutrient digestibility during critical growth phases and to reduce environmental ammonia and hydrogen sulphide emissions in commercial pig production. Consistent with these findings, dietary supplementation with fermented Chinese herbal medicine feed additives improved growth performance and intestinal microbiota composition in weaned piglets [95], and probiotics-fermented *Massa Medicata Fermentata* ameliorated weaning stress by restoring intestinal homeostasis [96].

In poultry, *Clostridium perfringens*-induced necrotic enteritis (NE) remains a major enteric disease, with

Table 2. Comparative effects of fermented herbal preparations in production animals

Fermented Preparation	Fermentation Strain/Inoculum	Animal Model (challenge, if any)	Primary Endpoint(s)	Key Outcome(s)	Ref.
Chinese herbal mixture (4 kg/t diet)	Not specified	Weaned piglets	Growth performance, diarrhoea incidence	↑ Final weight, ↑ ADG, ↓ F/G ratio, ↓ diarrhoea incidence	[85]
FHF (Banlangen, Lianqiao, Huangqin, Gancao; 0.6% diet)	Probiotic consortium	Weaned piglets	Growth, serum inflammation, intestinal barrier, caecal microbiota	↑ Final body weight, ↑ ADG, ↓ FCR, ↓ diarrhoea, ↓ serum TNF-α and DAO, ↑ jejunal tight junction proteins, ↑ <i>Limosilactobacillus</i> and <i>Lactobacillus</i>	[4]
Fermented herbal extract (FHE)	Not specified	Weaning and fattening pigs	Nutrient digestibility, faecal gas emissions	Improved digestibility, ↓ ammonia and hydrogen sulphide emissions	[88]
Fermented cottonseed meal (8% diet)	Not specified	Broilers	Weight gain	↑ Weight gain by 3-4%	[89]
Fermented rapeseed meal (50% soybean meal replacement)	Not specified	Broilers	Weight gain, FCR	↑ Weight gain by 6-24%, ↓ FCR by 6-18%	[89]
Thymol/carvacrol (plant-derived additives)	Not specified	NE-challenged broilers	NE lesion scores, mortality	↓ Lesion scores, ↓ mortality	[90,91]
Fermented Chinese herbal additive	Probiotic consortium	Beef cattle	ADG, FCR, faecal pathogens	↑ ADG, ↓ FCR, ↓ faecal pathogen loads	[92]

estimated annual economic impacts exceeding several billion US dollars [95,96]. Fermented feed ingredients -including cottonseed meal and rapeseed meal- have shown potential in ameliorating NE-associated growth depression and intestinal lesions [95]. Additionally, plant-derived bioactive compounds such as thymol and carvacrol, whether delivered directly or via fermentation matrices, appear to reduce NE lesion scores and mortality through antimicrobial and immunomodulatory actions [91]. It should be noted that most poultry-related findings were obtained using fermented feed ingredients, not fermented Chinese herbal medicines as the test material. Caution is therefore required when extrapolating results to other plant species. More directly, dietary supplementation with fermented *Astragalus* powder improved growth performance and health status in broiler chickens [97].

In ruminants, research on fermented Chinese herbal medicines remains comparatively limited. Existing studies suggest that probiotic-fermented herbal additives may improve average daily gain and feed conversion efficiency in weaned lambs, whilst favourably modulating serum biochemistry and the intestinal microbiome [98]. Butyrate-producing fermentation consortia have also been implicated in enhancing ruminal epithelial barrier function and attenuating systemic inflammatory markers. However, the evidence base is insufficient to draw definitive conclusions regarding rumen-gut axis inflammation, and further species-specific trials are warranted.

Collectively, these studies demonstrate that the efficacy of fermented herbal interventions is highly dependent on the specific botanical matrix, fermentation strain, animal model and endpoint examined (Table 2). Rather than

supporting a generalised claim that “fermented herbs are effective,” the data highlight the need for standardised, comparative evaluation of distinct preparations across defined production contexts.

Research Progress in Experimental Models of Enteritis

Experimental colitis models provide controlled conditions for mechanistic studies. The DSS colitis model is commonly used to evaluate epithelial damage, dysbiosis and inflammatory pathways [97]. Fermented *Astragalus* and its metabolites modulate inflammatory status and gut microbiota in DSS colitis mice, whilst repairing the intestinal barrier [51]. Fermented Pueraria root extract reduces pro-inflammatory cytokines and alleviates DSS colitis [50]. Zhang et al. [45] found that chebulagic acid ameliorates oxidative stress, inflammation and gut microbiota dysbiosis in DSS colitis.

Beyond the DSS model, the 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis model represents a well-established chemically-induced model that mimics key features of Crohn's disease, including transmural inflammation, Th1-dominant immune responses and granuloma formation [98]. In TNBS-induced colitis, rectal administration of TNBS in ethanol disrupts the colonic mucosal barrier and haptens colonic proteins, triggering a T-cell-mediated delayed-type hypersensitivity response characterised by elevated interferon-γ (IFN-γ), TNF-α and IL-12 production [99,100]. Fermented Chinese herbal medicines have demonstrated protective efficacy in this model through multiple mechanisms. Lian-Zhi-Fan (LZF) solution, a fermented preparation of Coptidis Rhizome, Gardeniae Fructus and alum, significantly ameliorates

body weight loss, disease activity index (DAI) score and colonic histopathological injury in TNBS-induced colitis rats ^[101]. Metabolomics analysis revealed that LZFM modulates amino acid metabolism (L-alanine, L-valine), primary bile acid biosynthesis (cholic acid, taurocholic acid) and nicotinate/nicotinamide metabolism, thereby restoring energy production and attenuating inflammatory responses ^[102]. Additionally, berberine, a representative alkaloid from *Coptidis Rhizome*, attenuates TNBS-induced colitis by inhibiting inflammatory responses and Th1/Th17 differentiation, concomitantly with modulation of intestinal microbiota composition ^[101]. In a *Lactobacillus brevis* AL0035 fermentation study, oral administration of the probiotic strain counteracted TNBS-induced body weight loss, reduced DAI and histological injury scores, and decreased mRNA and protein expression of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6, IL-12, IFN- γ) whilst enhancing IL-10 expression ^[103,104]. Furthermore, AL0035 restored microbial diversity depleted by TNBS administration, increased the relative abundance of *Lactobacillaceae* and *Lachnospiraceae*, and decreased *Proteobacteria* abundance, indicating that fermentation-derived metabolites alleviate TNBS colitis through coordinated regulation of inflammatory cytokines, tight junction protein expression (ZO-1) and gut microbiota composition ^[105]. These findings collectively demonstrate that fermented Chinese herbal medicines exert intestinal protective effects in the TNBS model via immunomodulation, barrier repair and microbiota restoration, consistent with mechanisms observed in DSS and other colitis models.

The LPS stimulation model, antibiotic-induced injury model, *Salmonella gastroenteritis* model and intestinal ischaemia-reperfusion model are also employed to evaluate intestinal inflammation. Bose et al. utilised LPS stimulation to validate the protective effects of fermented herbal preparations ^[49], and demonstrated in a separate study that herbal preparations counteract neomycin-induced intestinal injury ^[102]. These models illustrate the intestinal protective potential of Chinese herbal medicines and their fermentation products from various perspectives, including pathogen-induced damage, drug-induced injury and barrier ischaemia.

Comparison and Evaluation of the Application Effects of Fermented Herbal Medicines

Evaluation focus for fermented Chinese herbal medicines varies across models. Production animal studies emphasise growth performance, diarrhoea, gut health, microbiota and immune parameters, whereas experimental colitis models focus on histopathology, inflammatory cytokines, oxidative stress and barrier proteins. Piglet studies by Wang et al.^[4] and Chen et al.^[85] align more closely with practical animal production applications. DSS-

induced colitis research presents a more comprehensive mechanistic chain, demonstrating that fermented *Astragalus* simultaneously influences inflammation, microbiota and intestinal barrier integrity ^[51]. TNBS-induced colitis studies provide complementary evidence for Th1-mediated immune modulation and transmural inflammation, with fermented preparations such as LZFM solution and berberine demonstrating efficacy through metabolic pathway regulation and microbiota restoration ^[97]. Chebulagic acid studies, meanwhile, are more helpful in confirming the anti-inflammatory basis of specific *Terminalia chebula* components ^[45].

Medicinal herbs also differ in their fermentation outcomes. MMF research focuses on traditional fermented medicines, digestive function and microbiome regulation ^[106], as detailed in preceding sections. Fermented *Astragalus* research, meanwhile, emphasises polysaccharides, prebiotic effects, immunomodulation and barrier repair ^[51]. Co-fermented Chinese herbal medicine by-product research demonstrates that herbal residues yield antioxidant and anti-inflammatory benefits following appropriate fermentation ^[104]. Therefore, a blanket statement that 'fermented Chinese herbal medicines are effective' is inaccurate; rather, specific herbal materials, strains, metabolites, inflammatory models and evaluation endpoints must be clearly defined. Drawing on evidence from livestock and poultry production, experimental enteritis models and cellular studies, Fig. 5 summarises the distribution of research evidence for the intestinal protective effects of fermented Chinese herbal medicines.

SAFETY, STANDARDISATION AND COMMERCIAL APPLICABILITY

The safety profile of fermented Chinese herbal medicines requires explicit consideration before routine veterinary application. Herbal raw materials and improperly controlled fermentations are vulnerable to microbial contamination and mycotoxins, which remain endemic in feed supply chains and can impair animal health and enter the food chain ^[107,108]. Although fermentation can degrade antinutritional factors and certain mycotoxins ^[15,16], uncontrolled fermentation may equally generate toxic metabolites or biogenic amines, and batch-to-batch variability in herbal starting materials further complicates safety evaluation ^[92,107]. Adverse effects have rarely been reported in the studies reviewed here, but they have also rarely been monitored systematically; future trials should incorporate explicit safety endpoints, including mycotoxin screening, microbial limits and histopathological examination ^[107,108].

Standardisation is a prerequisite for reproducible efficacy. Future studies should report, as a minimum: (i)

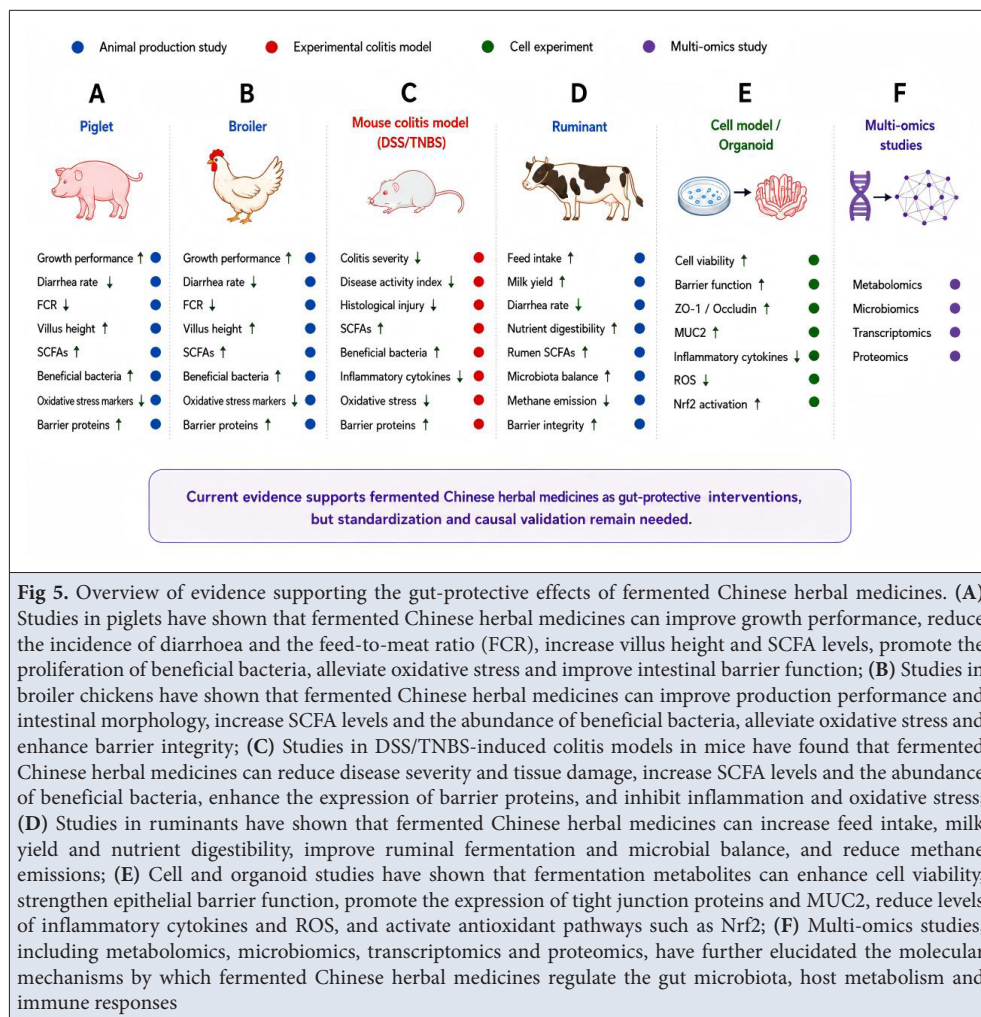


Fig 5. Overview of evidence supporting the gut-protective effects of fermented Chinese herbal medicines. (A) Studies in piglets have shown that fermented Chinese herbal medicines can improve growth performance, reduce the incidence of diarrhoea and the feed-to-meat ratio (FCR), increase villus height and SCFA levels, promote the proliferation of beneficial bacteria, alleviate oxidative stress and improve intestinal barrier function; (B) Studies in broiler chickens have shown that fermented Chinese herbal medicines can improve production performance and intestinal morphology, increase SCFA levels and the abundance of beneficial bacteria, alleviate oxidative stress and enhance barrier integrity; (C) Studies in DSS/TNBS-induced colitis models in mice have found that fermented Chinese herbal medicines can reduce disease severity and tissue damage, increase SCFA levels and the abundance of beneficial bacteria, enhance the expression of barrier proteins, and inhibit inflammation and oxidative stress; (D) Studies in ruminants have shown that fermented Chinese herbal medicines can increase feed intake, milk yield and nutrient digestibility, improve ruminal fermentation and microbial balance, and reduce methane emissions; (E) Cell and organoid studies have shown that fermentation metabolites can enhance cell viability, strengthen epithelial barrier function, promote the expression of tight junction proteins and MUC2, reduce levels of inflammatory cytokines and ROS, and activate antioxidant pathways such as Nrf2; (F) Multi-omics studies, including metabolomics, microbiomics, transcriptomics and proteomics, have further elucidated the molecular mechanisms by which fermented Chinese herbal medicines regulate the gut microbiota, host metabolism and immune responses

unambiguous strain identification, preferably based on whole-genome sequencing with deposition in recognised culture collections; (ii) fully defined fermentation parameters, including substrate composition, inoculum size, temperature, pH, moisture, oxygen regime and duration; (iii) chemical standardisation based on marker compounds or quality markers (Q-markers) linked to the intended bioactivity^[109]; and (iv) untargeted metabolomic fingerprinting to confirm batch consistency and product stability during storage^[32,33]. Only with such controls can dose-response relationships be compared meaningfully across studies and preparations.

From a commercial perspective, the doses used in experimental studies warrant critical appraisal. Inclusion levels reported in piglet trials (for example, 0.6% dietary inclusion of a fermented herbal formulation^[4]) are within ranges considered economically feasible for commercial feed formulation, whereas high-dose extract regimens or short-term intensive administration may be impractical under field conditions^[88]. Cost-effectiveness will depend on substrate availability, fermentation scalability and product stability; solid-state fermentation

of agro-industrial residues offers a low-cost production platform in this regard^[27,30]. Expressing doses in terms of defined marker-compound content rather than raw herb equivalents would further facilitate commercial translation and regulatory evaluation.

CONCLUSION

Existing research supports the intestinal anti-inflammatory potential of fermented Chinese herbal medicines, though evidence quality varies considerably. Early studies demonstrate protective effects against LPS- and neomycin-induced intestinal injury, yet lack in-depth analysis of ingredient biotransformation and microbiota-mediated mechanisms^[49]. Trials conducted in production animals fit practical breeding scenarios, but complex dietary and environmental confounding factors restrict the interpretation of causal relationship^[84]. Although murine DSS colitis models enable relatively complete mechanistic exploration, findings cannot be reliably extrapolated to porcine post-weaning diarrhoea due to interspecies difference^[51].

Studies on flavonoid glycoside hydrolysis and fermentation-induced phenolic remodelling provide representative

evidence for active ingredient biotransformation ^[41,43]. Nevertheless, numerous animal experiments lack quantitative compositional comparisons pre- and post-fermentation, obstructing the identification of truly bioactive compounds.

Mechanistically, most investigations converge on the “microbiota-barrier-inflammation” axis; however, widespread issues of strong correlation without confirmed causality remain unresolved. Alterations in gut microbiota may occur either prior to or secondary to inflammation, and elevated SCFA concentrations could exert direct barrier protection or merely accompany the enrichment of beneficial commensals. Rigorous validation via faecal microbiota transplantation, metabolite supplementation and receptor blockade experiments is required to establish definitive causal links ^[45]. Future high-quality research should adopt multi-omics screening to uncover candidate pathways, followed by targeted functional verification.

Industrial standardisation constitutes a major bottleneck. Large discrepancies in starter strains, substrate proportion, fermentation humidity, temperature, duration and drying protocols hinder cross-study comparison of product composition and biological efficacy ^[24]. It is urgent to build a systematic quality evaluation system covering raw material fingerprints, fermentation termination indicators, quantitative detection of active ingredients and safety assessment.

Future investigations should develop targeted fermentation strategies within the “strain-substrate-metabolite-host” conceptual framework. Researchers can screen functional strains capable of transforming specific herbal components and select tailored fermented products according to enteritis phenotypes and baseline intestinal microbiota profiles ^[101]. Microbial fermentation improves the bioaccessibility of herbal constituents via polysaccharide degradation, glycoside hydrolysis and phenolic compound release. The resulting fermented preparations regulate gut microbiota, reinforce intestinal barrier integrity, inhibit NF- κ B/NLRP3 signalling and mitigate oxidative stress. To fully exploit their application prospects in livestock production, the field needs to address inconsistent fermentation protocols, ambiguous strain selection criteria, insufficient evidence of component transformation, inadequate mechanistic validation and scarce large-scale field trials. Ultimately, a complete and reproducible evidence chain spanning strain, bioactive components, metabolites, gut microbiota, host responses and *in vivo* efficacy should be established. In summary, the most reliable effects of fermented Chinese herbal medicine in production animals are the combinatorial biotransformation of herbal components and the promotion of growth performance, along with mechanisms of barrier protection, anti-inflammation, and

microbiota regulation. Although biologically plausible and increasingly supported by evidence, these effects are still at an early stage and require verification in target animals through standardized and controllable dosage trials, while also clearly addressing issues such as safety, standardization, and commercial feasibility.

DECLARATIONS

Availability of Data and Materials: As this is a review article, no original datasets were generated or analyzed. All information discussed in this review is provided within the manuscript and its cited references.

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REVIEW ARTICLE

Exposure to Microplastics and Nanoplastics through Animal-Derived Foods: A One Health Perspective on the Convergence of Environmental and Health Risks

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Abstract

Microplastics (MPs) and nanoplastics (NPs) pose a significant One Health challenge that transcends the boundaries of the environment, animal and human health, due to their widespread presence in global food systems. This narrative review draws on peer-reviewed literature published up to May 2026 to assess environmental entry points, animal exposure pathways, veterinary health outcomes and impacts on human health. Our analysis confirms that there is strong evidence regarding environmental contamination and entry into aquatic food chains; however, it reveals significant knowledge gaps at the animal-to-human interface, particularly concerning terrestrial farm animal products such as meat, milk and eggs. Toxicological studies indicate that multi-organ pathology can occur at high exposure concentrations; however, inferences regarding real-world dietary exposure are limited by methodological constraints, such as the use of pure polystyrene spheres at doses up to 100.000 times higher than environmental levels and the absence of standardized analytical protocols. Crucially, there is no longitudinal epidemiological evidence for a causal relationship between dietary microplastic ingestion from animal-derived foods and specific clinical outcomes in humans. We propose an integrated One Health surveillance architecture that unites environmental monitoring, animal sentinels, and human biomonitoring. Prioritizing standardized methods, terrestrial product contamination data, and prospective cohort studies is essential for an evidence-based risk assessment that does not undermine the proven nutritional benefits of ADFs.

Keywords: Microplastics, Nanoplastics, One Health, Animal-derived foods, Food safety, Environmental contamination, Human health, Risk assessment

INTRODUCTION

Animal-derived foods (ADFs), including meat, dairy products, fish and eggs, remain important components of human diets. They provide high-quality protein and essential micronutrients, particularly for growing children, pregnant women, and malnourished populations. Low intake of these foods has been linked to stunted growth, anemia and poor bone development. However, ADFs can also act as pathways through which environmental pollutants enter the human food supply. When farm animals, poultry or fish are reared under production conditions where they come into contact with contaminated

feed, water or soil, pollutants can enter animal production systems and ultimately reach the human food chain^[1]. This dual role-nutritional value on the one hand and potential contaminant transfer on the other-makes ADFs a suitable framework for examining microplastic (MP) risks from a One Health perspective. MPs are generally defined as solid plastic particles ranging from 1 µm to 5 mm in size, whereas nanoplastics (NPs) comprise smaller plastic particles in the approximately 1-1000 nm size range^[2]. Although size boundaries may vary among classification frameworks, these ranges are widely used in current environmental and food-safety literature.



The One Health perspective is important because MPs move across the boundaries of the environment, animal health and human health [3]. They can pass from soil and water to plants, animals and, ultimately, human consumers. Addressing this movement requires collaboration between environmental scientists, veterinarians, food safety experts and public health researchers. Within this framework, indirect exposure pathways are just as important as direct ones. Ecosystem degradation, produce contaminated via fertilizers or irrigation, and the long-term accumulation of plastics in the oceans and on agricultural land are among these pathways. These processes may increase future exposure risks for both animals and humans [4,5].

Modern agriculture is heavily reliant on plastic materials. Plastic mulch films, sewage sludge used as fertilizer, and irrigation with wastewater introduce MPs into agricultural soils, where they can persist and accumulate over time. In some regions, concentrations of up to 80,000 particles per kilogram of soil have been reported [6]. These concentrations are of particular significance when assessed in terms of animal exposure pathways. Earthworms can ingest MPs from the soil and are subsequently consumed by poultry; this creates a direct, biologically mediated transfer pathway that bypasses the plant route [7]. Grasses, which are an important component of ruminant diets, may also be affected by MP contamination. Experimental studies show that MPs reduce grass seed germination by 17-30% and shoot biomass by approximately 21% [8]. Furthermore, MPs can be taken up via the roots and transported within the plant through the xylem vessels, potentially becoming part of the forage [9]. Olmo and Holman [10] detected between 36 and 300 MP particles per kilogram in animal feed and up to 7,900 particles per kilogram in surrounding soil. MPs have also been detected in honey; here, contamination may occur via pollen, airborne dust or broader environmental exposure [11]. These findings demonstrate that soil, air, water and plants are not separate compartments; rather, they are interconnected reservoirs that can directly supply animal production systems.

Entry into the environment is only the first stage. The critical question is whether environmental MPs are transferred from farms to animal products such as meat, milk and eggs, and through which mechanisms this transfer occurs. The transfer can take place via various direct and indirect routes. Animals may ingest MPs via contaminated drinking water and feed; free-range poultry, meanwhile, may be indirectly exposed by consuming earthworms that have accumulated soil-borne plastic particles [12]. Secondary contamination may arise from residues carried by the wind from feed packaging bags, irrigation hoses, silage covers, bale netting and nearby landfill sites. The agricultural equivalents of polyethylene

packaging -which can break down into MPs and leach into food [13] are found in the plastic materials used in feed storage and processing. Once inside the animal's body, small particles-particularly those under 150 µm can pass through the intestinal wall, enter the bloodstream and accumulate in organs such as the liver, kidneys and muscles [14]. MPs have been detected in meat, liver, kidneys, raw milk and eggs [15,16]. Consequently, at a mechanistic level, the pathway from agricultural plastic waste to potential human dietary exposure appears plausible. However, this should not be taken as evidence of a public health crisis on a real-world scale. Interpreting the current evidence is complex. The detection of MPs in ADFs is not sufficient to classify the issue as a confirmed One Health risk. Many experimental studies use exposure concentrations that are hundreds or thousands of times higher than typical real-world exposure levels. These studies often rely on pure plastic spheres rather than the worn and irregular particles found in food systems. Analytical inconsistencies -such as the lack of standardized methods, inconsistent reporting units and the risk of procedural contamination-also limit interpretation. There is still no validated and rapid test procedure for MPs in food. Although evidence increasingly suggests that MPs are widespread, can move through food chains and can cause harm in laboratory animals at very high doses, a clear causal link between the consumption of contaminated meat or milk and specific human diseases has not yet been established.

This review addresses this gap by tracing the chain of evidence from environmental contamination through to animal exposure and human dietary intake. It identifies which links are well-established, which remain weak, and which systemic barriers limit the ability to draw stronger conclusions. Given their position at the intersection of environmental, veterinary and human health, the review focuses specifically on food of animal origin as a route of exposure. In doing so, it demonstrates how the widespread use of plastics challenges the One Health perspective, whilst also highlighting where stronger scientific evidence is required before regulatory decisions or risk communication strategies can be developed.

DATA SOURCES AND SEARCH STRATEGY

This narrative review is based on a comprehensive literature search conducted in the Web of Science, Scopus and PubMed databases. The search focused on studies addressing MP contamination in food of animal origin and the associated food chain pathways. No restriction was applied to the starting publication year, and all relevant publications available up to 1 May 2026 were considered for inclusion in the review. Prior to the main

search, a preliminary review was conducted to identify the most commonly used terminology in the field. Search terms combined concepts related to MPs, NPs and polymer identification with descriptors pertaining to ADFs and animal production systems. These included “microplastic”, “nanoplastic”, “polymer”, “polymer type”, “polymer identification”, “polymer composition”, “animal-derived food”, “livestock”, “poultry”, “cattle”, “sheep”, “goat”, “pig”, “meat”, “milk”, “eggs”, “dairy”, “seafood”, “aquaculture”, “fish”, “shellfish”, “food safety”, “animal health”, “One Health” and “trophic transfer”. Boolean operators were used to combine these terms and, where necessary, to broaden or narrow the search.

This review focuses on studies reporting the detection, quantification or characterization of MP in ADFs, including meat, milk, eggs, seafood, dairy products and related matrices. Studies on animal feed were considered where they provided information on the transfer of MP to food products intended for human consumption. Research on aquatic organisms was included where these organisms form part of the human food chain. Studies on environmental matrices, plant systems or toxicological mechanisms were utilized where they contributed to the interpretation of food chain transfer or the broader One Health context. Studies on honey were also considered where they related to ADFs and environmental exposure pathways. Original research articles formed the main basis of the review. Review articles, conference proceedings, books and book chapters were utilized where they provided background information, a conceptual framework or methodological context. Publications in languages other than English were excluded. After duplicate records were removed, the publications were assessed for their relevance to the objectives of the review by examining the title, abstract and, where necessary, the full text. The selected literature was categorized thematically in terms of food type, contamination levels, polymer composition, analytical methods, potential sources of contamination, food safety implications and its relevance to One Health.

ONE HEALTH EVIDENCE-CHAIN FRAMEWORK

A structured evidence-chain framework is needed to connect environmental contamination, animal exposure and human dietary intake in the assessment of MP and NP risks through ADFs. Environmental contamination leads to animal exposure through a wide array of pathways, including direct ingestion, inhalation, trophic transfer, and the movement of biological and environmental vectors. Food-producing animals may be exposed to MPs and NPs through ingestion or inhalation of contaminated environmental matrices, including soil, water, air and

commercial feed ^[4,15]. Particles smaller than 150 µm may cross biological barriers, including the intestinal and pulmonary epithelia, and enter the bloodstream ^[17]. Larger particles are often excreted in feces, whereas smaller internalized particles may travel via the bloodstream and lymphatic system to various organs and tissues ^[17,18].

MPs may be present in ADFs intended for human consumption. They have been detected in edible tissues such as meat, liver and kidneys of livestock and poultry ^[19]. In milk-producing animals, these particles may pass into the mammary glands and enter raw milk. In poultry, they can enter developing eggs before they are laid ^[15,16]. Post-harvest contamination can also occur when food contacts plastic materials during production, processing, handling and packaging. Plastic equipment, worker gear and packaging may release MPs or additives into food, especially in fatty products where leaching is higher ^[20].

Exposure to MPs and NPs may contribute to health hazards through several interconnected pathways. These particles may enter circulation and accumulate within organs such as the liver, kidneys, lungs, brain and reproductive tissues after passing through biological barriers such as the intestinal, placental and blood-brain barriers. At the cellular level, exposure has been associated with endocrine disruption, oxidative stress, inflammation, microbiome imbalance, reproductive toxicity and neurotoxicity ^[19,21]. Additionally, pathogens and antibiotic resistance genes may be carried by MPs, raising the risk of microbial infections ^[21,22].

WELL-ESTABLISHED LINKS IN THE EVIDENCE CHAIN

Environmental contamination and entry into aquatic food chains are the most mature and substantiated parts of the current evidence chain. MPs are widely detected in major environmental matrices, including air, water and soil ^[23,24]. Consequently, the ingestion of MPs by marine and freshwater organisms, including commercially important fish and shellfish, is a recognized pathway for entry into the human food system ^[25]. This makes aquatic food-chain entry one of the strongest links in the current evidence chain. A second well-established link is the role of MPs as microbial scaffolds. A growing body of research shows that MPs may enrich microbial communities carrying antibiotic resistance genes (ARGs), representing an emerging One Health concern ^[26]. Third, the molecular mechanisms associated with MP-induced damage, particularly oxidative stress, chronic inflammation and cellular apoptosis, have been demonstrated in model organisms such as mice and zebrafish ^[17,27]. However, these studies have typically been conducted under controlled

laboratory conditions using virgin polystyrene (PS) beads. Finally, the presence of MP in human biological samples, including blood, placenta, feces and lung tissue, has been documented in analytical studies [21]. These findings support the view that environmental plastic particles can enter the human body. Taken together, these established links indicate that MP can enter the human food system via aquatic animals; however, evidence regarding terrestrial livestock products remains more limited.

PERSISTENT GAPS AND UNDERSTUDIED DOMAINS

Despite some established links, significant gaps remain in our understanding of the interfaces between environmental reservoirs, ADFs and clinical outcomes in humans. Compared with aquatic systems, the presence of MP in terrestrial farm animal tissues -including fresh meat, raw milk and eggs- and in raw ADFs has still not been sufficiently investigated [15,28]. The physiological mechanisms governing the transfer of MP from feed or contaminated soil to edible organs, milk and eggs are also not yet fully understood [27].

Most toxicity data are derived from studies using MP concentrations well above environmentally relevant levels. This limits the validity of these findings for real-world dietary exposure assessments [24,29]. The literature is also skewed; over 96% of studies focus on pure polystyrene spheres. This narrow focus fails to adequately represent other polymer types, such as polyethylene, polyvinyl chloride and polyethylene terephthalate, as well as the fragmented and fibrous morphologies commonly found in real food systems [13].

Another significant gap concerns the effects of food processing methods -including cooking, pasteurization and high-temperature treatments- on the physical properties, chemical composition and toxicological significance of MP in ADFs [4]. Although MP's have been detected in human biological samples, definitive evidence linking dietary MP exposure to specific clinical outcomes in humans is still lacking [24,29]. With regard to ADFs such as meat, milk and eggs, these gaps mean that real-world risk cannot yet be accurately quantified.

SYSTEMIC BARRIERS UNDERPINNING THE INCOMPLETE EVIDENCE CHAIN

There are several systemic barriers contributing to gaps in the chain of evidence. Unless these barriers are addressed, any assessment of MP contamination in ADFs will continue to be incomplete. First, no universally accepted protocol for the extraction and identification of MPs exists. Various analytical instruments, including Fourier-transform

infrared spectroscopy (FT-IR), Raman spectroscopy, and pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS), produce inconsistent results regarding particle size and polymer composition [13,17,30]. Second, current quantification procedures typically overlook the most biologically active particles. Conventional filters and detection thresholds are inadequate for capturing NPs smaller than 1 micrometer or small MPs smaller than 150 micrometers [30,31]. Third, incompatible reporting units, such as particles per kilogram and milligrams per kg, hinder quantitative meta-analyses throughout the food chain [32]. Fourth, research endeavors continue to be divided between environmental ecotoxicologists, who primarily examine ecosystem loads, and biomedical clinical researchers, who focus on cellular reactions. This results in limited multidisciplinary integration in study design [33]. Fifth, at the institutional level, environmental health has only recently been formally incorporated into the Tripartite, now Quadripartite, One Health alliance. Thus, environmental surveillance infrastructure notably lags behind its human and animal clinical equivalents [25,34].

STRATEGIC ADVANTAGES OF THE ONE HEALTH EVIDENCE-CHAIN FRAMEWORK FOR FUTURE RISK ASSESSMENT

Despite the gaps and barriers noted above, the One Health evidence-chain framework itself offers several strategic advantages for future risk assessment. Implementation science approaches, exemplified by the CLEAN procedure (Cut, Lock, Extract, Amend, Network), move beyond descriptive environmental surveys. They offer actionable roadmaps that directly connect scientific evidence to policy initiatives and source control [25].

The framework facilitates the use of sentinel species, such as honeybees, as bioindicators of air and soil MP levels. These can function as early warning systems for human dietary exposure, allowing for pre-emptive intervention before contaminants enter the food chain [3].

The approach advocates for cumulative exposome evaluation through comprehensive dietary and environmental exposure modelling. This integrates combined loads from water, food and inhalation, rather than depending on discrete, compartmentalized studies [33].

It requires standardized data collection across air, water, soil, food and human tissue matrices. This is essential for pinpointing areas where MP pollution and antimicrobial resistance risks intersect [25,31]. By defining MPs as a population-level environmental and occupational hazard, the framework facilitates anticipatory governance. Nations

and regulatory bodies can tackle emerging threats through proactive design and manufacturing modifications instead of solely relying on reactive medical interventions ^[25].

ENVIRONMENTAL ENTRY POINTS INTO ANIMAL FOOD SYSTEMS

Environmental entry points of MPs and NPs into animal food systems are extensive, occurring at every stage from the abiotic environment to post-harvest processing. The main environmental entry points are outlined below.

Primary Environmental Sources of Microplastics

Primary MPs are intentionally manufactured at microscopic sizes for specific commercial and industrial purposes, with common examples including microbeads in personal care products such as exfoliating scrubs and toothpaste, industrial abrasives used in sandblasting and machinery cleaning, and plastic pellets known as nurdles, which serve as the raw material for producing nearly all plastic goods ^[35]. Unlike secondary MPs, which form from the environmental breakdown of larger plastic items via mechanical abrasion, photooxidation, and biological degradation, these primary MPs are deliberately created at a small scale, making them particularly problematic for wastewater treatment systems, especially microbeads, which can flow directly into oceans and waterways ^[28,36]. While many countries have banned rinse-off microbeads in cosmetics, nurdles and industrial abrasives continue to be released during manufacturing, transport, and industrial processes, contributing significantly to global plastic pollution. These sources are relevant to ADFs when released particles enter wastewater, agricultural soils, irrigation systems, feed production environments or aquatic food chains.

Entry Points into Aquatic and Terrestrial Environments

MPs enter aquatic and terrestrial environments through a variety of routes, which are becoming increasingly well documented, although they are still poorly regulated in comparison to traditional pollutants. MPs enter aquatic systems primarily through wastewater treatment plant (WWTP) effluents, surface runoff from contaminated land, and atmospheric deposition ^[9,37]. Although WWTPs can remove up to 99% of particles, large numbers of MPs may still be discharged into rivers and oceans each day ^[37,38]. Similarly, soils are contaminated through intensive farming practices, including the application of plastic mulch films, sewage sludge, organic compost containing plastic residues, and irrigation with contaminated wastewater ^[39,40].

The Role of Wastewater and Sewage Sludge

WWTPs efficiently trap MPs in the solid fraction, concentrating them in sewage sludge (biosolids), which is frequently applied to farmlands as a nutrient-rich

fertilizer ^[37]. Sewage sludge is considered a major driver of soil MP pollution, with some estimates suggesting that up to 430,000 tons of plastic are deposited on European farmlands annually via this route ^[28,41]. Plastic particles in wastewater and sludge have high surface areas that readily adsorb toxic organic and inorganic pollutants, such as heavy metals (e.g., cadmium), pesticides, and polycyclic aromatic hydrocarbons (PAHs) ^[9,42]. Reclaimed wastewater used for irrigation can introduce MPs directly onto crop surfaces and into the soil matrix.

Impact of Plasticulture and Agricultural Films

The term “plasticulture” refers to the systematic use of plastic materials in agricultural production to enhance crop yield, extend growing seasons, improve water use efficiency, and manage pests and weeds. Modern plasticulture is overwhelmingly dependent on three primary categories of plastics: mulch films, greenhouse covers, and protective nets ^[43].

Ramanayaka et al.^[44] reported that the degradation of non-biodegradable plastic mulch films over time leads to an increase in the release of plastic additives and MPs into agricultural soils, with di(2-ethylhexyl) phthalate (DEHP) detected at concentrations as high as 25.2 mg/kg in agricultural soils, indicating potential risks of uptake, translocation, and accumulation in plants. Greenhouse covers, constructed from polyethylene, polycarbonate, or ethylene-vinyl acetate (EVA), create controlled microenvironments that protect crops from extreme weather, but the weathering of these covers due to ultraviolet radiation leads to the gradual release of MP particles into underlying soils. Sorasan et al.^[45] demonstrated that naturally aged greenhouse cover films release more nano- and MP particles than new films under simulated sunlight exposure, with Raman spectroscopy confirming the lower crystallinity of released polyethylene NPs compared to new films. Protective nets, including anti-insect nets, bird nets, and shade nets made from high-density polyethylene or polypropylene, are deployed as physical barriers to exclude pests while reducing the need for chemical pesticides; however, these nets undergo UV degradation and mechanical abrasion during extended field use, generating MPs that are subsequently mobilized by wind, rain and irrigation. Consequently, there is a fundamental trade-off between short-term agricultural productivity and long-term soil health and environmental contamination due to modern plasticulture’s reliance on these three plastic forms ^[46]. This highlights the need for biodegradable alternatives, improved retrieval technologies and updated agricultural guidelines.

Exposure Pathways for Food-Producing Animals

Food-producing animals such as cattle, sheep, goats, pigs and poultry are exposed to environmental MPs via three

main ingestion routes: contaminated water, contaminated feed and biological vectors. Livestock often drink surface water from rivers, ponds and reservoirs contaminated by MPs from agricultural wastewater, wastewater discharge and atmospheric deposition ^[47]. In locations where treated wastewater is used for irrigation, grazing animals may consume MPs via pasture vegetation and standing water, and the presence of MPs in drinking water sources in areas of intensive agriculture has been quantified ^[48].

The second pathway, contaminated feed, occurs through two main mechanisms. Grazing animals often ingest contaminated soil in addition to the plants they consume, particularly soil-bound MPs that have accumulated from plasticulture practices and sewage sludge application ^[40]. Processed fodder and silage may also be contaminated during storage and handling through plastic packaging, silage wraps and bale nets ^[49].

The third important channel is biological vectors, which provide an indirect MP transmission pathway from soil to animal systems. Soil fauna, such as earthworms (*Eisenia fetida* and *Lumbricus terrestris*), ingest MPs from contaminated soils while digging and feeding, resulting in the build-up of particles in their gut tissues ^[12]. Free-range poultry, including chickens, turkeys and guinea fowl, may ingest MPs directly when they consume earthworms as a natural protein source, thereby introducing soil-derived plastic particles into the avian digestive tract. Compelling evidence for this sequence was provided by Huerta Lwanga et al. ^[12], who showed that chickens fed MP-contaminated earthworms accumulated significant loads of MPs in their gastrointestinal tracts, with MP concentrations in feces reaching up to 129.8 particles per gram dry weight. The soil-earthworm-chicken pathway is important because it demonstrates trophic transfer of MPs from soil invertebrates to higher vertebrates, suggesting that terrestrial food chains may be susceptible to MP transfer even in the absence of direct plastic exposure ^[50].

Contributions from Aquaculture Systems

Aquaculture systems serve as significant environmental entry points for MPs and NPs into animal food systems, acting as both a source of contamination and a conduit for trophic transfer. These contributions occur through the degradation of infrastructure, operational practices and the use of contaminated feed materials. Modern aquaculture relies heavily on plastic materials for fish cages, pens, pond linings and tanks; for example, gravity cages use high-density polyethylene pipes for structural frameworks, which can fragment due to UV exposure and mechanical stress ^[18]. Expanded polystyrene floats and buoys are also major contributors to MP pollution, with fragments from aquaculture debris accounting for over 90 per cent of MPs found on nearby shorelines in some regions.

Once introduced, contaminants are amplified and stabilized by specific environmental matrices within the farm environment. MPs serve as microbial scaffolds that may selectively enrich ARGs and antibiotic-resistant bacteria (ARB), while these “microplastomes” can facilitate horizontal gene transfer across different microbial species and environmental matrices ^[22,51]. Additionally, pond sediments function as long-term repositories for antibiotic residues and resistance genes, and these determinants can be resuspended into the water column during storms or routine farm operations, continuously re-exposing cultured animals ^[52].

The transmission from the environment into the food system occurs through several direct and indirect routes. Cultured species such as tilapia ingest MPs and associated resistant pathogens directly from the water and sediment, and research has tracked the movement of these resistomes from pond ecosystems into edible fish guts, indicating a potential risk for human dietary safety ^[14]. Therefore, the integration of these contaminants into the food system requires a source-to-table surveillance approach, as current governance often fails to address how environmental dynamics in aquaculture translate into human dietary exposure risks. The principal production environments, animal groups, ADFs consumed by humans, exposure sources and high-risk MP contamination sources are summarized in *Table 1*.

POTENTIAL HEALTH IMPLICATIONS AND TOXICOLOGICAL UNCERTAINTY

Consequences on Animal Health

Building upon established environmental exposure pathways, the ingestion of MPs and NPs by livestock, poultry and aquatic species may induce systemic physiological stress. In vivo studies in terrestrial and aquatic organisms suggest that exposure can contribute to multi-organ toxic effects ^[4,10,17,19,27]. Owing to their small size, high surface-area-to-volume ratios and chemical persistence, these particulate contaminants may cross physiological barriers, translocate into tissues and contribute to systemic or organ-specific pathological changes ^[4,17,19]. These effects are relevant not only to veterinary health and animal welfare, but also to agricultural biosecurity ^[10,19,27].

Gastrointestinal Damage and Microbiome Dysbiosis

As the primary portal of entry in food-producing animals, the gastrointestinal tract is a key site of MP exposure and potential accumulation. Ingested plastics may exert mechanical stress on the intestinal mucosa, leading to villus damage, enterocyte degradation and disruption of the intestinal vascular barrier ^[57,58]. These alterations may increase intestinal permeability and

Table 1. Environmental exposure pathways and highrisk sources of MP contamination in animal production systems

Production or Exposure Environment	Source Animal Groups	Animal-Derived Foods Consumed by Humans	Common Environmental Feed and Exposure Sources	High-Risk Sources for MP Contamination	References
Terrestrial Livestock Systems	Cow, buffalo, goat, sheep, camel, yak, rabbit, pig	Beef, veal, buffalo meat, goat meat, mutton, lamb, pork, rabbit meat, milk, cheese, yogurt, butter, ghee, cream, curd, liver, kidney, heart, tongue, tripe, bone broth, animal fat, gelatin, collagen	Grasses, forbs, leaves, hay, silage, crop residues, straw, grains, roots, tubers, agricultural by-products, commercial feed, soil particles, and drinking water	Plastic mulch-contaminated soil, sewage sludge-amended soil, wastewater irrigation, contaminated grasses and forbs, feed packaging bags, plastic ropes, irrigation pipes, drinking water, airborne dust, and landfill-derived debris	[8,12,53]
Poultry Production Systems	Chicken, duck, turkey, goose, quail, pigeon	Chicken meat, duck meat, turkey meat, goose meat, quail meat, pigeon meat, eggs, liver, heart, gizzard, poultry fat, poultry broth	Grains, seeds, grasses, small plants, insects, worms, snails, kitchen scraps, commercial feed, soil particles, litter material, and drinking water	Contaminated commercial feed, plastic feed sacks, poultry litter, soil and dust particles, plastic bedding materials, contaminated drinking water, synthetic fibres from farm materials, and degraded plastic equipment	[27,53,54]
Freshwater Aquatic Systems	Freshwater fish, prawn, crab, snail	Freshwater fish, dried fish, smoked fish, fermented fish, fish roe, fish oil, prawn, freshwater crab, edible snails	Phytoplankton, zooplankton, algae, aquatic plants, detritus, insects, larvae, worms, small fish, sediment-associated organic matter, aquaculture feed, and freshwater	Polluted rivers, wastewater discharge, aquaculture ponds, plastic fragments in sediment, contaminated aquaculture feed, fishing nets, plastic ropes, plastic bags, domestic waste, and industrial effluents	[27,53,55]
Marine Aquatic Systems	Marine fish, shrimp, crab, lobster, squid, octopus, cuttlefish, shellfish	Marine fish, canned fish, salted fish, dried fish, fish roe, caviar, fish liver, cod liver oil, shrimp, crab, lobster, squid, octopus, cuttlefish, mussels, oysters, clams, scallops, abalone	Phytoplankton, zooplankton, algae, seaweed, detritus, small fish, crustaceans, mollusks, marine worms, suspended organic particles, sediment organic matter, and seawater	Marine plastic debris, MP-contaminated seawater, fishing nets, ropes, plastic packaging, ship waste, coastal wastewater discharge, contaminated sediments, and filter-feeding exposure in shellfish	[17,18,56]
Coastal and Brackish-Water Systems	Shrimp, prawn, crab, brackish-water fish, oysters	Shrimp, prawn, crab meat, brackish-water fish, oysters, dried shrimp, shrimp paste, fish sauce	Algae, phytoplankton, zooplankton, detritus, organic-rich sediment, small aquatic organisms, aquatic plants, aquaculture feed, brackish water, and coastal sediment	Coastal plastic waste, aquaculture pond liners, plastic nets, plastic ropes, contaminated sediment, wastewater runoff, agricultural runoff, plastic feed bags, and degraded fishing materials	[18,55,56]
Insect and Hive-Based Systems	Bees, crickets, grasshoppers, locusts, silkworms, ants, termites	Honey, royal jelly, bee pollen, propolis, crickets, grasshoppers, locusts, silkworm pupae, ants, termites	Nectar, pollen, plant leaves, grasses, crop plants, tree sap, fruits, seeds, decaying organic matter, fungi, wood material, plant residues, and environmental dust	Airborne MP particles, contaminated pollen, polluted plant surfaces, plastic mulch residues, agricultural dust, plastic greenhouse films, contaminated leaves, and degraded plastic materials near farms	[8,30]

suppress mucus secretion, thereby weakening normal gut barrier function ^[59,60]. MPs may also alter the diversity and taxonomic composition of the gut microbiome ^[60]. Experimental studies in avian and rodent models have reported reductions in beneficial, anti-inflammatory taxa, including *Clostridium* and *Faecalibacterium*, together with increases in opportunistic microorganisms ^[36,58]. In livestock and poultry, the combined effects of mucosal injury and microbial dysbiosis may impair nutrient digestion and assimilation. These disturbances may consequently be reflected in reduced final body weight, lower daily weight gain and poorer feed conversion ratios,

linking gastrointestinal toxicity directly to animal health and production performance ^[10,15].

Oxidative Stress and Programmed Cell Death

Following ingestion and systemic translocation, plastic particles may induce intracellular redox imbalances. MP exposure may trigger the overproduction of reactive oxygen species (ROS) while simultaneously exhausting endogenous antioxidant defenses. This is marked by a significant downregulation of key enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) ^[10]. The resulting oxidative

stress may cause damage to cellular DNA, proteins and lipids. This damage can activate programmed cell death pathways, including apoptosis, necrosis and pyroptosis, across multiple organs such as the liver, kidneys, spleen and heart ^[10,27].

Immunotoxicity and Chronic Inflammation

The host immune system may recognize MPs and NPs as foreign bodies, provoking sustained immunological responses ^[4]. Exposure may drive pathological alterations within primary and secondary lymphoid organs, particularly the thymus and spleen, resulting in structural tissue disruption and inflammatory cell infiltration ^[10,27].

Particle exposure may activate key inflammatory cascades, including the NF- κ B and NLRP3 inflammasome pathways. This activation can trigger an overproduction of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF- α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1 β) ^[10,61]. Over time, chronic inflammation may contribute to immune dysregulation and increased susceptibility to secondary infections ^[61].

Hepatic Toxicity and Metabolic Disruption

As a major metabolic and detoxification organ, the liver may accumulate translocated plastic particles and undergo metabolic disruption ^[4,57]. MPs may disrupt hepatic lipid and energy homeostasis by altering the transcription of critical metabolic genes, such as peroxisome proliferator-activated receptor gamma (PPAR γ), and by contributing to intracellular ATP depletion. This metabolic shift may promote the aberrant accumulation of intracellular lipid droplets, cholestasis and parenchymal damage. The presentation has been compared with the progression of non-alcoholic fatty liver disease (NAFLD) in mammalian models ^[4,10].

Reproductive and Transgenerational Toxicity

In experimental animal models, exposure to MPs and NPs has been associated with reproductive toxicity in both sexes. In male animals, accumulation within testicular tissue has been linked to spermatogenic cell death, testicular inflammation and interstitial fibrosis, together with reductions in sperm count, viability and motility ^[4,31]. In female animals, exposure has been associated with ovarian fibrosis, depletion of primordial and mature follicle pools and alterations in reproductive hormone levels, including reduced estradiol and progesterone concentrations ^[4,62]. Experimental evidence also indicates that NPs may cross the placental barrier during gestation. Maternal exposure has been associated with vertical transfer to fetal tissues and with adverse developmental outcomes, including intrauterine growth restriction, altered offspring sex ratios and metabolic disturbances in subsequent generations ^[4,61,62]. These

findings indicate that reproductive toxicity may have implications not only for individual animal health but also for breeding performance and population sustainability.

Neurological and Cardiovascular Disorders

Beyond metabolic and reproductive systems, plastic particles may exert toxic effects on the neuro-cardio-vascular axis. NPs capable of traversing the blood-brain barrier may accumulate within central nervous system tissues. This localized accumulation may be associated with microglial activation, cerebral and cerebellar hemorrhages, acetylcholinesterase (AChE) inhibition and neurobehavioral changes ^[4,10,32]. Concurrently, the myocardium may be sensitive to MP-induced oxidative stress. Reported cardiovascular outcomes include myocardial dysplasia, bradycardia, cardiomyocyte apoptosis and progressive cardiac fibrosis ^[10,27,61].

Environmental Vectors for Pathogens and Xenobiotics

Beyond their intrinsic particle toxicity, MPs may act as environmental vectors because of their hydrophobic surfaces and high specific surface areas, increasing the risks associated with co-occurring contaminants ^[4,10]. As chemical carriers, MPs can partition and concentrate heavy metals, including cadmium and lead, persistent organic pollutants (POPs), polycyclic aromatic hydrocarbons (PAHs), and veterinary antibiotics such as chlortetracycline from the surrounding environment ^[63,64]. When co-ingested, these contaminants may exacerbate toxic effects. For instance, avian models co-exposed to polyvinyl chloride (PVC) MPs and cadmium show compounded hepatic atrophy and necrotic damage compared with single-contaminant exposure ^[65,66]. Additionally, weathering plastics may leach intrinsic manufacturing additives, including endocrine-disrupting phthalates and bisphenol A (BPA) ^[67].

MPs may also act as biological vectors within the “plastisphere,” providing a stable substrate for the formation of complex microbial biofilms. These biofilms may harbor and transport zoonotic pathogens, including *Vibrio* spp., *Helicobacter pylori* and *Salmonella*, potentially contributing to gastric infections and mucosal damage upon ingestion ^[68,69]. The dense microbial ecology of the plastisphere may also facilitate horizontal gene transfer of ARGs, accelerating the emergence and dissemination of multidrug-resistant bacteria within intensive livestock operations ^[1,10].

Ultimately, multi-organ damage, systemic inflammation and the accumulation of xenobiotics within edible animal tissues may present a dual concern. These effects can reduce agricultural yield and animal welfare, while also contributing to the movement of plastic particles and associated chemical or biological contaminants through

the food chain. These veterinary consequences may contribute to human exposure through the consumption of ADFs containing plastic particles and associated contaminants.

IMPLICATIONS FOR HUMAN HEALTH

Human exposure to MPs and NPs is now widespread, occurring primarily through three main routes: ingestion of contaminated food and water, inhalation of airborne particles and dust, and dermal contact [32]. Under certain conditions, these particulate contaminants may cross biological barriers and enter systemic circulation, where they may contribute to multi-organ effects [17,70,71].

Gastrointestinal Toxicity and Gut Microbiome Dysbiosis

In humans, the gastrointestinal tract represents a major site of exposure to ingested plastic particles and may serve as an important interface between dietary exposure and systemic effects. Particles smaller than 150 µm may penetrate the intestinal mucus layer, while smaller fractions can be internalized through endocytosis, including uptake mediated by specialized microfold cells (M-cells) located within the Peyer's patches of the gut-associated lymphoid tissue [61]. Persistent exposure to MPs within the intestinal lumen may alter both the taxonomic composition and metabolic activity of the gut microbiota [60]. Such alterations may contribute to dysbiosis and compromise intestinal barrier integrity by increasing gut permeability. Unlike the predominantly experimental evidence available for food-producing animals, human evidence is currently based mainly on observational findings. Patients with inflammatory bowel disease have been reported to exhibit significantly higher levels of MPs in fecal samples than healthy controls [72,73]. Although this does not establish causality, it suggests a possible association between intestinal MP burden, microbiome disturbance and chronic intestinal inflammation.

Damage to the Respiratory and Pulmonary Systems

Beyond dietary ingestion, inhalation of airborne MPs, particularly synthetic microfibers shed from textiles and suspended indoor dust, represents another important exposure route for the human respiratory system [74]. Inhaled particles may penetrate deep into the lower airways and provoke a pathological response known as "dust overload". Under these conditions, alveolar macrophages may be unable to clear the foreign material effectively, contributing to chronic airway inflammation, alveolitis and progressive interstitial lung disease [61,75]. In vitro models using human lung epithelial cells indicate that MP exposure may downregulate α1-antitrypsin expression, a biomarker associated with increased COPD risk. These particles may also induce DNA damage, which

could contribute over time to malignant transformation under certain exposure conditions [61,69].

Systemic Translocation and Cardiovascular Risks

Once MPs and NPs cross the intestinal or pulmonary epithelial barriers, they may enter systemic circulation, as suggested by their detection in human blood samples [70,71]. Circulating plastic particles may contribute to systemic inflammatory cascades, cytotoxic effects on blood cells and vascular occlusion [17]. Recent studies have identified MPs and NPs embedded within human thrombi (blood clots) and atheromas (arterial plaques) [76]. The presence of these particles within vascular tissues has been associated with an increased risk of cardiovascular events, including coronary artery disease, myocardial infarction and stroke [75,76].

Cellular Damage, Oxidative Stress, and Genotoxicity

At the cellular level, the large surface area and structural reactivity of NPs may disrupt baseline metabolic homeostasis. Intracellular accumulation of plastic particles may result in the overproduction of reactive oxygen species (ROS) while exhausting endogenous antioxidant pools. This redox imbalance can cause oxidative damage to cellular proteins and membrane lipids [67,77].

Oxidative stress may also contribute to DNA damage, mitochondrial dysfunction and activation of programmed cell death pathways. These processes can promote downstream cellular changes that may contribute to carcinogenesis under sustained or high-exposure conditions [78,79].

Reproductive Toxicity and Transgenerational Effects

Evidence concerning reproductive effects in humans remains more limited than the experimental animal literature. MPs and NPs have been detected in several human reproductive matrices, including placenta, amniotic fluid, breast milk, semen and testicular tissue, demonstrating that reproductive organs and pregnancy-related tissues may be exposed to these particles [80]. Emerging studies have also raised concerns regarding possible effects on male reproductive parameters and the integrity of the blood-testis barrier [81,82]. However, much of the evidence concerning ovarian damage, hormonal disruption and developmental effects of prenatal exposure is still derived from experimental models rather than direct human clinical studies [83-85]. Accordingly, the detection of plastic particles in human reproductive tissues supports biological plausibility, but current evidence remains insufficient to establish causal relationships with specific reproductive or transgenerational outcomes in humans.

Neurotoxicity and Cognitive Effects

Due to their small size, NPs may cross the blood-brain barrier (BBB) and enter the central nervous system.

Accumulation within cerebral tissues may trigger microglial activation. This activation can drive elevated ROS generation and sustained neuroinflammation ^[32,86].

NPs have been implicated in structural neuronal damage, amyloid-beta (A β) oligomer formation and abnormal protein aggregation. These findings raise concerns that chronic low-dose exposure may contribute to the pathogenesis or progression of neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease and multiple sclerosis ^[13,87].

Biological and Chemical Transport of Hazardous Pollutants

MPs may act as environmental vectors for co-occurring hazardous contaminants and may increase their toxicological relevance inside human tissues. Due to their hydrophobic surfaces, MPs can partition and concentrate persistent organic pollutants (POPs), including polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs), from surrounding aquatic and terrestrial environments ^[50].

MPs may also leach intrinsic manufacturing additives, including bisphenol A (BPA), phthalates and brominated flame retardants. These compounds may interfere with endocrine signaling pathways and have been associated with adverse metabolic, reproductive and developmental effects. In addition, MPs provide a stable substrate for the colonization of complex microbial biofilms. This "plastisphere" may host and transport virulent human pathogens, including *Vibrio* spp. and *Helicobacter pylori*, potentially contributing to gastrointestinal infection, mucosal damage and localized inflammation upon ingestion ^[61].

WELL-CALIBRATED RISK INTERPRETATION

Although numerous *in vitro* and animal studies support the biological plausibility of MP and NP toxicity, the extrapolation of these data to real-world human health hazards must be approached with a high degree of caution and balance. To date, no empirical evidence demonstrates a causal link between the ingestion of animal products contaminated with MPs and specific adverse human health outcomes.

The Gap in Environmental Relevance in Laboratory Studies

A significant challenge in current toxicological risk assessment is the wide disparity between laboratory experimental designs and real-world exposure scenarios. Many laboratory toxicity experiments expose animals or cell cultures to MP concentrations that are 2 to 7 orders of magnitude, up to 100,000 times, higher than those

normally detected in pristine or urban environments ^[24]. In addition, the vast majority of experimental research relies on pristine, perfectly spherical, synthetic polystyrene (PS) beads ^[60,88]. In reality, environmental MPs consist of a diverse mixture of irregular fragments, fibers and films composed of different polymers, including polyethylene (PE), polypropylene (PP) and polyethylene terephthalate (PET), which may have undergone prolonged weathering and microbial colonization within the plastisphere ^[89]. Therefore, idealized laboratory models may not fully reflect actual environmental exposure scenarios.

The Vector Effect and Chemical Contribution Dynamics

While MPs can adsorb and accumulate persistent organic pollutants (POPs), heavy metals and manufacturing additives, their contribution to total human chemical exposure through dietary intake appears limited. Worst-case exposure models for the consumption of highly contaminated seafood, such as bivalves and mussels, indicate that chemical transfer from ingested MPs accounts for less than 0.1% of total human dietary intake for toxicants such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), DDT, bisphenol A (BPA), and polybrominated diphenyl ethers (PBDEs) ^[18].

Baseline exposure to harmful chemicals from non-plastic food matrices, drinking water, indoor dust and conventional environmental pathways may therefore represent a larger contribution than the quantities introduced via MPs in both human and animal hosts ^[24].

Bioavailability, Clearance, and the Gastrointestinal Barrier

Not all consumed plastics accumulate within host tissues, as the gastrointestinal tracts of humans and animals function as selective protective barriers. The majority of ingested MPs, particularly those with dimensions exceeding 150 μm , are unable to cross the intact intestinal epithelium and are cleared from the body via fecal excretion ^[10]. For sub-micron plastic fractions, including NPs, that are small enough to breach the intestinal mucosal barrier, the estimated rate of systemic translocation into the bloodstream remains low, falling below 0.3% ^[90].

The Nanoplastic Analytical Blind Spot

NPs are theoretically expected to carry the highest toxicological risk because of their capacity to cross restrictive biological barriers and penetrate intracellular compartments ^[91]. However, this remains a substantial blind spot in conventional risk assessment. Characterizing and quantifying particles below the 1 μm threshold using standard spectroscopic techniques, such as micro-

Fourier Transform Infrared (μ -FTIR) and micro-Raman spectroscopy, remains highly challenging.

Consequently, reliable and validated data on the actual occurrence, steady-state concentration and chemical composition of NPs in complex food matrices such as meat, milk and shellfish are still lacking. Without advanced, high-resolution analytical tools, true systemic NP risk remains difficult to quantify epidemiologically.

Methodological Inconsistencies and Lack of Standardization

The broader field of MP research is constrained by methodological variation, which limits cross-study comparisons and weakens the basis for regulatory conclusions ^[31,53]. Differences in filter pore sizes, chemical digestion agents, including enzymatic and alkaline protocols, and reporting units, such as particles per gram and micrograms per liter, make results difficult to compare across studies ^[92].

Procedural contamination also remains an important concern. Many published studies have lacked rigorous procedural blanks, creating a risk of false positives from background airborne microfibers or laboratory equipment during sample preparation ^[93]. These limitations help explain the current absence of legally enforced and scientifically validated safety thresholds for MPs in human tissues or commercial food supplies.

Absence of Longitudinal Epidemiological Proof

The current body of literature supporting MP toxicity relies almost exclusively on short-term, acute animal exposure models or high-dose *in vitro* cell lines. To date, there are no long-term, prospective cohort, or longitudinal epidemiological studies evaluating the chronic health impacts of low-dose MP exposure on human populations across multiple decades ^[91].

Balanced Nutritional Public Health Perspectives

From a public health and nutritional standpoint, the potential hazards of dietary MP exposure should be weighed against the established nutritional benefits of these foods. Animal-derived whole foods -including fish, lean meat, and dairy products- are critical sources of highly bioavailable proteins, essential micronutrients (e.g., vit. B₁₂, iron, zinc), and bioactive fatty acids vital for human growth, neurological function, and cardiovascular health. Current evidence suggests that the nutritional and socio-economic benefits of consuming these products should be weighed against the low-level and still insufficiently quantified risks associated with dietary exposure to MPs.

ONE HEALTH SURVEILLANCE AND GOVERNANCE SYSTEM

Plastic pollution has interconnected impacts across ecological matrices, wildlife populations, and human public health. To respond effectively to this escalating global crisis, governance structures must transition from theoretical conceptualization to rigorous implementation science-the systematic translation of empirical evidence into realistic, cross-sectoral initiatives, regulatory frameworks, and operational practices.

Integrated Cross-Sectoral Surveillance

An operational One Health paradigm requires continuous and coordinated surveillance across environmental health, animal production, food safety and public health domains. Historically, the environmental domain has received disproportionately low priority within the One Health triad. However, ecosystems serve as the ultimate primary pathway, sink and reservoir for MPs and NPs ^[34]. The formal incorporation of the United Nations Environment Programme (UNEP) into the Quadripartite One Health coalition underscores the need to integrate environmental monitoring into One Health surveillance. Continuous monitoring of aquatic bodies, terrestrial soils, air, municipal wastewater streams and agricultural sludge is vital for establishing responsive early-warning networks ^[34,61].

Domesticated livestock and wild populations act as sensitive environmental sentinels for anthropogenic contamination ^[24]. Long-term monitoring of companion animals and livestock exposed to pastures, commercial feed and agricultural water supplies can provide early-warning indicators of environmental contamination. Tracking these animal cohorts allows researchers to map MP bioaccumulation patterns and systemic toxicities before clinical manifestations become evident in human populations ^[24,91].

Surveillance must also encompass the entirety of the global food supply chain to generate accurate dietary exposure assessments and support advanced human biomonitoring strategies, including the screening of human blood, placental tissues and fecal matter ^[91]. Standardized and validated analytical techniques are needed across public health and veterinary laboratories to ensure cross-domain data comparability, harmonized reporting and data interoperability.

PILLARS OF A ONE HEALTH GOVERNANCE FRAMEWORK

Human medicine, veterinary sciences and environmental management have historically operated separately. This fragmentation creates structural silos that impede

multi-directional data exchange and limit institutional coordination ^[24]. Overcoming these barriers requires the formalization of multi-sectoral coordination mechanisms, such as dedicated inter-ministerial task forces or specialized One Health Secretariats, to explicitly delineate organizational roles, institutionalize accountability and cultivate cross-stakeholder trust ^[94]. Environmental, zoonotic and toxicological surveillance networks must be technologically integrated. Establishing unified, secure data-sharing infrastructure enables real-time epidemiological analysis by linking environmental mass spectrometry data, animal necropsy records and human clinical admissions. This cross-referencing facilitates coordinated and targeted interventions during contamination events or periods of elevated exposure ^[95].

Cultivating a competent One Health workforce requires unified, multidisciplinary educational pathways. Joint training frameworks, such as cross-disciplinary Field Epidemiology Training Programmers (FETPs) that bring together medical clinicians, veterinary pathologists and environmental toxicologists, are essential. These initiatives can reduce communication barriers, align scientific terminology and support collaborative problem-solving from the outset ^[96].

Collaborative governance is heavily constrained by an ongoing deficiency in dedicated financial resources; historical funding streams have remained highly reactive and crisis-driven ^[34,97]. Operationalizing a resilient One Health architecture demands pooled, non-siloed and sustainable budget allocations. Secure funding provides separate state departments with the systemic motivation to collaborate transparently rather than compete for limited, isolated financial resources.

COMPREHENSIVE LEGISLATION AND MITIGATION POLICIES

Ultimately, cross-sectoral surveillance data must be directly leveraged to drive proactive, binding public policy. Robust national and international legislation is required to mitigate MP pollution at its source.

The One Health Mitigation Strategy

Transitioning to an integrated, systems-based framework requires a multi-tiered policy approach. National policies should promote sustainable waste management strategies grounded in circular economy principles, including Refuse, Rethink, Reduce, Reuse and Recycle ^[98].

Legislation should support the gradual phase-out of non-essential single-use plastics, restrict intentional MP additives in commercial goods and encourage the development of marine-degradable bioplastics. Governance frameworks should also prioritize targeted

funding for innovative engineering practices, including microbially mediated plastic bioremediation and advanced wastewater filtration technologies capable of capturing sub-micron particles.

By shifting away from isolated food-safety testing and toward this integrated systems approach, global governance can link environmental source tracking, animal sentinel data and human biomonitoring to support sustainable, cross-sectoral legislative reform ^[91,97].

CONCLUSION

This review shows that MPs and NPs represent an emerging One Health concern at the intersection of environmental contamination, animal production and human dietary exposure. The evidence is strongest for environmental contamination, aquatic food chains and the biological plausibility of particle translocation. However, the evidence remains more limited for terrestrial ADFs, particularly meat, milk and eggs. This gap is important because these products occupy a central place in human nutrition and may also serve as pathways for environmental contaminants.

Current toxicological studies indicate that MPs and NPs can contribute to oxidative stress, inflammation, microbiome disruption, reproductive effects and multi-organ toxicity under experimental conditions. However, many of these studies rely on high exposure concentrations, pristine polymer particles and laboratory models that do not fully represent real-world dietary exposure. At present, there is no longitudinal epidemiological evidence establishing a causal relationship between MP exposure from ADFs and specific human diseases.

Future research should therefore prioritize standardized analytical protocols, comparable reporting units, reliable detection of NPs, and contamination data from terrestrial ADFs. Particular attention should be given to the effects of food processing, post-harvest contamination, animal feed, and the transfer of MPs from environmental matrices into edible tissues and secondary products. Prospective cohort studies and integrated biomonitoring will be necessary to clarify whether dietary exposure from ADFs has measurable long-term health consequences.

A balanced One Health approach is essential. MP and NP contamination should be addressed as a genuine food-safety and environmental health concern, but the current evidence does not justify overstating the risk or undermining the established nutritional value of ADFs. The most appropriate path forward is integrated surveillance linking environmental monitoring, animal sentinels, food-chain testing and human biomonitoring. Such an approach can support evidence-based regulation while avoiding unnecessary alarm.

DECLARATIONS

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RESEARCH ARTICLE

Biochemical and Pharmacological Evaluation of Selected *Artemisia* Species with Antioxidant and Antimicrobial Activities

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Abstract

The genus *Artemisia* is recognized for its diverse secondary metabolites and therapeutic potential; however, evaluating species from the Nakhchivan region is significant for mapping their regional bioactive profiles. This study investigated the chemical characteristics, antioxidant activity, and antimicrobial effects of three *Artemisia* species collected from the Nakhchivan Autonomous Republic to reveal their potential pharmacological benefits. Ethanolic extracts of *Artemisia lerchiana*, *Artemisia absinthium*, and *Artemisia dracunculus* were prepared and total phenolic content (TPC) and total flavonoid content (TFC) were determined. Antimicrobial activity was assessed against *Bacillus cereus*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. The TPC value of the *Artemisia* species ranged from 19.620 to 21.875 mg GAE/g plant, with *A. dracunculus* L. exhibited the highest TFC (6.642±0.076 mg QE/g plant). According to the FRAP assay, *A. absinthium* L. showed the highest antioxidant activity (3.551±0.220 µmol TE/g plant), whereas *A. lerchiana* and *A. dracunculus* exhibited the lowest SC₅₀ values in the DPPH assay. The extracts displayed varying degrees of antimicrobial activity, with *A. dracunculus* being the most effective, particularly against *B. cereus* and *C. albicans*, yielding inhibition zones of 12.05±0.05 mm and 14.48±0.03 mm, respectively. Gram-negative bacteria exhibited higher resistance to the tested extracts than Gram-positive species. These findings suggest that *Artemisia* species from the Nakhchivan region are promising sources of natural antioxidant and antimicrobial agents.

Keywords: *Artemisia*, Phenolic Compounds, Antimicrobial, Antioxidant, Environment

INTRODUCTION

Plants are one of the richest natural components for human health and well-being. Therefore, it is very necessary to cultivate, use and study valuable plants in detail on a scientific basis. In modern times, the comprehensive study of the gene pool of the world, identification and protection of its resources is one of the priority directions. Currently, because of active and intensive use of natural resources, significant reductions in vegetation are observed [1]. If vegetable plants are widely used properly, various diseases spreading among the population will be significantly reduced, some diseases caused by vitamin deficiency will

be eradicated, and several diseases that cannot be cured now will be prevented.

In this regard, the widespread consumption of vegetables and leafy greens plays a crucial role in promoting and maintaining human health. Identifying the sources of biologically active substances for the creation of new therapeutic preparations is crucial. Among naturally occurring medicinal compounds, terpenoids, particularly sesquiterpene lactones and coumarins, have attracted considerable scientific interest in the last 40-50 years [2]. These compounds are commonly found in the plant world, and as natural substances, they have great pharmacological



effects [3]. Species belonging to the *Artemisia* genus (wormwood) are considered important natural reservoirs of these bioactive constituents.

A. dracunculus L. is a small, shrub-shaped, perennial plant [4]. It typically grows on dry slopes, grasslands, and open fields is widely distributed in Japan, Europe, India, North America, Iran, and China [5]. In the literature, *A. dracunculus* L. is known by the common name “Tarragon” [2] and is also known as “Tarkhun”; it is a valuable medicinal plant in Iranian culture [6]. Other *Artemisia* species, including *A. absinthium* L. and *A. lerchiana*, are widely distributed in Azerbaijan, particularly in the Greater and Lesser Caucasus regions, the Samur-Shabran Plain and the Kura-Aras Plain [7]. This genus contains chemical compounds such as monoterpenes, sesquiterpenes, sesquiterpenes, coumarins, sesquiterpene lactones, flavonoids, polyacetylenes and sterols [4].

Among the medicinal products currently in use, preparations obtained from plants are predominant. Thus, in the second half of the 20th century, 40% of the medicines used in clinical practice in the region were herbal medicinal preparations, and several *Artemisia* species (*A. absinthium* L., *A. annua* L., *A. scoparia* Waldst. et Kit., *A. vulgaris* L.) were included in the pharmacopoeia as official medicinal plants because of their bioactive components (phenolic acids, flavonoids, essential oils, etc.) [8].

Based on these considerations, this study aims to provide a comprehensive evaluation of the chemical composition, antioxidant capacity, and antimicrobial potential of three *Artemisia* species collected from the Nakhchivan Autonomous Republic, thereby elucidating their suitability for medicinal and functional applications.

MATERIAL AND METHODS

Ethical Statement

This study did not involve any procedures requiring ethical approval.

Plant Material and Extraction

Wormwood (*Artemisia absinthium* L.), Lerchian wormwood (*Artemisia lerchiana* weber ex stechm.) and tarragon (*Artemisia dracunculus* L.) were collected during June

2024 from different locations within the Nakhchivan Autonomous Republic and prepared for subsequent analyses. *Artemisia absinthium* and *Artemisia lerchiana* were collected from Babek District of Nakhchivan Autonomous Republic, whereas tarragon species were collected from backyards in Nakhchivan city. Information regarding the collection localities, geographic coordinates, and sampling dates of the species is summarized in Table 1.

Voucher specimens are stored in the herbarium collection of the Department of Biology at Nakhchivan State University, Nakhchivan, Azerbaijan.

The sample preparation and extraction procedure is illustrated in Fig. 1. Briefly, the plants were dried at room temperature, ground, and prepared for analysis. The samples were extracted with 50% ethanol-water at a concentration of 0.23 g/mL and agitated at 150 rpm for 24 h in an incubator (Microtest MCI 55) at 25°C. All chemicals and reagents used in the study were purchased from Sigma and Merck companies.

Determination of Total Phenolic Content (TPC)

The Folin-Ciocalteu method was used to determine the TPC values of the samples [9] as gallic acid equivalents (GAE). To summarize the analysis, 20 µL of the sample, 680 µL of pure water, and 400 µL of the 0.2 N Folin Ciocalteu reagent were mixed. After, the mixture was kept in the dark for 5 min and then 400 µL of sodium carbonate solution (10%) was added. Absorbance values versus concentration were measured at 760 nm using a UV-Vis spectrophotometer (Biochrom Libra) after 2 h. The results were reported as milligrams of gallic acid equivalents per gram of dry sample (mg GAE/g plant) [10].



Fig 1. Schematic sample preparation and extraction procedure for *Artemisia* species

Table 1. Information about the locality, coordinates and collections date of selected *Artemisia* species collected from Babek district and Nakhchivan city of Nakhchivan AR, Azerbaijan

<i>Artemisia</i> Samples/Herbarium Number	Collections Locality	Coordinate	Collection Date
<i>Artemisia dracunculus</i> L./1024	Nakhchivan AR, Nakhchivan City	N39.203'517"EO45.426057	02.06.2024
<i>Artemisia absinthium</i> L./ 1025	Nakhchivan AR Babek District, Gahab Village	N38015'15"EO 45030'01"	08.06.2024
<i>Artemisia lerchiana</i> weber ex stechm./1026	Nakhchivan AR Babek District, Gahab Village	N39016'16"EO45030'28"	08.06.2024

Ferric Reducing Antioxidant Power (FRAP)

FRAP assay is based on reducing the Fe^{+3} tripyridyltriazine complex to a blue-colored Fe^{2+} chelate in the presence of antioxidant compounds. The FRAP reagent was prepared by mixing 0.3 M acetate buffer solution (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) (prepared with 40 mM HCl), and 20 mM FeCl_3 solutions in a 10:1:1 ratio, respectively. Briefly, 50 μL of sample and 1.5 mL of FRAP reagent were added to the test tube and incubated in the dark at 37°C for 4 min. The absorbance of reaction mixture was measured at 593 nm using a spectrophotometer. Trolox was used as the standard, and values are given as μmol Trolox equivalent (TE) per gram sample [11].

Scavenging Activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical

The DPPH free radical scavenging activity of the samples was determined according to Degirmenci et al. [12]. Briefly, 750 μL of sample and 750 μL of DPPH (prepared in 100 μM methanol) were mixed and incubated at room temperature in the dark for 50 min. The absorbances were measured at 517 nm at the conclusion of the period. The result was determined by calculating the SC_{50} (mg/mL), which is defined as the concentration of the sample that causes a 50% decrease in the concentration of DPPH radicals.

Determination of Total Flavonoid Content (TFC)

The TFC was determined using the aluminum chloride colorimetric method. A 60 μL of extract was diluted with 540 μL of methanol and subsequently mixed with 600 μL of 2% (w/v) AlCl_3 solution. Following a 30 min of incubation at room temperature, the absorbance values were read at 415 nm. The results are given in mg quercetin equivalent (QAE) per gram sample [13].

Determination of Phenolic Compositions via HPLC-PDA

The phenolic profiles of the plant samples were assessed according to the methodology described by for 25 phenolic compounds [14]. A 10 mL of extract was taken to adjust pH values of 2 and then it was submitted to liquid-liquid extraction using diethyl ether twice, with volumes of 10 mL and 5 mL, respectively. The extraction was carried out at a stirring speed of 200 rpm for 15 min at room temperature. The identical procedure was replicated using ethyl acetate, and the resulting organic phases from each extraction step were pooled into a pre-weighed flask. The solvent in the organic phase was removed using a rotary evaporator at 40°C . The remaining substance was dissolved in 2 mL of methanol, filtered through a 0.45 μm membrane filter, and then injected into the RP-HPLC-PDA system.

The phenolic substances profile was determined using Shimadzu Corporation LC 20AT HPLC device, which

can be analyzed simultaneously in the wavelength range of 200-800 nm, equipped with a photo diode array (PDA) detector (M30A) and a C18 column (250 mm $\times$ 4.6 mm, 5 μm ; GL Sciences). The mobile phase was carried out with a gradient program consisting of acetonitrile, water and acetic acid. The injection volume was 20 μL , the flow rate was maintained at 1.0 mL/min, and the column temperature was set to 30°C . The concentrations of the phenolic compounds were determined using standard calibration curves, and the results were represented in $\mu\text{g/g}$.

Determination of Antimicrobial Activity

The antimicrobial activity of plant extracts was investigated using the agar diffusion method [15]. The antimicrobial effects of plants were tested against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 35218, *Bacillus cereus* RSKK 709, *Staphylococcus epidermidis* ATCC 12228, and *Candida albicans* ATCC 10231. All microbial suspensions were adjusted to 0.5 McFarland standard for bacteria and 2 McFarland standard for yeast and then inoculated onto Mueller-Hinton agar using sterile swabs. Wells were opened on the medium with a sterile mushroom borer (6 mm diameter), and 75 μL of each extract (0.23 g/mL) was added to the wells.

The plates were incubated at 37°C for bacteria and 25°C for yeast for 24 h. After incubation, the inhibition zone diameters (mm) formed around the cavities were measured [16]. In addition, ofloxacin (1 mg/mL) and chlorhexidine (1 mg/mL) were employed as positive controls to evaluate and compare the antimicrobial activities of the tested agents.

Statistical Analysis

The experimental data were analyzed using the SPSS version 20.00 (SPSS Inc., Chicago, IL, USA) program. All measurements were performed in triplicate, and the results are reported as mean $\pm$ standard deviation. Group comparisons were evaluated using One-way analysis of variance (ANOVA) and Duncan's multiple comparison test was subsequently applied to determine significant differences between the mean values ($P \leq 0.05$).

RESULTS

Total Phenolic Content, Total Flavonoid Content and Antioxidant Activity

TPC, TFC and antioxidant activity (via FRAP and DPPH) analyzes were performed to determine the potential of these *Artemisia* species are natural antioxidant sources (Fig. 2). As indicated in Fig 3-A, the TPC of the plant extracts ranged from 19.620 ± 0.389 to 21.875 ± 1.659 mg GAE/g plant. Comparatively, *A. dracuncululus* L. showed statistically lowest TPC value with 19.620 ± 0.389 mg GAE/g plant ($P \leq 0.05$). All extracts exhibited significant

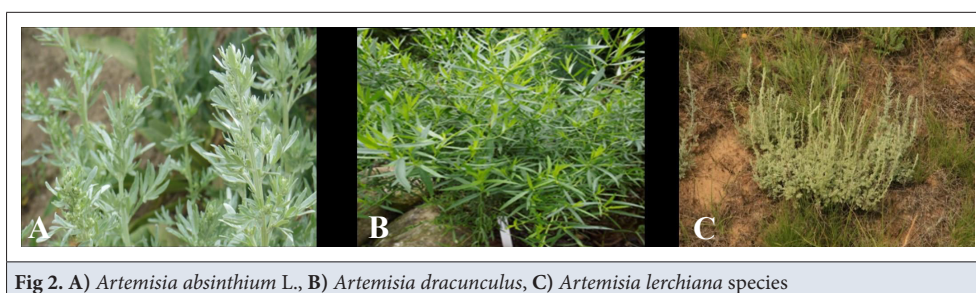


Fig 2. A) *Artemisia absinthium* L., B) *Artemisia dracunculus*, C) *Artemisia lerchiana* species

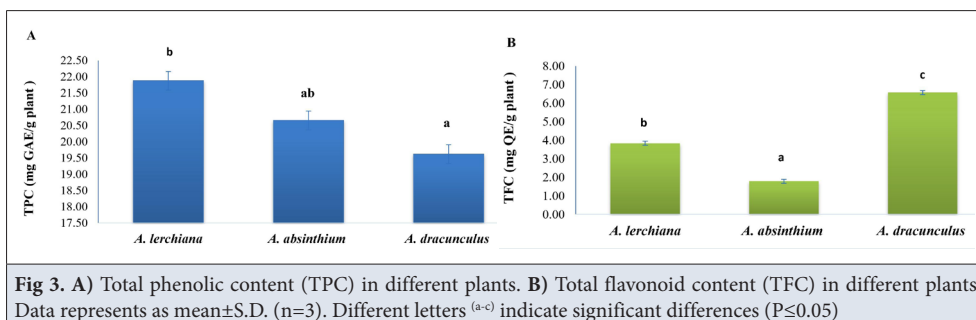


Fig 3. A) Total phenolic content (TPC) in different plants. B) Total flavonoid content (TFC) in different plants. Data represents as mean±S.D. (n=3). Different letters ^(a-c) indicate significant differences ($P \leq 0.05$)

variations in their total flavonoid levels (Fig. 3-B), and the *A. dracunculus* L. plant displayed the highest total flavonoid value of 6.642 ± 0.076 mg QE/g plant.

The FRAP values of the extracts ranged from 1.929 ± 0.13 to 3.551 ± 0.220 $\mu\text{mol TE/g}$. Among the three evaluated species, *A. absinthium* L. extract exhibited the statistically highest ferric reducing capacity, reaching 3.551 ± 0.220 $\mu\text{mol TE/g plant}$ (Fig. 4-A). In the DPPH radical scavenging assay, the SC_{50} values for extracts ranged from 0.373 ± 0.015 to 0.453 ± 0.009 mg/mL (Fig. 4-B). A higher level of activity in scavenging radicals is indicated by lower SC_{50} values. This revealed that *Artemisia lerchiana* weber ex stechn and *Artemisia dracunculus* L. had statistically the highest antioxidant activity versus free radical scavenging activity.

Quantification of Phenolic Compounds in Plant Samples

Phenolic compounds in the *Artemisia* species were analyzed by HPLC-PDA using 25 phenolic standards. The plant extracts investigated were found to contain the following phenolic acids: *p*-hydroxybenzoic acid,

protocatechuic acid, syringic acid, chlorogenic acid, rutin, ellagic acid, ferulic acid, apigenin, chrysin, pinocembrin and vanillic acid, all of which were quantified. The remaining standards, namely epicatechin, caffeic acid, *p*-coumaric acid, catechin hydrate, myricetin, daidzein, luteolin, quercetin, *t*-cinnamic acid, hesperetin, ramnetin, caffeic acid phenethyl ester, galangin and naringenin were not detected in any of the analyzed samples.

The concentrations of the identified phenolic compounds are presented in Table 2. Ferulic acid and chrysin were detected and quantified in all the examined plant samples. Rutin and ellagic acid were detected only in *A. absinthium* L., with concentrations of 2244.667 ± 11.66 and 46.681 ± 1.01 $\mu\text{g/g plants}$, respectively. Syringic acid was found only in the *A. lerchiana* plant at a concentration of 218.909 ± 6.54 $\mu\text{g/g plant}$. Similarly, vanillic acid was exclusively found in the *A. dracunculus* L. plant, with a concentration of 97.153 ± 4.58 $\mu\text{g/g plant}$.

Antimicrobial Activity

The antimicrobial activity of the ethanolic extracts of three *Artemisia* species was evaluated using the agar

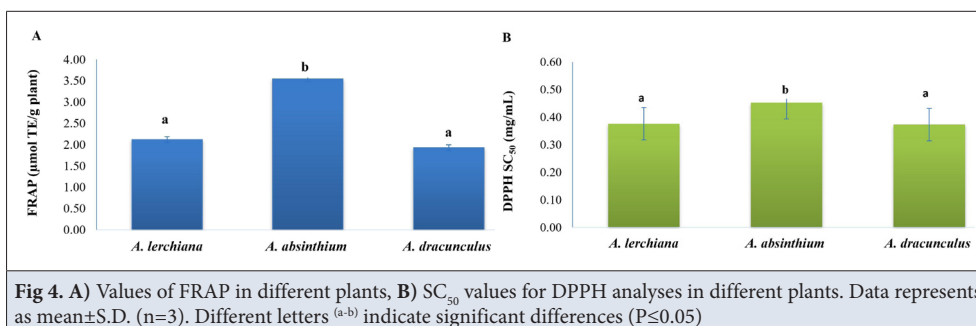


Fig 4. A) Values of FRAP in different plants, B) SC_{50} values for DPPH analyses in different plants. Data represents as mean±S.D. (n=3). Different letters ^(a-b) indicate significant differences ($P \leq 0.05$)

Table 2. The phenolic profiles of the samples using the HPLC-PDA

Content of Phenolic Acids (µg/g plants)	Types of Plant		
	<i>A. lerchiana</i>	<i>A. absinthium</i> L.	<i>A. dracunculus</i> L.
Protocatechuic acid	1.715±0.01	n.d.	200.662±4.22
Chlorogenic acid	56.471±0.21	n.d.	694.017±1.41
<i>p</i> -OH benzoic acid	39.489±1.98	n.d.	54.308±7.09
Syringic acid	218.909±6.54	n.d.	n.d.
Rutin	n.d.	2244.667±11.66	n.d.
Ellagic acid	n.d.	46.681±1.01	n.d.
Ferulic acid	117.656±3.41	113.899±6.32	76.478±3.55
Apigenin	21.029±1.61	n.d.	44.695±2.42
Krisin	22.293±0.95	69.259±1.13	28.703±3.11
Pinocembrin	n.d.	37.066±2.18	367.817±8.76
Vanillic acid	n.d.	n.d.	97.153±4.58

Mean values from three repetitions ± standard deviations. n.d, not detected

Table 3. Antimicrobial activity of the ethanol extracts of *A. lerchiana*, *A. absinthium* L., *A. dracunculus* L.

Samples	Inhibition Zone (mm)				
	<i>B. cereus</i>	<i>S. epidermidis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>C. albicans</i>
<i>A. lerchiana</i>	10.10±0.10 ^b	15.5±0.10 ^c	12.07±0.06 ^a	-	11.10±0.10 ^b
<i>A. absinthium</i> L.	9.07±0.06 ^a	9.57±0.06 ^a	-	-	10.05±0.05 ^a
<i>A. dracunculus</i> L.	12.05±0.05 ^c	14.03±0.06 ^b	-	-	14.48±0.03 ^c
Ofloxacin	33.83±0.29 ^d	36.67±0.29 ^d	35.5±0.50 ^b	36.5±0.50	-
Chlorhexidine					21.67±0.29 ^d

^{a-c} Different letters in the same column indicate statistical difference (P≤0.05)

well diffusion method at a concentration of 0.23 g/mL. The inhibition zone diameters obtained against five test microorganisms, namely *B. cereus*, *S. epidermidis*, *S. aureus*, *E. coli*, and *C. albicans* were provided in Table 3. Among the tested extracts, *A. lerchiana* showed an inhibition area of 10.10±0.10 mm against *B. cereus*; 12.07±0.06 mm against *S. aureus*; 15.5±0.10 mm against *S. epidermidis*, and 11.10±0.10 mm against *C. albicans*. Notably, *A. lerchiana* was the only species that demonstrated activity against *S. aureus* and showed the strongest inhibitory effect against *S. epidermidis*.

Statistical analysis revealed that *A. dracunculus* L. showed the highest activity against *B. cereus* and *C. albicans*, with zone diameters of 12.05±0.05 and 14.48±0.03 mm, respectively. In contrast, none of the extracts showed any inhibitory effect on *E. coli*. *A. dracunculus* L. exhibited the highest antimicrobial activity against *B. cereus* and *C. albicans*, while *A. lerchiana* showed the highest activity against *S. epidermidis* and was the only species effective against *S. aureus*. As expected, the positive controls exhibited substantially greater antimicrobial activity than the plant extracts. Ofloxacin produced inhibition zones of

33.83 mm against *B. cereus*, 35.50 mm against *S. aureus*, and 36.67 mm against *S. epidermidis* while chlorhexidine generated 21.67 mm zone of inhibition against *C. albicans*.

DISCUSSION

The present study demonstrated that the investigated *Artemisia* species possess considerable antioxidant and antimicrobial potential, despite exhibiting relatively similar total phenolic contents. This finding suggests that the biological activities of these species cannot be explained solely by the overall quantity of phenolic compounds. Rather, the observed differences are more likely associated with species phytochemical composition, including the diversity, relative abundance, and possible synergistic interactions of individual phenolics. Therefore, the biological properties of *Artemisia* species appear to depend not only on total phenolic content but also on qualitative differences in their phytochemical profiles [17].

Consistent with this interpretation, all investigated *Artemisia* species exhibited comparable total phenolic contents, indicating that they represent valuable natural

sources of phenolic antioxidants. Previous studies have also demonstrated that *Artemisia* species possess considerable phenolic content, although the reported levels vary substantially among studies. Such variations are most likely attributable to differences in plant species, geographical origin, environmental conditions, harvesting period, plant organ, extraction solvent, and extraction methodology [18]. Therefore, direct comparisons of absolute phenolic values should be interpreted with caution, while the overall evidence consistently supports the rich phenolic composition and antioxidant potential of *Artemisia* species [19]. Accordingly, the relatively high phenolic content observed in the present study provides a biochemical basis for the antioxidant properties of the extracts; however, the subsequent HPLC-PDA analysis clearly indicates that biological activity is more closely associated with qualitative phytochemical composition than with total phenolic concentration alone [20].

A similar trend was observed for total flavonoid content (TFC), with significant differences detected among the investigated *Artemisia* species (Fig. 3-B). These findings indicate species-specific variation in flavonoid accumulation, with *A. dracunculus* exhibiting the greatest flavonoid enrichment among the studied taxa [21]. Previous studies have likewise reported substantial variability in flavonoid content across *Artemisia* species, which has been attributed to differences in species, environmental conditions, plant developmental stage, geographical origin, and extraction procedures. Consequently, the observed differences are consistent with the well established phytochemical diversity of the genus and further support the importance of species selection when evaluating the biological potential of *Artemisia* plants [22]. Although absolute values differ among studies, these findings collectively indicate that flavonoid accumulation is strongly influenced by species characteristics and extraction conditions. Accordingly, the relatively high flavonoid contents observed in the present study further support the antioxidant potential of the investigated *Artemisia* species. Flavonoids are known to donate hydrogen atoms or electrons to reactive oxygen species, stabilize free radicals through resonance, and modulate oxidative stress-related signaling pathways. Therefore, the relatively high flavonoid levels detected, particularly in *A. dracunculus*, may partially explain its pronounced antioxidant performance.

Since phenolic and flavonoid compounds are major contributors to antioxidant activity, the antioxidant capacities of the extracts were subsequently evaluated using both FRAP and DPPH assays [23]. Interestingly, the ranking of the investigated species differed between these assays. While *A. absinthium* exhibited the highest ferric reducing power, *A. lerchiana* and *A. dracunculus*

demonstrated stronger DPPH radical scavenging activities. This discrepancy indicates that antioxidant activity is mediated through multiple mechanisms rather than a single mode of action. For example, the FRAP assay primarily reflects electron transfer capacity, whereas the DPPH assay mainly evaluates hydrogen atom donation and radical scavenging efficiency. Consequently, phytochemicals possessing different redox characteristics may perform differently depending on the assay employed, explaining why species ranking varied between FRAP and DPPH analyses. Similar assay dependent differences have also been reported for other *Artemisia* species [24,25]. Taken together, these findings suggest that antioxidant capacity is influenced not only by the overall amount of phenolic compounds but also by the composition and relative abundance of individual phytochemicals [26]. This interpretation agrees with previous reports demonstrating that antioxidant activity is not always directly proportional to the total concentration of bioactive compounds [27].

To better explain these differences in antioxidant behavior, the phenolic composition of the investigated extracts was characterized by HPLC-PDA analysis. Although the total phenolic contents of the species were relatively similar, their individual phenolic profiles differed markedly. Each species exhibited a distinct distribution of phenolic acids and flavonoids, suggesting that qualitative rather than quantitative differences may account for the observed biological activities [28]. *A. absinthium* was characterized by a remarkably high rutin content, whereas *A. dracunculus* contained the highest concentrations of chlorogenic acid and pinocembrin and was the only species in which vanillic acid was detected [29]. In contrast, syringic acid was the predominant phenolic compound in *A. lerchiana*. Previous investigations have similarly identified chlorogenic acid, ferulic acid, caffeic acid, pinocembrin, and rutin as major phenolic constituents of *Artemisia* species [30]. Consistent with these reports, chlorogenic acid, ferulic acid, rutin, and pinocembrin were also detected in the present study. However, additional compounds including protocatechuic acid, apigenin, chrysin, pinocembrin, and vanillic acid were identified, revealing a more comprehensive phenolic profile. Furthermore, the chlorogenic acid content of *A. dracunculus* (694.017 µg/g plant) was considerably higher than that reported previously [31]. Chlorogenic acid has been widely reported to exhibit strong antioxidant activity through hydrogen atom donation, transition metal chelation, inhibition of lipid peroxidation, and modulation of cellular oxidative stress pathways. Likewise, rutin possesses remarkable radical scavenging capacity owing to its multiple hydroxyl groups and has additionally been associated with anti-inflammatory and antimicrobial activities. Pinocembrin has been shown to disrupt bacterial membrane integrity,

interfere with microbial energy metabolism, and alleviate oxidative damage, whereas syringic acid contributes to antimicrobial activity through membrane destabilization and oxidative stress induction [32]. Therefore, the distinct biological activities observed among the investigated *Artemisia* species are likely attributable to the combined actions and synergistic interactions of these phenolic constituents rather than to the predominance of a single compound. These findings further support the concept that the biological properties of *Artemisia* extracts depend not only on total phenolic concentration but also on the identity and relative abundance of individual phenolic constituents.

The differences observed in phytochemical composition also appeared to influence antimicrobial activity. Plant secondary metabolites, particularly phenolics, flavonoids, tannins, saponins, and steroids, are known to contribute to antibacterial activity [33]. Therefore, species-specific variations in phenolic composition would be expected to produce corresponding differences in antimicrobial efficacy. In agreement with previous reports [34], the investigated *Artemisia* extracts exhibited species-dependent antimicrobial activities. *A. dracunculus* generally displayed the strongest inhibitory effects against most tested microorganisms, whereas *A. lerchiana* showed the highest activity against *S. aureus* and *S. epidermidis*. Moreover, *A. lerchiana* was the only species exhibiting antibacterial activity against *S. aureus*. These findings are consistent with the HPLC-PDA results and suggest that the elevated chlorogenic acid and pinocembrin contents of *A. dracunculus* may have contributed to its superior antimicrobial performance, whereas the antibacterial activity of *A. lerchiana* may be associated with its high syringic acid content. Conversely, none of the investigated extracts inhibited Gram negative *E. coli*. Although this observation is commonly attributed to the outer membrane acting as an effective permeability barrier, additional factors should also be considered. The antimicrobial efficacy of plant extracts depends on the polarity of active constituents, their ability to diffuse through bacterial envelopes, extract composition, and the concentrations of bioactive molecules reaching intracellular targets. Moreover, the lipopolysaccharide-rich outer membrane of Gram negative bacteria restricts the penetration of many hydrophobic phenolic compounds, thereby substantially reducing their antibacterial effectiveness. Consequently, the absence of activity against *E. coli* probably reflects the combined influence of bacterial structural resistance and the physicochemical characteristics of the extracted phytochemicals rather than a complete lack of antibacterial constituents.

Similar antimicrobial activities have previously been reported for *A. absinthium* and *A. lerchiana* [35]. Behbahani

et al. [36] observed inhibitory activities of *A. lerchiana* against *S. aureus*, *B. cereus*, *E. coli*, and *C. albicans*. Likewise, the ethyl acetate extract of *A. absinthium* inhibited *E. coli* and *S. aureus* [37], whereas Ali et al. [38] reported no antibacterial activity against either microorganism, consistent with the present findings. Such discrepancies are most likely attributable to differences in geographical origin, environmental conditions, plant material, harvesting period, and extraction methodology, all of which influence phytochemical composition. It should also be considered that crude plant extracts represent complex phytochemical mixtures in which synergistic or antagonistic interactions among individual metabolites may substantially influence antimicrobial efficacy. Therefore, biological activity cannot always be directly predicted from the concentration of a single compound. Although the inhibition zones observed in the present study were generally smaller than those produced by the reference antimicrobial agents, the detected activities nevertheless demonstrate biologically relevant antimicrobial potential.

Overall, the present findings demonstrate that relatively similar total phenolic contents do not necessarily translate into similar biological activities. Instead, the observed antioxidant and antimicrobial properties appear to be governed primarily by the qualitative composition and relative abundance of individual phenolic constituents identified by HPLC-PDA analysis. The integration of comprehensive phytochemical profiling with multiple biological assays represents one of the major strengths of the present study, enabling a more robust interpretation of the relationship between chemical composition and biological function.

Nevertheless, several limitations should also be acknowledged. Since biological activities were evaluated using crude extracts, the individual contributions of specific phenolic compounds and their synergistic interactions could not be directly determined. Furthermore, only *in vitro* antioxidant and antimicrobial assays were performed, and the molecular mechanisms responsible for the observed biological effects remain to be elucidated.

The present findings demonstrate that the investigated *Artemisia* species constitute promising natural sources of bioactive compounds with notable antioxidant and antimicrobial properties. The observed biological activities, together with the phytochemical profiles, indicate that these species have considerable potential for further pharmacological and functional applications. Future studies should focus on the isolation and characterization of the compounds responsible for these activities, elucidation of their molecular mechanisms of action, assessment of possible synergistic interactions among phytochemicals, and validation of their efficacy and safety in appropriate *in vivo* models. Such investigations

will contribute to a more comprehensive understanding of the therapeutic potential of *Artemisia* species and support their development as natural bioactive ingredients for food, nutraceutical, and pharmaceutical applications.

DECLARATIONS

Availability of Data and Materials: The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Ethical Statement: This study does not require ethical approval.

Conflict of Interest: The author declares that there are no competing interests.

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Author Contributions: SR: investigation, sample harvesting, and writing original draft preparation. GMB: methodology, visualization, writing-original draft preparation, review and editing. OY: methodology, conceptualization and supervision. FT: conceptualization and supervision. EA and ZS sample harvesting, review and editing. All authors have read and agreed to the published version of the manuscript.

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RESEARCH ARTICLE

Comparative Development of Promastigote- and Amastigote-Based Cutaneous Leishmaniasis Models in BALB/c Mice

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Abstract

Cutaneous leishmaniasis (CL) is a significant public health problem caused by parasites of the genus *Leishmania*. Experimental animal models developed for cutaneous leishmaniasis are of great importance for understanding the disease process, the mechanisms of disease development, and evaluating treatment approaches. The aim of our study was to compare lesion development and the clinical course of infection in experimental cutaneous leishmaniasis models created in BALB/c mice using promastigote and amastigote forms of *Leishmania major*. The promastigote form of *L. major* was cultured in NNN and RPMI-1640 media. The amastigote form was previously obtained from the THP-1 cell line and stored by cryopreservation. The parasite forms were applied intradermally to the right foot of the experimental animals. The experimental animals were treated with 10⁵ promastigote/mL, 10⁵ amastigote/mL, and sterile saline. Lesion development was monitored weekly for 12 weeks. Lesion development was assessed by measuring footpad thickness. Infection was successfully induced in both groups. After 12 weeks, the average footpad thickness was 3.32 mm in the amastigote group and 2.33 mm in the promastigote group. Amastigotes were observed in Giemsa-stained touch preparations, and promastigotes were found to grow from the lesions in inoculation onto NNN medium. The findings of this study demonstrate that the experimental cutaneous leishmaniasis model created with the amastigote form is reliable and applicable, allowing faster results in therapeutic and immunological studies.

Keywords: Amastigote, Cutaneous leishmaniasis, Experimental model, *Leishmania major*, Promastigote

INTRODUCTION

Leishmaniasis is an important parasitic infection affecting both humans and animals, caused by protozoa of the genus *Leishmania* and classified as a neglected tropical disease. *Leishmania* has two main morphological forms throughout its life cycle: the flagellated promastigote form, which develops in the gut of female sandflies (*Phlebotomus* spp.), and the non-flagellate amastigote form, which lives intracellularly within macrophages in mammalian hosts [1,2]. Although both forms exhibit different morphological features, they share basic cellular structures such as the nucleus, kinetoplast, and basal body.

Leishmaniasis is transmitted through the bite of infected female sandflies. Three main clinical forms are seen: cutaneous leishmaniasis, visceral leishmaniasis (VL), and mucocutaneous leishmaniasis (MKL). More than 20 *Leishmania* species have been identified that cause disease in humans and animals [3]. Canine leishmaniasis, particularly that caused by *Leishmania infantum*, is prevalent in many endemic regions, including the Mediterranean

Basin. Infected dogs play a central role as reservoir hosts in the epidemiology of the disease [4]. Therefore, leishmaniasis is considered an important zoonosis from a veterinary public health perspective.

Türkiye is one of the endemic countries for leishmaniasis. The vast majority of reported CL and VL cases are caused by *L. tropica* and *L. infantum* [5]. Factors such as climate change, inadequate vector control, increased migration, and insecticide resistance have been reported to affect the epidemiology of the disease [5].

The experimental animal models developed are critical for understanding disease pathogenesis, host-parasite relationships, host immune responses, and the development of new treatment strategies. Mouse models are preferred because of their low cost, defined genetic structures, and the ability to macroscopically assess the infection process. BALB/c mice are widely used in cutaneous leishmaniasis studies because of their high susceptibility to *L. major* infection and their ability to develop significant cutaneous lesions [6].



In experimental cutaneous leishmaniasis models, the promastigote form is the most commonly used because it mimics vector-borne transmission. Studies on experimental cutaneous leishmaniasis induced using the amastigote form also exist. However, the clinical course of infections initiated by promastigote and amastigote forms under the same experimental conditions remains insufficiently characterized.

Accordingly, in this study, experimental cutaneous leishmaniasis models were established in BALB/c mice using *L. major* promastigotes and amastigotes, and the clinical progression of infection was evaluated comparatively. We hypothesized that infection with the amastigote form of *L. major* would result in earlier lesion onset and more rapid disease progression compared to the promastigote form in the BALB/c mouse model.

MATERIAL AND METHODS

Ethical Statement

This study was approved by the Manisa Celal Bayar University Animal Experiments Local Ethics Committee (Approval no: 319, Date: 15/04/2025).

Cultivation of *Leishmania major* Promastigotes

In our study, we used the *L. major* isolate coded MHOM/TR/2013/CBUALA17, which was isolated from a patient living in Türkiye with no history of travel abroad, subsequently cryopreserved after species identification, and stored in the Parasite Bank of Manisa Celal Bayar University, Faculty of Medicine. The isolate was removed from the liquid nitrogen tank and thawed in a 37°C water bath for approximately two min. Before use, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% gentamicin were added to RPMI-1640 medium. Five mL of RPMI-1640 medium was added to a sterile Falcon tube. Then, the entire suspension from the cryo tube after thawing was transferred into the Falcon tube. The Falcon tube was centrifuged at 1200 rpm for 5 min. After centrifugation, the supernatant was discarded, and the remaining pellets were transferred to NNN (Novy-MacNeal-Nicolle) medium. The NNN medium was incubated in an oven at 26°C.

The growth status of *Leishmania* parasites was monitored by examining a sample taken from the culture medium every two days using an inverted microscope. Promastigotes from the NNN medium showing growth were inoculated into RPMI-1640 medium, which had been previously supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% gentamicin. The RPMI-1640 medium containing promastigotes was incubated at 26°C. Parasite growth was monitored every two days using an inverted microscope. When the parasites reached the logarithmic phase, they

were counted using trypan blue stain on a Thoma slide. A suspension with a final concentration of 10^5 promastigotes/mL was prepared for use in our study.

Preparation of *Leishmania major* Amastigote Suspension

The infected THP-1 cell line [7] was first treated with 0.05% sodium dodecyl sulfate (SDS) for 30 sec to disrupt the cell membrane. Then, PBS was added to neutralize the effect of SDS, yielding a suspension. The resulting suspension was passed three times through a 27 G infector needle. A density gradient was created in the suspension using 45% Percoll and 100% Percoll to obtain pure amastigotes. The prepared density gradient was centrifuged at $3500 \times g$ for 30 min, and amastigotes were obtained [8]. The obtained amastigotes were washed three times with RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% gentamicin and cryopreserved. In our study, we used a cryopreserved *L. major* strain (MHOM/TR/2013/CBUALA17) stored in liquid nitrogen. The cryo tube, removed from the liquid nitrogen tank, was thawed by holding it in a 37°C water bath for 2 min. The suspension in the cryo tube was transferred to a sterile Falcon tube containing 5 mL of RPMI-1640 medium. The Falcon tube was centrifuged at 1200 rpm for 5 min. After centrifugation, the supernatant was discarded. One mL of medium was added to the remaining pellet to create a suspension. Viability and amastigote count were determined on a Thoma slide using trypan blue staining. A suspension with a final concentration of 10^5 amastigotes/mL was prepared.

Experimental Animals and Group Allocation

In our study, three groups were formed to create an experimental cutaneous leishmaniasis model. Fifteen male BALB/c white mice, aged 3-4 months and weighing 20-25 g, obtained from the Manisa Celal Bayar University Experimental Animal Application and Research Center, were randomly divided into three groups of five. The experimental animals were housed in polycarbonate cages on sawdust bedding under controlled environmental conditions with a 12-h light/12-h dark cycle. The experimental animals were fed a standard laboratory diet and provided with unlimited drinking water.

The experimental groups were defined as follows:

L. major promastigote group

L. major amastigote group

Control group

Establishment of the Cutaneous Leishmaniasis Model

Fifty μ L of the prepared 10^5 promastigote/mL *L. major* promastigote suspension was administered intradermally

to the right footpad of each mouse in the promastigote group. 50 µL of the 10^5 amastigote/mL *L. major* amastigote suspension was administered to the right footpad of each mouse in the amastigote group. Mice in the control group received 50 µL of saline intradermally to their right footpads.

Follow-up of the Model, Sampling, and Study Termination

Experimental animals were monitored weekly for 12 weeks. Footpad measurements were taken weekly at the same time of day by the same researcher using the same digital caliper for 12 weeks. To ensure measurement consistency, the same researcher measured the footpad thickness three times consecutively from the same anatomical region of each experimental animal. The average of these measurements was used for statistical analysis. At the end of the study, the experimental animals were sacrificed, and their right hind footpad was removed. Tissue samples were prepared and inoculated into NNN medium to investigate the presence of parasites.

Statistical Analysis

The normality of model residuals was assessed using the Shapiro-Wilk test. Because repeated measurements were obtained from the same experimental animals over 12 weeks, a mixed-design repeated-measures ANOVA was performed using R (version 4.6.0; R Foundation for Statistical Computing, Vienna, Austria). Experimental group (promastigote, amastigote, and control) was included as the between-subject factor, whereas time (week) was included as the within-subject factor. The sphericity assumption was evaluated using Mauchly's test. Since the assumption of sphericity was violated, Greenhouse-Geisser-corrected degrees of freedom and p-values were reported. Partial eta squared (η^2) was calculated as the measure of effect size. Pairwise comparisons based on estimated marginal means were performed using Tukey-adjusted P-values where appropriate. As a sensitivity analysis, the longitudinal data were additionally analyzed using a linear mixed-effects model (LMM) with mouse included as a random intercept, group and week as fixed effects, and Kenward-Roger approximation for significance testing. Marginal and conditional R^2 values were calculated to evaluate the explanatory power of the mixed model. Statistical significance was defined as $P < 0.05$.

The sample size ($n=5$ mice per group) was determined in accordance with previous cutaneous leishmaniasis modeling using BALB/c mice and the ethics committee's principle of reducing the use of animals in animal experiments. No a priori power analysis was performed to determine the sample size. A post-hoc sensitivity power analysis was performed using G*Power software (version 3.1.9.7) to assess the statistical sensitivity of the study.

RESULTS

Leishmania major Promastigote Culture

Microscopic examination following the inoculation of the *L. major* promastigote form into NNN medium revealed the initiation of parasite proliferation on the fifth day. Monitoring of liquid cultures using an inverted microscope demonstrated that the promastigotes reached the logarithmic growth phase on the sixth day. Based on counts performed during the logarithmic phase, a suspension with a concentration of 10^5 promastigotes/mL was obtained for use in experimental applications.

Preparation of *Leishmania major* Amastigote Suspension

Based on counts performed after removal of *L. major* amastigotes from the liquid nitrogen tank, a suspension with a concentration of 10^5 amastigotes/mL was prepared for use in experimental infections.

Promastigote Group Findings

Weekly footpad thickness measurements were recorded in the promastigote group following infection. The mean measurement in the first week was 1.39 mm, and values gradually increased in subsequent weeks. By the fourth week, the mean measurement reached 1.67 mm, and the increase continued during the later stages of follow-up in parallel with the progression of inflammation (Fig. 1, Fig. 2).

At the end of the twelve-week follow-up period, the mean footpad thickness in the promastigote group was recorded as 2.33 mm. The gradual increase observed in weekly measurements indicated that the lesion thickness increased regularly throughout the follow-up period (Table 1).

Amastigote Group Findings

In the amastigote group, weekly footpad thickness measurements were recorded to monitor lesion development. A gradual increase in mean footpad

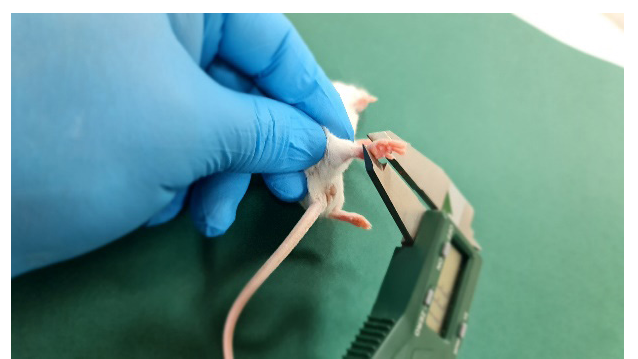


Fig 1. Footpad thickness measurements



Fig 2. Footpad of the *Leishmania major* promastigote group

thickness was observed during the first four weeks of follow-up, with the rate of increase accelerating significantly after the fourth week.

The mean footpad thickness reached 2.90 mm in the fifth week, and measurements continued to increase in the following weeks, reaching 3.32 mm at the end of the twelfth week (Table 2). When comparing the groups, higher footpad thickness values were observed in the amastigote group than in the promastigote group from the fourth week onwards (Fig. 3).

Control Group Findings

In the control group treated only with saline solution, footpad thickness measurements remained unchanged throughout the 12-week follow-up period. Footpad thickness in control group mice varied between 1.33-1.34 mm on average over 12 weeks (Table 3). No significant change in footpad thickness was observed in control group mice during the study period. No increase suggestive of lesion development was detected.

Statistical Comparison of Footpad Thickness Measurements Between Groups

A mixed-effects repeated-measures ANOVA showed significant effects of group ($F(2,12) = 2812.74$, $P < 0.001$, $\eta^2 = 0.998$, 95% CI [1.00, 1.00]), time (Greenhouse-Geisser correction: $F(1.80, 21.58) = 3811.16$, $P < 0.001$, $\eta^2 = 0.997$, 95% CI [1.00, 1.00]) and group $\times$ time interaction (Greenhouse-Geisser correction: $F(3.60, 21.58) = 1502.92$, $P < 0.001$, $\eta^2 = 0.996$, 95% CI [1.00, 1.00]). These findings demonstrate that footpad thickness changes significantly over time and that the infection pattern differs between experimental groups.

Almost identical results were obtained when applying the linear mixed-effects (LMM) model. Group ($F(2,12) = 2812.7$, $P < 0.001$), week ($F(11,132) = 3811.2$, $P < 0.001$) and group $\times$ week interaction ($F(22,132) = 1502.9$, $P < 0.001$) showed significant fixed effects. The model explained almost all of the observed variability in footpad thickness (marginal $R^2 = 0.998$, 95% CI 0.996-0.998; conditional $R^2 = 0.999$, 95% CI 0.999-0.999).

Tukey-corrected pairwise comparisons based on estimated marginal means revealed no significant difference between the control and promastigote groups during week 1 ($P = 0.065$). However, all remaining pairwise comparisons became statistically significant from week 2 onwards (all $P < 0.001$). A clear distinction emerged between the promastigote and amastigote groups at week 5, consistent with lesion development observed in amastigote-infected mice (Fig. 4).

A sensitivity power analysis was performed using G*Power software (version 3.1.9.7) for a repeated-measures between-groups interaction design. Considering a significance

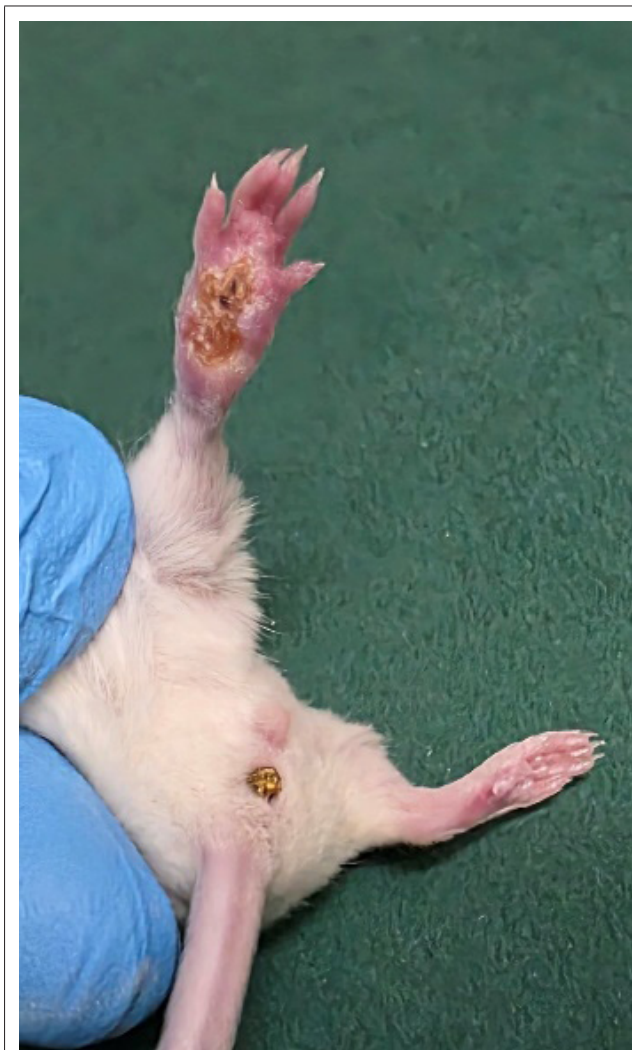
Table 1. Weekly footpad thickness (mm) in the *Leishmania major* promastigote group

Week	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mean $\pm$ SD
Week 1	1.39	1.40	1.38	1.38	1.39	1.39 $\pm$ 0.01
Week 2	1.49	1.49	1.47	1.47	1.48	1.48 $\pm$ 0.01
Week 3	1.60	1.59	1.56	1.56	1.58	1.58 $\pm$ 0.02
Week 4	1.70	1.68	1.65	1.65	1.67	1.67 $\pm$ 0.02
Week 5	1.81	1.78	1.74	1.74	1.76	1.77 $\pm$ 0.03
Week 6	1.91	1.87	1.83	1.83	1.86	1.86 $\pm$ 0.03
Week 7	2.02	1.97	1.92	1.92	1.95	1.96 $\pm$ 0.04
Week 8	2.12	2.06	2.01	2.01	2.04	2.05 $\pm$ 0.05
Week 9	2.23	2.16	2.10	2.10	2.14	2.15 $\pm$ 0.05
Week 10	2.33	2.25	2.19	2.19	2.23	2.24 $\pm$ 0.06
Week 11	2.38	2.31	2.23	2.25	2.26	2.29 $\pm$ 0.06
Week 12	2.44	2.35	2.28	2.28	2.32	2.33 $\pm$ 0.07

Table 2. Weekly footpad thickness (mm) in the *Leishmania major* amastigote group

Week	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mean
Week 1	1.44	1.44	1.46	1.44	1.45	1.45±0.01
Week 2	1.56	1.56	1.56	1.56	1.57	1.56±0.00
Week 3	1.68	1.68	1.66	1.68	1.69	1.68±0.01
Week 4	1.80	1.80	1.76	1.80	1.81	1.79±0.02
Week 5	2.92	2.93	2.85	2.87	2.93	2.90±0.04
Week 6	2.99	2.98	2.90	2.92	2.96	2.95±0.04
Week 7	3.06	3.02	2.95	2.97	2.99	3.00±0.04
Week 8	3.13	3.06	3.00	3.02	3.02	3.05±0.05
Week 9	3.20	3.10	3.05	3.07	3.05	3.09±0.06
Week 10	3.27	3.14	3.10	3.12	3.08	3.14±0.07
Week 11	3.30	3.22	3.18	3.22	3.20	3.22±0.05
Week 12	3.33	3.30	3.31	3.32	3.32	3.32±0.01

level of 0.05, statistical power of 80%, three experimental groups, 12 repeated measurements, a total of 15 animals, a

**Fig 3.** Footpad of the *Leishmania major* amastigote group

correlation coefficient of 0.50 between repeated measures, and the Greenhouse-Geisser sphericity correction factor ($\epsilon = 0.1635$) obtained from the existing data, the minimum detectable effect size was calculated as Cohen's $f = 0.49$. According to Cohen's classification, this value represents a large effect size.

Tissue Samples and NNN Culture Positivity Findings

Microscopic examination of touch preparations prepared from tissue samples obtained from the right hind footpads of experimental animals sacrificed at the end of the study clearly demonstrated the presence of amastigote forms in tissues from both the promastigote- and amastigote-infected groups.

Following inoculation of the tissue samples onto NNN

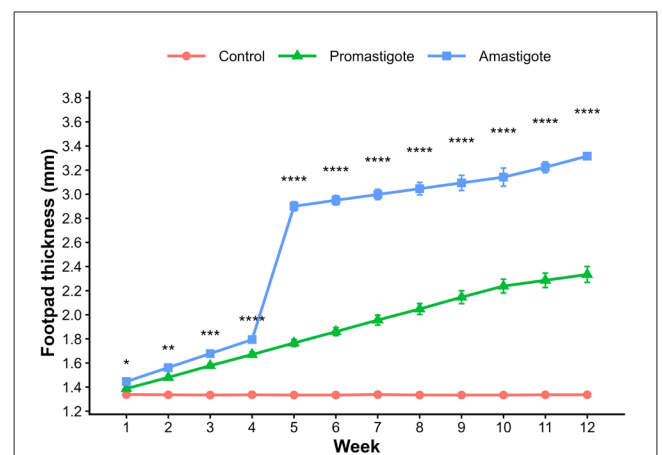
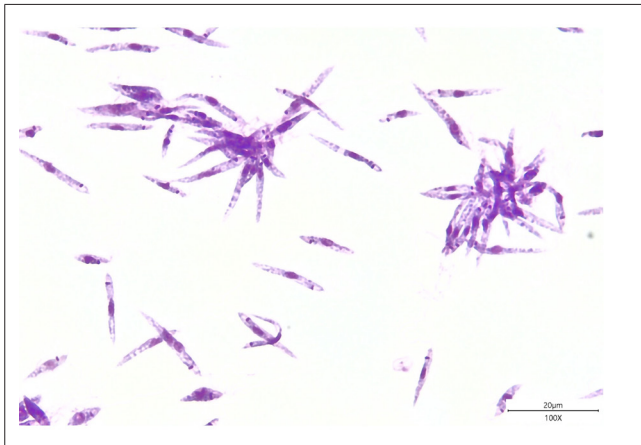
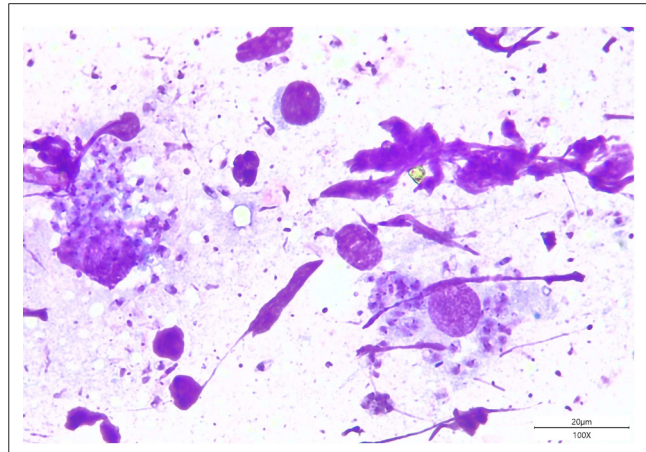


Fig 4. Footpad thicknesses of *Leishmania major* promastigote and amastigote group mice and control group mice over a 12-week follow-up period. Data are presented as mean $\pm$ standard deviation (SD; $n = 5$ mice per group). Asterisks indicate Tukey-corrected pairwise comparisons between promastigote and amastigote groups based on estimated marginal means: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$

Table 3. Weekly footpad thickness (mm) in the control group

Week	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mean
Week 1	1.33	1.34	1.34	1.34	1.34	1.34±0.00
Week 2	1.34	1.33	1.34	1.33	1.34	1.34±0.01
Week 3	1.33	1.34	1.33	1.34	1.33	1.33±0.01
Week 4	1.33	1.33	1.34	1.34	1.34	1.34±0.01
Week 5	1.34	1.33	1.34	1.33	1.33	1.33±0.01
Week 6	1.33	1.34	1.33	1.34	1.33	1.33±0.01
Week 7	1.34	1.33	1.34	1.34	1.34	1.34±0.00
Week 8	1.33	1.34	1.33	1.34	1.33	1.33±0.01
Week 9	1.34	1.33	1.34	1.33	1.33	1.33±0.01
Week 10	1.33	1.34	1.33	1.33	1.34	1.33±0.01
Week 11	1.34	1.33	1.34	1.34	1.33	1.34±0.01
Week 12	1.33	1.34	1.33	1.34	1.34	1.34±0.01

**Fig 5.** Giemsa-stained microscopic appearance of *Leishmania major* promastigotes growing on NNN medium**Fig 6.** Giemsa-stained microscopic view of a mouse footpad touch preparation from the amastigote group

medium, transformation into the promastigote form and active parasite proliferation were detected in both infected groups, and the cultures were found to be positive (Fig. 5, Fig. 6). In contrast, no amastigote forms were observed in preparations prepared from tissue samples of the control group, and no promastigote growth was detected in NNN cultures inoculated with control group samples.

DISCUSSION

Cutaneous leishmaniasis is a common parasitic infection both worldwide and in Türkiye. The most important causative agent of cutaneous leishmaniasis in Türkiye is *L. tropica*. In recent years, cases of cutaneous leishmaniasis caused by *L. major* have also been observed. In cutaneous leishmaniasis, where treatment options are limited, treatment success is decreasing and treatment durations may be prolonged due to drug resistance reported in recent years. Therefore, experimental animal models are of great importance for understanding the development

mechanism, biology, host-parasite relationship, and developing new treatment strategies for cutaneous leishmaniasis [6,9]. BALB/c mice are one of the commonly used experimental models in cutaneous leishmaniasis research due to their high susceptibility to *L. major* infection and Th2-dominant immune response [10]. This study aims to comparatively evaluate infections induced by promastigote and amastigote forms in the BALB/c model and thus explain the infection dynamics of different life cycle stages of the parasite.

Our findings demonstrated that both promastigote and amastigote forms successfully induced infection in BALB/c mice. The cutaneous leishmaniasis model induced by the amastigote form showed a more pronounced pathological course from the fifth week onwards compared to the cutaneous leishmaniasis model induced by the promastigote form. This difference may be related to the biological characteristics of the amastigote form that are compatible with the mammalian host; however,

further immunological and molecular studies are needed to elucidate these possible mechanisms.

It has been reported that the promastigote and amastigote forms of the *Leishmania* parasite differ not only in shape but also in surface molecules, metabolism, gene expression, and virulence factors [11]. Promastigote forms express high levels of lipophosphoglycan (LPG) and the surface protease gp63, while these are expressed less in amastigote forms. It has also been reported that cysteine protease expression is low in promastigote forms and high in amastigote forms [12,13]. These differences lead macrophages to perceive promastigote and amastigote forms as different targets and to develop different cellular responses to each. In addition, promastigote and amastigote forms do not use the same mechanism to suppress the immune response. Studies have shown that promastigotes activate two phosphatases, SHP-1 and PTP-1B, together to suppress macrophage defense mechanisms upon initial entry into the host. Together, these molecules suppress pro-inflammatory signaling pathways such as JAK2, STAT1, NF- κ B, AP-1, and TNF- α , consequently reducing macrophage microbicidal activity [13]. By contrast, amastigote forms evade the immune response by activating only SHP-1 and are reported to suppress NF- κ B, STAT-1, and AP-1 more strongly than promastigotes upon initial interaction with macrophages [13]. In our study, the differences observed between promastigote and amastigote groups are thought to stem from their different mechanisms for entry into the host cell and the development of intracellular infection. After inoculation of promastigotes, they first encounter the complement system and phagocytic cells, then enter the cell and transform into the amastigote form. This transformation process, which requires adaptation to the host cell environment, suggests that intracellular proliferation and lesion development may begin later in infections initiated by promastigotes. By contrast, amastigotes are already adapted to the intracellular environment. They can begin intracellular proliferation after being taken up by phagocytic cells. Therefore, it is thought that infections caused by amastigotes lead to an earlier increase in parasite load and faster lesion development compared to infections caused by promastigotes.

The high susceptibility of BALB/c mice to *L. major* infection is associated with their development of a Th2-dominant immune response in which IL-4 and IL-10 production is predominant [10]. In the present study, the acceleration of inflammation and the regular increase in footpad thickness observed particularly from weeks 4-5 onward were consistent with the susceptible phenotype of the BALB/c model described in the literature. The relatively limited increase in footpad thickness during the early weeks and the delayed manifestation of infection

in the promastigote-infected group may be explained by the requirement of promastigotes to undergo differentiation into amastigotes following phagocytosis in the mammalian host. Although equal inoculum doses were used numerically in both groups, the requirement for *in vivo* differentiation of the promastigote form into the amastigote stage should be considered a fundamental biological factor that may partially explain the delayed lesion development observed in this group. It has previously been reported that this biological transition process can slow infection kinetics [14].

The observation that the amastigote form induced earlier and more severe pathology is consistent with studies reporting that amastigote inoculation is more effective in initiating infection. It has been demonstrated that axenic amastigotes of *L. infantum* establish infection more rapidly and reach detectable parasite burdens earlier than promastigotes [15]. Similarly, amastigotes have been reported to possess metabolic and proteomic advantages in host adaptation and to proliferate without requiring transition to the intracellular phase [16]. In the present study, the marked inflammation observed in the amastigote group, particularly from weeks 4-5 onward, and the higher footpad thickness measurements recorded at week 12 compared to the promastigote group are consistent with this evidence.

From a practical standpoint, these findings provide researchers with useful information for selecting an experimental model to develop antileishmanial treatments. The earlier onset and faster progression of lesions observed in the amastigote-based model may better reflect the intracellular stage responsible for pathology in human infections. An experimental cutaneous leishmaniasis model created with the amastigote form could provide a practical experimental platform, particularly for studies focusing on compounds that target intracellular amastigotes. The shorter time required for lesion development may contribute to more efficient evaluation of new molecules and active substances.

Although both parasite forms are used in experimental cutaneous leishmaniasis modeling, comparisons conducted under the same experimental conditions remain incomplete. The results show that the choice of parasite form affects infection kinetics, lesion severity, and experimental duration, thereby improving the experimental model. This study can guide researchers in selecting the most suitable experimental model for their research objectives.

The use of repeated-measures analysis controlled within-subject variability in time-dependent measurements obtained from the same animals and allowed for a more reliable evaluation of differences in lesion progression

among groups. As a result of these analyses, both the promastigote and amastigote groups were found to reach significantly higher footpad thickness values throughout the follow-up period compared to the control group.

The demonstration of amastigotes in Giemsa-stained touch-up preparations and the growth of promastigotes from footpad lesions in NNN medium at the end of the study confirmed the successful occurrence of *in vivo* infection. The absence of both microscopic and culture positivity in the control group supports the idea that the observed pathological changes are specific to the experimental infection.

The contribution of this study to the literature can be summarized in two main points: (i) the promastigote and amastigote forms of *L. major* isolate exhibit different kinetics in infection severity and development; and (ii) the amastigote form allows the experimental model to be established in a shorter time, enabling earlier evaluation of therapeutic agents. Amastigote injection has been reported to reliably and reproducibly create lesions in the BALB/c model [17]. Our study offers a feasible and time-efficient approach for testing drugs, vaccines, and immunomodulatory agents.

The low biological variation observed among individual animals in this study is likely due to the good standardization of experimental conditions. The same *L. major* isolate was used in both experimental groups; the same amount of parasite was applied to the footpads; all experimental animals were BALB/c mice of the same age and sex, and they were monitored under the same environmental conditions. Since the footpad thickness measurements were performed by the same researcher, from the same anatomical region, and using the same digital caliper, technical variability between measurements was reduced. The fact that the measurements were not performed blinded can be considered one of the limitations of the study. The genetically homogeneous BALB/c mouse strain and the selection of all experimental animals of the same age and sex may also have contributed to the recorded low biological variation.

Our study was designed as a comparative experimental study. Therefore, immunological evaluations and quantitative parasitological analyses were not included in our study. Our study encompasses the clinical findings of two different models, with microscopic and culture evaluations performed to confirm the infection. For future studies, it is recommended to strengthen the model through further histopathological evaluations, monitoring immune response dynamics using cytokine profiling, quantitative measurement of parasite load, and integration of treatment strategies. Time-course analysis of Th1/Th2 response polarization may help elucidate the

interaction between the immune system and the more aggressive course observed in amastigote infections. In addition, comparing different *Leishmania* species with different doses will increase the value of the developed amastigote model.

In our study, an experimental cutaneous leishmaniasis model was created on the paw pads of BALB/c mice using both promastigote and amastigote forms of *L. major*. The created experimental cutaneous leishmaniasis model was monitored for 12 weeks. The experimental cutaneous leishmaniasis model created with both parasite forms was successfully established. In the modeling with the amastigote form, lesion development accelerated significantly from the fifth week onwards, and higher lesion measurements were obtained. In the modeling with the promastigote form, lesion development showed a slower and more stable progression.

Microscopic examination and culture results provided biological confirmation of the infection. The findings show that the developed experimental model has been successfully implemented.

The fact that the experimental cutaneous leishmaniasis model created with the amastigote form appeared approximately 5-6 weeks earlier than the model created with the promastigote form led to a significant shortening of the study period. The earlier initiation of the model provides a significant advantage in reducing animal care time and associated costs. The experimental cutaneous leishmaniasis model created with the amastigote form could be an ethically and practically advantageous alternative, as it can be established approximately 50% faster than the promastigote model and reduces care burden and costs.

Modeling experimental cutaneous leishmaniasis using the amastigote form of the *Leishmania* parasite affords several benefits. This model reduces the time required for lesion development, shortens the evaluation time for candidate molecules or compounds, and consequently increases efficiency in workflow planning. In experimental cutaneous leishmaniasis modeling with the amastigote form, the transformation process from promastigote to amastigote is eliminated, enabling faster observation of the resulting lesion.

In conclusion, the BALB/c cutaneous leishmaniasis model established using the amastigote form appears to be a reliable and applicable experimental model for evaluating the efficacy of drugs, vaccines, and immunotherapeutic agents, while offering notable advantages with respect to time efficiency, cost reduction, and animal welfare.

When all findings are considered, the model created in BALB/c mice with the amastigote form of the *Leishmania* parasite could be a practical and time-efficient

experimental model for future studies evaluating new antileishmanial strategies.

DECLARATIONS

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

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Competing of Interest: The authors declared that there is no competing of interests.

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

Ethical Compliance Declaration: The authors declare that the study was conducted in accordance with all applicable animal welfare and research ethics principles, institutional and national/international regulations, and the requirements of the relevant ethics committees and funding/supporting institutions, where applicable. The authors accept full responsibility for the ethical integrity of the study.

Author Contributions: IC and AO contributed to the study's design, methodology, experimental execution, data analysis, and interpretation. IC wrote the article, and AO critically reviewed it. Both authors have read and approved the final version of the article.

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RESEARCH ARTICLE

Immunomodulatory Effects of Encapsulated IgY on Antibody Titers and Bursa Fabricius Integrity in Broilers Infected with *Salmonella Pullorum*

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Abstract

Passive immunization using immunoglobulin yolk (IgY) has attracted considerable attention as a prominent antibiotic alternative in poultry health management. However, its practical application is constrained by vulnerability to gastrointestinal degradation, limiting its immunoprotective potential against enteric pathogens like *Salmonella Pullorum*. To address this gap, this study investigated the protective efficacy of encapsulated IgY on bursa of Fabricius integrity and antibody responses in challenged Cobb 500 broilers. Under a completely randomized design, the chickens were allocated into a negative control group, a positive control group, and three treatment groups receiving encapsulated IgY at doses of 0.11, 0.22, and 0.34 mL/day, respectively. Encapsulated IgY administration was initiated at 8 days of age and continued for 14 consecutive days until the challenge infection was performed at 22 days of age via intramuscular injection into the pectoralis major muscle using a bacterial dose of 1.5×10^8 CFU. AGPT analysis demonstrated delayed antibody kinetics post-infection. Crucially, encapsulated IgY significantly countered *Salmonella*-induced lesions through distinct structural protections: minimizing follicular depletion (P1: 2.00 ± 0.37 vs K⁺: 2.80 ± 0.14), reducing lymphoid cell necrosis (P3: 1.36 ± 0.17 vs K⁺: 2.60 ± 0.20), and dose-dependently restoring plica diameter up to 243.02 ± 9.5 μ m (P1) and 224.78 ± 20 μ m (P2) at the highest dose. Although IgY technology shows immense therapeutic value, its encapsulated potential in veterinary medicine remains underexplored. This study provides a scientific rationale for utilizing encapsulated IgY as a physiological protective factor, advancing sustainable health management in livestock production, which directly aligns with current priority areas in veterinary research.

Keywords: Broiler Chickens, Bursa of Fabricius, Encapsulation, Immunoglobulin Yolk, *Salmonella Pullorum*

INTRODUCTION

Pullorum disease, caused by *Salmonella enterica* serovar Pullorum, continues to pose a major challenge to poultry production worldwide, particularly in developing regions [1]. This Gram-negative bacterium is highly host-specific and primarily infects young chicks, causing systemic illness characterized by anorexia, white diarrhea, dehydration, reduced growth, and high mortality rates [2]. One of the main organs targeted by *Salmonella Pullorum* infection is the Bursa of Fabricius, a lymphoid organ unique to avian species that is essential for the development and maturation of B lymphocytes [3]. Structural damage or atrophy of this

organ can impair humoral immune function and reduce the bird's resistance to secondary infections.

The growing concern regarding antimicrobial resistance has encouraged the poultry industry to explore alternative strategies that can control pathogenic infections while maintaining immune competence. One promising approach is the utilization of Immunoglobulin Y (IgY), a specific antibody naturally deposited in egg yolks of hens immunized with particular antigens. IgY possesses several advantages, including high antigen specificity, safety, and minimal cross-reactivity with mammalian immune systems, making it a potential candidate for passive immunotherapy in poultry [4].



Previous investigations have demonstrated the antibacterial potential of IgY against various enteric pathogens^[5,6] such as *Escherichia coli*, *Salmonella* spp., and *Clostridium perfringens*. These studies indicate that IgY can provide protective effects against intestinal infections. Nevertheless, most research has focused mainly on its antimicrobial activity *in vitro* or within the intestinal lumen. Limited information is available regarding its systemic immunological effects, particularly its influence on primary lymphoid organs such as the Bursa of Fabricius.

A major limitation of orally administered IgY is its susceptibility to degradation by gastric enzymes, which may reduce its biological activity. To address this issue, encapsulation technologies have been developed to protect IgY during passage through the gastrointestinal tract and enhance its stability and bioavailability^[7]. Chitosan, a natural polysaccharide derived from chitin, has received considerable attention as a drug delivery material due to its biocompatibility, biodegradability, low toxicity, and chemical stability^[8,9]. Furthermore, the presence of reactive functional groups allows chitosan to be modified into various drug delivery systems such as nanoparticles, microparticles, and hydrogels capable of protecting bioactive compounds from harsh gastrointestinal conditions^[9].

Despite advances in encapsulation technology, information regarding the effects of encapsulated IgY on both serological immune responses and histopathological changes in the Bursa of Fabricius remains limited, particularly in broiler chickens challenged with *S. Pullorum*. Since antibody titers reflect the activity of the humoral immune system, which is closely related to the integrity of the Bursa of Fabricius, understanding this relationship is essential.

Therefore, this study aimed to evaluate the influence of orally administered encapsulated IgY on antibody titers and the histological condition of the Bursa of Fabricius in broiler chickens experimentally infected with *S. Pullorum*. Through combined serological and histopathological analyses, this research seeks to clarify the role of IgY in strengthening humoral immunity while preserving lymphoid tissue architecture.

MATERIAL AND METHODS

Ethical Statement

This study obtained ethical clearance from the Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Medicine, Airlangga University (Approval No. 1.KEH.099.07.2024). All experimental procedures were carried out in compliance with established guidelines for the ethical use and welfare of experimental animals.

Animal Preparation

Day-old Cobb 500 broiler chicks (Cobb-Vantress) were maintained in a closed-house system and acclimatized for seven days. During the adaptation period, birds were provided with uniform feed and water. Feed was administered at a controlled amount of 23 g per bird per day, while drinking water was supplied *ad libitum*. Environmental conditions, particularly brooder temperature, were monitored hourly and adjusted according to chick behavior to ensure optimal comfort.

IgY Extraction and Purification

IgY extraction was performed using hyperimmune egg yolks specific to *Salmonella Pullorum*. Egg yolks were separated from albumen and diluted with distilled water at a ratio of 1:8 (v/v). The solution was adjusted to pH 5.0 and subsequently stored in a freezer for 24 h. After thawing, the mixture was centrifuged at 13,500 rpm for 15 min at 4°C to obtain the water-soluble fraction (WSF). The WSF was then adjusted with NaCl solution to pH 4.0 and centrifuged again to obtain the precipitate, which was subsequently dissolved in phosphate-buffered saline (PBS) and adjusted to pH 7.0^[10].

IgY Encapsulation

Encapsulation was performed using calcium chloride (CaCl₂), chitosan, sodium alginate, and purified IgY/yolk preparation at a volume ratio of 1:1:1:1. For each 100 mL formulation, 3.2% CaCl₂ (w/v; approximately 0.29 M), 2% chitosan (w/v), 0.1% sodium alginate (w/v), and 2% purified IgY (w/v) were used. Purified IgY was gradually added to the alginate-CaCl₂-chitosan mixture using a micropipette and homogenized with a magnetic stirrer. The resulting suspension was filtered, rinsed with distilled water, and further homogenized for 15-20 min to obtain encapsulated IgY particles^[10].

Propagation of *Salmonella Pullorum* Colonies

The *S. Pullorum* isolate (strain QJ-2D-Sal) was subcultured on *Salmonella*-Shigella Agar (SSA) and incubated for 24 h^[11]. Colonies were harvested using a sterile inoculating loop and suspended in PBS. The suspension turbidity was adjusted to match McFarland standard I (3×10⁸ CFU) and subsequently diluted to achieve a final concentration of 1.5×10⁸ CFU^[12].

Treatment of Experimental Animals

Twenty-five broiler chickens aged seven days were randomly allocated into five groups. The negative control group (K-) received neither IgY nor infection, while the positive control group (K+) was infected with *Salmonella Pullorum* without IgY treatment. The remaining groups (P1, P2, and P3) received encapsulated IgY based on the average body weight of the chickens on day 7, which was

162 grams with an effective IgY dosage of 700 mg/kg^[13]. The administered doses were 700 mg/kg BW for P1, 1400 mg/kg BW for P2, and 2100 mg/kg BW for P3. These body-weight-based doses were converted using a stock solution (1000 mg/mL) into absolute volumetric doses of 0.11 mL, 0.22 mL, and 0.34 mL per bird per day, respectively, followed by bacterial infection. IgY administration was performed orally each morning after feeding. Infection with *Salmonella Pullorum* was performed at 22 days of age via intramuscular injection into the *musculus pectoralis major* using a bacterial dose of 1.5×10^8 CFU^[14,15].

Organ Sample Collection

Sampling was performed when the chickens reached 25 days of age. Birds were euthanized by cervical dislocation, and the Bursa of Fabricius was collected through dissection and preserved in 10% formalin solution for histological examination. Blood samples were obtained from the brachial vein using sterile syringes and stored in EDTA tubes.

Agar Gel Precipitation Test (AGPT)

The AGPT method was used to detect the presence of specific antibodies in serum samples. A 1% agarose gel prepared in PBS was poured into Petri dishes and allowed to solidify. Wells with a diameter of 3 mm were created in a radial arrangement. Antigen was placed in the central well, while serum samples were added to the surrounding wells. Plates were incubated in a humid chamber at room temperature for 24-48 h. The appearance of a precipitation line between antigen and antibody wells indicated a positive reaction^[16].

Histopathological Observation

Histological sections of the Bursa of Fabricius were examined under a light microscope at 100× magnification across five different fields. Observed parameters included follicular cell depletion, lymphoid cell necrosis, and plica diameter. Histopathological scoring followed the method described by Lukert and Saif. Follicular depletion and necrosis were categorized into four levels: score 0 (no lesion), score 1 (mild; 1-30%), score 2 (moderate; 31-50%), and score 3 (severe; ≥51%).

Statistical Analysis

Non-parametric methods were employed as histopathological scores represent ordinal data and do not follow a normal distribution. Specifically, histopathological score data were analyzed using the Kruskal-Wallis test. When significant differences ($P < 0.05$) were detected, pairwise comparisons were conducted using the Mann-Whitney test. Quantitative statistical analyses were performed using SPSS, version 29.0 (IBM Corp), while qualitative observations were interpreted descriptively.

RESULTS

Agar Gel Precipitation Test (AGPT)

The AGPT evaluation was conducted at 7, 14, 21 and 28 days following the administration of encapsulated yolk immunoglobulin (IgY) (Fig. 1). As presented in Table 1, the extracted IgY demonstrated the capability to generate a specific antibody response against *Salmonella Pullorum*, with antibody titers reaching $2^4 \times 10^5$.

Depletion Follicular

The analysis of follicular depletion revealed significant variation among the experimental groups (K-, K+, P1, P2, and P3) in the Bursa of Fabricius (Fig. 2). As shown in Table 2 statistical analysis indicated no significant differences ($P < 0.05$) in follicular depletion between several group comparisons, including K- (1.80±0.14) with P1 (2.00±0.37), K- with P2 (1.80±0.20), K- with P3 (1.56±0.26), P1 with P2, P1 with P3, and P2 with P3. In contrast, significant differences ($P > 0.05$) were identified between the negative control group (K-; 1.80±0.14) and the positive control group (K+; 2.80±0.14), as well as between the positive control (K+) and all treatment groups, including P1 (2.00±0.37), P2 (1.80±0.20), and P3 (1.56±0.26).

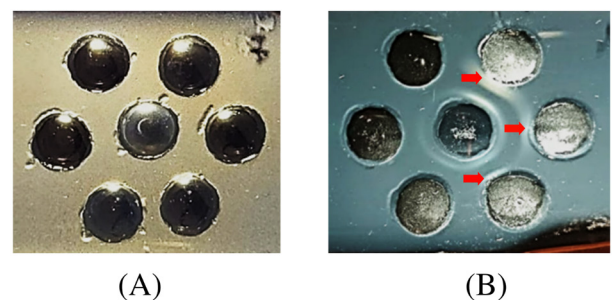


Fig 1. AGPT Results (A) IgY Extraction and (B) IgY Encapsulated IgY Post 4 weeks. The red arrow indicates the presence of precipitation signs near the AGPT test well

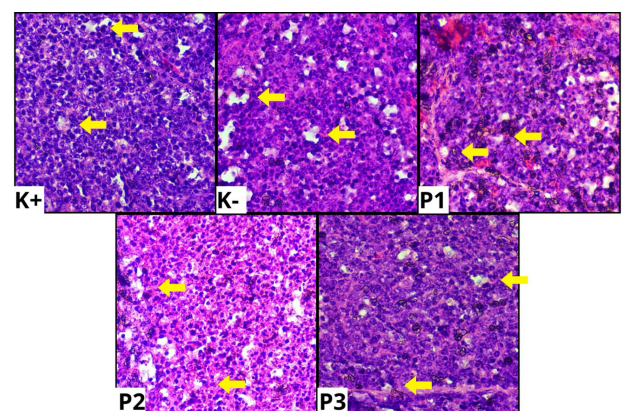


Fig 2. Depletion of Bursa Fabricius. The yellow arrow is depletion of Bursa Fabricius

Table 1. The results titer agar gel precipitation test

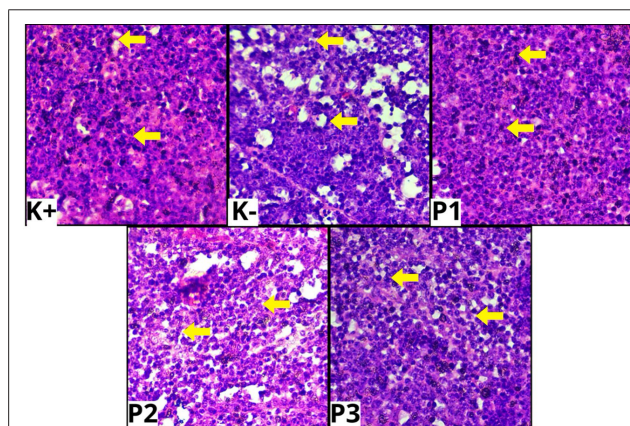
Time	Well	Dilution	Precipitation Line	Titer
24 hours	A1	1:1	No	-
	A2	1:2	No	-
	A3	1:4	No	-
	A4	1:8	No	-
	A5	1:16	No	-
	A6	1:32	No	-
1 week	B1	1:1	Yes	-
	B2	1:2	Yes	-
	B3	1:4	Yes	-
	B4	1:8	No	-
	B5	1:16	No	-
	B6	1:32	No	-
2 weeks	C1	1:1	Yes	-
	C2	1:2	Yes	-
	C3	1:4	Yes	-
	C4	1:8	No	-
	C5	1:16	No	-
	C6	1:32	No	-
3 weeks	D1	1:1	Yes	-
	D2	1:2	Yes	-
	D3	1:4	Yes	-
	D4	1:8	No	-
	D5	1:16	No	-
	D6	1:32	No	-
4 weeks	E1	1:1	Yes	200.000
	E2	1:2	Yes	400.000
	E3	1:4	Yes	1.600.000
	E4	1:8	No	-
	E5	1:16	No	-
	E6	1:32	No	-

Necrosis

Analysis of the necrosis parameter yielded a P-value of <0.05 , indicating the presence of significant differences among the experimental groups (K-, K+, P1, P2, and P3) in terms of lymphoid cell necrosis (Fig. 3). To determine which groups differed significantly, a Mann-Whitney post hoc test was performed. The results showed that there were no statistically significant differences ($P>0.05$) between the K- group (1.72 ± 0.27) and either the P1 (1.92 ± 0.11) or P2 (1.64 ± 0.17) groups. However, significant differences ($P>0.05$) were detected between K- (1.72 ± 0.27) and K+ (2.60 ± 0.20), as well as between K- and P3 (1.36 ± 0.17). Additional significant differences ($P>0.05$) were also identified between the positive control group (K+; 2.60 ± 0.20) and all treatment groups: P1 (1.92 ± 0.11), P2 (1.64 ± 0.17), and P3 (1.36 ± 0.17). Furthermore, comparisons among the treatment groups themselves (P1 vs P2, P1 vs P3, and P2 vs P3) also demonstrated statistically significant differences among the groups ($P>0.05$).

Plica Diameter

The analysis of the plica diameter parameter produced a P-value of >0.05 , indicating significant differences among the experimental groups (K-, K+, P1, P2, and P3) with respect to the plica diameter of the Bursa of Fabricius (Fig.

**Fig 3.** Necrosis of Bursa Fabricius. The yellow arrow is necrosis cell of Bursa Fabricius**Table 2.** Mean scores of follicular depletion, necrosis, and plica diameter in the bursa of Fabricius of the control and treatment groups

Group	Mean Depletion Value $\pm$ SD	Mean Necrosis Value $\pm$ SD	Mean Plica Diameter Value $\pm$ SD (μ m)
Negative Control (K-)	1.80 ± 0.14^a	1.72 ± 0.27^{ac}	232.85 ± 6.59^{ac}
Positive Control (K+)	2.80 ± 0.14^b	2.60 ± 0.20^b	297.71 ± 20.70^b
Treatment 1 (P1)	2.00 ± 0.37^a	1.92 ± 0.11^c	243.02 ± 9.50^c
Treatment 2 (P2)	1.80 ± 0.20^a	1.64 ± 0.17^a	224.78 ± 20.07^a
Treatment 3 (P3)	1.56 ± 0.26^a	1.36 ± 0.17^d	161.59 ± 35.24^d

Data are presented as mean $\pm$ SD. K-, negative control; K+, positive control; P1, P2, and P3, treatment groups receiving 0.11, 0.22, and 0.34 mL/day of encapsulated IgY, respectively. Different superscript letters (a, b, c, d) within a column indicate significant differences based on the Mann-Whitney test ($P<0.05$)

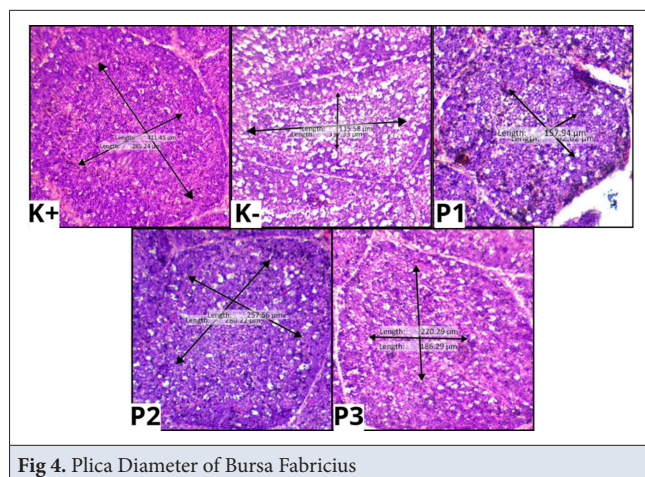


Fig 4. Plica Diameter of Bursa Fabricius

4). Further pairwise comparisons revealed that there were no significant differences ($P < 0.05$) between the K- group ($232.85 \pm 6.59 \mu\text{m}$) and the P1 group ($243.02 \pm 9.50 \mu\text{m}$), nor between K- and P2 ($224.78 \pm 20.07 \mu\text{m}$). In contrast, significant differences ($P > 0.05$) were observed between K- ($232.85 \pm 6.59 \mu\text{m}$) and K+ ($297.71 \pm 20.70 \mu\text{m}$), as well as between K- and P3 ($161.59 \pm 35.24 \mu\text{m}$). Significant differences were also found between the positive control group (K+; $297.71 \pm 20.70 \mu\text{m}$) and each treatment group P1 ($243.02 \pm 9.50 \mu\text{m}$), P2 ($224.78 \pm 20.07 \mu\text{m}$), and P3 ($161.59 \pm 35.24 \mu\text{m}$). Moreover, comparisons among the treatment groups themselves (P1 vs P2, P1 vs P3, and P2 vs P3) also showed statistically significant differences ($P > 0.05$).

DISCUSSION

As shown in Fig. 1, administration of encapsulated IgY 24 hours after treatment did not produce a detectable increase in antibody titers in the blood of broiler chickens. A similar pattern was observed on days 7, 14, and 21, with no precipitation lines detected on the AGPT agar medium, indicating that a measurable specific immune response against *S. Pullorum* had not yet developed. On day 28 (Table 1), an increase in the specific antibody titer against *S. Pullorum* was observed. These findings correspond with the report of Schat et al.^[17], who stated that antibody production following infection with *S. Pullorum* generally becomes detectable only after approximately three weeks. This condition may be associated with the biological nature of encapsulated IgY, which acts as a passive antibody. Such antibodies become functionally active primarily when the pathogen is present in the host system. In contrast, active immunization typically requires repeated booster administration to stimulate the host immune system and induce endogenous antibody production that can be detected serologically. Unlike conventional vaccines, which introduce antigens to stimulate an active immune response, orally administered encapsulated IgY acts

within the gastrointestinal lumen through mechanisms such as inhibition of microbial adhesion, bacterial agglutination or immobilization, and neutralization of toxins and virulence factors, thereby reducing pathogen colonization and burden within the gastrointestinal tract. According to Rahman et al.^[18], orally administered IgY is not translocated across the intestinal epithelium into the bloodstream in its intact molecular form; therefore, the primary effects of oral IgY are considered to be localized within the gastrointestinal tract. Accordingly, in the present study, the protective effect against *Salmonella Pullorum* infection can primarily be explained by the local interaction of IgY with the pathogen within the gastrointestinal tract.

The administration of encapsulated IgY demonstrated a protective effect by reducing follicular cell depletion in broiler chickens challenged with *S. Pullorum*. In the P1 treatment group, which received encapsulated IgY at a dose of 0.11 mL per chicken together with bacterial infection, the mean depletion score was 2.00 ± 0.37 . In comparison, the positive control group (K+), which was infected with *Salmonella Pullorum* without IgY treatment, exhibited a higher depletion score of 2.80 ± 0.14 . These observations are consistent with the findings reported by Wu et al.^[19], indicating that infection with *Salmonella Pullorum* can progressively disrupt lymphoid tissue architecture. The administration of IgY appears to mitigate such damage in infected chickens. This protective effect occurs because IgY functions as a passive antibody that becomes active when pathogenic microorganisms invade the host, thereby helping to limit the extent of lymphoid tissue injury.

The lowest mean necrosis score was observed in the P3 treatment group, which received encapsulated IgY at a dose of 0.34 mL per chicken in combination with *S. Pullorum* infection (1.36 ± 0.17) (Table 2). These results are supported by the work of Carlander et al.^[20], who reported that IgY administration can downregulate the expression of inflammatory-related genes and contribute to the stabilization of systemic immune responses. Encapsulated IgY may suppress excessive inflammatory reactions within the host, thereby preventing the progression of infection and limiting tissue damage. In addition, the ability of IgY to stabilize systemic immune responses may also contribute to improved tissue repair and accelerated recovery processes in infected chickens.

In the P1 and P2 treatment group (Table 2), which received dose of encapsulated IgY (0.11 mL and 0.22 mL per chicken) along with *S. Pullorum* infection, the plica diameter was recorded, with an average value of $243.02 \pm 9.5 \mu\text{m}$ (P1) and $224.02 \pm 20 \mu\text{m}$ (P2). Statistical analysis indicated that this group differed significantly ($P > 0.05$) from the negative control group (K-). The improvement in plica diameter suggests structural recovery of the lymphoid tissue mediated

by the protective activity of IgY antibodies. Liu et al.^[21] also reported that effective suppression of inflammation can facilitate tissue restoration and return the organ to its normal physiological state. Furthermore, a positive association was observed between increasing doses of encapsulated IgY and improved immunological outcomes, including reduced inflammation and regeneration of the plica structure in the Bursa of Fabricius. Encapsulated IgY works by providing natural passive antibodies that strengthen host defense mechanisms against pathogenic invasion. The encapsulation process further enhances this effect by protecting IgY during passage through the digestive tract and enabling targeted release at the appropriate site, thereby improving its ability to neutralize pathogens and reduce tissue injury^[22]. Increasing doses of encapsulated IgY were positively correlated with greater immunological benefits, including reduced lymphoid cell necrosis and enhanced regeneration of lymphoid tissues within the Bursa of Fabricius of broiler chickens infected with *S. Pullorum*. Even the lowest IgY dose (0.11 mL) administered in the P1 group was able to provide a protective effect by decreasing necrotic lesions, although the effect was less pronounced compared with higher doses. These findings support the presence of a dose-dependent relationship between IgY concentration and its immunoprotective capacity^[23]. Nevertheless, the relationship between dose and therapeutic efficacy is not always linear, as increasing the dose will enhance the therapeutic response only up to a certain optimal level.

Administration of encapsulated IgY in broiler chickens challenged with *Salmonella* Pullorum resulted in improved immunological responses and reduced histopathological damage in the Bursa of Fabricius compared with untreated infected chickens. The effective protective response was observed at a dose of 0.11 mL and 0.22 mL per chicken per day, indicating a dose-dependent immunoprotective effect of encapsulated IgY.

Several limitations should be considered when interpreting the findings of the present study. First, the relatively small sample size ($n = 5$ per group) may limit the statistical power and generalizability of the findings. Second, the relatively short observation period, with infection performed at 22 days of age and organ sampling conducted at 25 days of age, represents an additional limitation. A 3-day post-infection period may capture the early pathological response but may not be sufficient to fully characterize the progression of bursal atrophy or subsequent tissue regeneration. Therefore, the findings should be interpreted within the context of the experimental timeline. Future studies involving larger sample sizes and longer post-infection observation periods, including multiple sampling time points, are warranted to better characterize

the progression of bursal lesions and the subsequent tissue recovery following infection.

DECLARATIONS

Availability of Data and Materials: The datasets generated and analysed during the current study are available from the corresponding author (LM) on reasonable request res.

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

Ethical Compliance Declaration: This study obtained ethical clearance from the Animal Care and Use Committee (ACUC), Faculty of Veterinary Medicine, Airlangga University (Approval No. 1.KEH.099.07.2024). All experimental procedures were carried out in compliance with established guidelines for the ethical use and welfare of experimental animals

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RESEARCH ARTICLE

A Canine Inflammatory-Metabolic Risk Index Across ACVIM Stages of Myxomatous Mitral Valve Disease: A Composite Biomarker Approach

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Abstract

Myxomatous mitral valve disease (MMVD) is associated with progressive structural cardiac remodeling and systemic inflammatory and metabolic alterations. This study aimed to develop and preliminarily evaluate Canine Inflammatory-Metabolic Risk Index (CIMRI) as a composite biomarker, assess its differences across ACVIM stages and associations with echocardiographic indices, and explore its ability to discriminate dogs with current or previous congestive heart failure (CHF) from those without CHF. Fifty client-owned dogs were retrospectively enrolled and equally distributed across five disease stages. CIMRI was calculated using routinely available laboratory variables reflecting immune response, metabolic balance, and electrolyte status, normalized to total protein concentration. Associations with echocardiographic variables were assessed using Spearman's correlation analysis, and discriminatory performance was evaluated by receiver operating characteristic (ROC) analysis. CIMRI differed significantly across ACVIM stages, with the highest values observed in Stage D ($P<0.001$), and correlated positively with LA/Ao ($\rho = 0.631$, $P<0.001$) and LVIDdN ($\rho = 0.561$, $P<0.01$). ROC analysis yielded an AUC of 0.929 (95% CI: 0.837-1.000) for discriminating dogs with current or previous CHF (Stages C-D) from those without current or previous CHF (Stages A-B2). At the optimal cut-off of >5.8 , sensitivity and specificity were 95.0% and 90.0%, respectively. These findings support further evaluation of CIMRI as an exploratory composite biomarker associated with MMVD severity and CHF status in larger independent, prospective, and longitudinal populations.

Keywords: Canine, Composite Index, Echocardiography, Inflammatory-metabolic index, Myxomatous mitral valve disease

INTRODUCTION

Myxomatous mitral valve disease (MMVD) is the most common acquired cardiac disorder in dogs. The disease is characterized by progressive degeneration of the mitral valve apparatus, leading to mitral regurgitation, left atrial (LA) enlargement, and eventually congestive heart failure (CHF) [1,2]. Although the staging system proposed by the American College of Veterinary Internal Medicine (ACVIM) provides a useful framework, substantial heterogeneity exists within each stage regarding progression rates, clinical deterioration, and survival outcomes [3]. Consequently, improved tools are needed to more precisely characterize disease severity and biological heterogeneity among affected dogs.

Traditionally, MMVD progression has been attributed to hemodynamic overload resulting from chronic mitral regurgitation. Echocardiographic indices such as the left atrial-to-aortic root ratio (LA:Ao), left ventricular (LV) dimensions, and systolic function parameters are widely used to quantify cardiac remodeling and disease

severity [1,2,4-6]. However, growing evidence suggests that MMVD is not solely a mechanical disorder but involves complex systemic processes. While individual biomarkers provide valuable information, each reflects only a single aspect of the disease pathophysiology [7]. Composite indices that integrate multiple complementary physiological domains have shown potential clinical utility in both veterinary and human medicine. For instance, the hemoglobin, albumin, lymphocyte, and platelet (HALP) score predicts short-term mortality in dogs with CHF secondary to MMVD [8], and the Inflammatory Metabolic Index (IMI) and Metabolic-Inflammatory Stress Index (MISI) are effective in human clinical conditions [9]. These approaches suggest that a multidimensional metric can provide a more comprehensive representation of systemic disease burden than isolated parameters [7,10].

Several inflammatory, metabolic, and neurohormonal biomarkers have been individually investigated in canine MMVD; however, they are typically evaluated in isolation and may provide only a partial representation of the complex systemic processes underlying disease



progression [8,11-13]. MMVD is increasingly recognized as a multisystemic disorder, wherein inflammatory activation, metabolic dysregulation, neurohormonal disturbances, and systemic physiological status interact to influence cardiac remodeling and clinical outcomes [7,9,14]. However, integrating these complementary domains into a single quantitative index may provide a more comprehensive assessment of the systemic alterations associated with MMVD. A composite metric could therefore improve disease characterization and provide a more holistic assessment of systemic involvement associated with MMVD severity.

Based on this rationale, we developed the Canine Inflammatory-Metabolic Risk Index (CIMRI). Similar to previously described composite inflammatory and metabolic indices, CIMRI was designed as a pragmatic integrative score that combines complementary biological domains using routinely available laboratory variables. Specifically, the index incorporates inflammatory (neutrophil-to-lymphocyte ratio [NLR]) [15,16], acute-phase response (C-reactive protein-to-albumin ratio [CRP/Alb]) [17,18], metabolic (total cholesterol-to-glucose ratio [Chol/Glu]) [19], neurohormonal-electrolyte (sodium-to-potassium ratio [Na/K]) [20-21] and systemic protein status (total protein [TP]) into a single composite metric. Total protein was incorporated as a normalization factor to account for inter-individual variation in circulating protein concentrations and overall serum protein milieu, thereby providing contextual scaling of the combined inflammatory-metabolic signal rather than functioning as an independent disease marker.

We hypothesized that CIMRI would capture systemic alterations associated with MMVD severity. Therefore, this study aimed to develop and preliminarily evaluate CIMRI as a composite biomarker integrating routinely available inflammatory, metabolic, and electrolyte-related variables in dogs with MMVD. Specifically, we assessed differences in CIMRI across ACVIM stages, examined its associations with selected echocardiographic indices, and explored its ability to discriminate dogs with current or previous CHF (Stages C-D) from those without current or previous CHF (Stages A-B2).

MATERIAL AND METHODS

Ethical Statement

This retrospective study was conducted using previously collected clinical data obtained during routine veterinary care. The Bursa Uludağ University Animal Experiments Local Ethics Committee (HADYEK, Bursa, Türkiye) reviewed the study protocol and confirmed that formal ethical approval was not required because the study was retrospective and based exclusively on archived clinical

records (Decision No: B.30.2.ULU.0.8Z.00.00; Date: 22 June 2026). Written informed consent covering diagnostic procedures and the use of clinical data had been obtained from the owners of all dogs upon admission to the veterinary hospital.

Study Population

This retrospective study was conducted between March 2019 and March 2026 at the Veterinary Hospital. A total of 50 client-owned dogs of various breeds, ages, body weights, and both sexes were enrolled. Dogs were staged according to the ACVIM consensus classification for MMVD [3]:

- **Stage A (healthy/controls, n=10):** Dogs without clinical or echocardiographic evidence of MMVD.
- Asymptomatic dogs with an audible heart murmur were further stratified into Stage B1 and B2 according to the presence or absence of structural cardiac remodeling.
 - **Stage B1 (n=10):** Asymptomatic dogs without structural remodeling of the heart (no radiographic cardiomegaly, normal LA/Ao and LV dimensions on echocardiography).
 - **Stage B2 (n=10):** Asymptomatic dogs showing structural remodeling of the heart (echo-cardiographic LA and/or LV enlargement) without clinical signs of heart failure.
- **Stage C (n=10):** Dogs with current or past clinical signs of heart failure associated with MMVD, responsive to standard therapy.
- **Stage D (n=10):** Dogs with refractory heart failure, showing clinical signs despite optimized therapy.

Blood Sampling and Laboratory Analyses

Complete blood count (CBC) analysis provided 24 hematologic parameters (HM5 analyzer, Abaxis, USA). The NLR was manually calculated for this study. Routine serum biochemistry panels were measured in all dogs, and specific parameters relevant to this study included glucose, cholesterol, sodium (Na), potassium (K), C-reactive protein (CRP), albumin (Alb), and total protein (TP) (serum biochemical analyzer, Fuji Dri-Chem NX600V IC), as described previously [14,23].

Echocardiographic Assessment

Cardiac evaluation was performed in a quiet examination room without sedation, with precautions taken to minimize sound and motion artifacts. Conventional echocardiography was conducted using standard right parasternal short-axis (RPSAx) and right parasternal long-axis (RPLAx) views in accordance with ACVIM

recommendations [3]. Two-dimensional imaging together with pulsed-wave (PW), continuous-wave (CW), and color Doppler modalities were used (Vivid S60N Ultra, GE Healthcare, Norway) as reported in our previous studies [14,16,23].

The LA/Ao was measured at the aortic valve level from the RPSAx view. Left ventricular internal diameter in diastole (LVIDd) was obtained from RPLAx M-mode recordings. To account for body size differences, LVIDd was normalized using an allometric scaling method to derive the normalized left ventricular internal diameter in diastole (LVIDdN), calculated according to the formula [3]:

$$\text{LVIDdN} = \text{LVIDd (cm)} / \text{BW (kg)}^{0.294}$$

After entering the patient's body weight into the echocardiographic system and measuring LVIDd, the normalized value was automatically generated during offline analysis within the RPLAx LV M-mode measurements by the echocardiographic software (Vivid EchoPack, GE Healthcare, Version 204).

Left ventricular systolic function was assessed using M-mode measurements obtained from the RPLAx view, and ejection fraction (EF) and fractional shortening (FS) were calculated using the Teichholz method. Mitral annular plane systolic excursion (MAPSE) was measured from the septal insertion of the mitral annulus in the apical four-chamber view using M-mode. Mitral inflow velocities were recorded from the apical four-chamber view using ECG-guided pulsed-wave Doppler to determine the early (E) and late (A) diastolic velocities, and the mitral E/A ratio (MV E/A) was calculated [24]. All measurements were averaged over three consecutive cardiac cycles.

These parameters were selected because they can be readily obtained using standard echocardiographic equipment and are widely used in clinical practice and research to evaluate cardiac structure and function. Although several additional morphological and functional variables were recorded during routine echocardiographic examinations, only those relevant to the objectives of the present study were included in the final analysis.

Inclusion and Exclusion Criteria

Dogs diagnosed with MMVD were considered for inclusion if complete hematologic, biochemical, and echocardiographic data were available. Young (≤ 3 years) and senior (> 10 years) dogs were excluded to minimize age-related variability. Dogs with comorbidities such as pulmonary hypertension, hypothyroidism, cancer, or any other systemic disease were also excluded. Animals that had received medications including corticosteroids, non-steroidal anti-inflammatory drugs, antibiotics, positive inotropes, diuretics, or vaccinations within 10 days before evaluation were excluded, as these treatments could affect

hematologic and biochemical profiles. An exception was made for dogs in Stage D MMVD, as these animals were already refractory and exhibited CHF signs despite standard therapy, making recent medication use clinically unavoidable [7,14,23].

To ensure reliable echocardiographic and laboratory measurements, dogs exhibiting excessive excitement, stress, or respiratory distress were excluded, as these factors could induce stress leukogram patterns, transient hyperglycemia, and interfere with accurate cardiac assessment. Only dogs meeting all inclusion criteria and with high quality, artifact free echocardiographic images were enrolled in the study.

CIMRI Calculation and Integration

CIMRI was calculated as follows:

$$\text{CIMRI} = (\text{NLR} + \text{CRP}/\text{Alb} + \text{Chol}/\text{Glu} + \text{Na}/\text{K})/\text{TP}$$

This composite index integrates four complementary pathophysiological dimensions: systemic inflammation (NLR), acute-phase protein balance (serum CRP/Alb), metabolic homeostasis (serum Chol/Glu), and neurohormonal-electrolyte regulation (Na/K). Total protein was incorporated as a scaling factor to account for inter-individual variation in circulating protein concentrations and overall serum protein milieu. Similar normalization approaches are commonly employed in biomarker research to reduce variability related to differences in protein content and biological matrix composition. Rather than representing an independent disease marker, TP was included to contextualize the composite inflammatory-metabolic signal within the individual's systemic protein status. By combining these parameters into a single metric, CIMRI was designed to provide an integrative representation of biological processes associated with MMVD progression. The additive structure of CIMRI was intentionally chosen to maintain clinical simplicity and facilitate bedside applicability using routinely available biomarkers. Equal weighting was applied to minimize model overfitting given the relatively limited sample size. Weighted approaches, such as regression-derived or machine-learning-based coefficients, typically require large training datasets to yield stable and generalizable weight estimates; applying such methods to a modest, single-center cohort would risk overfitting and reduce the reproducibility of the index in independent populations. Furthermore, equal weighting avoids assumptions about the relative pathophysiological importance of each component, which remains incompletely defined in canine MMVD, and preserves a transparent, easily interpretable formula that can be readily calculated in clinical practice without specialized statistical software. This pragmatic approach is consistent with previously described composite inflammatory

and metabolic indices in both human and veterinary medicine^[9,27], which have similarly prioritized simplicity and clinical applicability over statistically optimized weighting in their initial development stages. Consistent with previously proposed composite inflammatory and metabolic indices, the present formulation was intended as a pragmatic integrative score rather than a mechanistic mathematical model. The CIMRI conceptual framework was illustrated in *Fig. 1*.

Statistical Analysis

The study included five groups: one healthy control group and four groups of dogs with varying severity of MMVD. Distributional assumptions for all continuous variables were assessed using the Shapiro-Wilk test. As CIMRI scores were not normally distributed across the groups, the non-parametric Kruskal-Wallis test was used to compare CIMRI values between the groups, followed by Dunn's multiple comparisons test for post hoc pairwise comparisons. Sex distribution among groups was compared using the Pearson chi-square test. The relationships between CIMRI scores and echocardiographic parameters were evaluated using Spearman's rank correlation analysis. Spearman's rank correlation coefficients (ρ) were reported with their 95% confidence intervals (CIs) and exact P values. As these analyses were exploratory, no adjustment for multiple testing was applied; therefore, the corresponding P values should be interpreted accordingly. Receiver operating characteristic (ROC) curve analysis

was performed to evaluate the ability of CIMRI to discriminate between dogs with current or previous CHF (Stages C-D, n=20) and those without CHF (Stages A-B2, n=30). Statistical significance was set at $P < 0.05$. All analyses were performed using SigmaPlot Statistical and Graphing Software (California, USA) and MedCalc (version 20.218, Ostend, Belgium). Data are presented as mean $\pm$ standard deviation (SD).

RESULTS

Study Population

A total of 50 small to medium breed dogs were included, distributed evenly across five groups: one healthy control group and four MMVD severity groups. The overall population had a mean age of 7.3 ± 2.4 years, with 26 males and 24 females. Breed distribution included Cavalier King Charles Spaniel (n=18), Terrier (n=8), Toy Poodle (n=8), Pug (n=6), Pomeranian (n=4), Yorkshire Terrier (n=2), and mixed breeds (n=4). Clinical characteristics and echocardiographic measurements according to ACVIM stages are presented in *Table 1*. Age, body weight, sex distribution, EF, FS, MAPSE, and MV E/A ratio did not differ significantly among groups ($P > 0.05$). LA/Ao and LVDdN differed significantly among ACVIM stages, with higher values observed in the dogs with CHF (Stages C and D; *Table 1*).

Inflammatory and metabolic parameters across different stages of MMVD are summarized in *Table 2*. The NLR and CRP/Alb differed significantly among ACVIM stages, with the highest values observed in Stage D ($P < 0.05$). Chol/Glu, Na/K ratio, and serum TP concentration did not differ significantly among groups ($P > 0.05$).

CIMRI Scores Across Groups

CIMRI values differed among ACVIM stages (*Fig. 2*). Mean scores were 5.3 ± 0.1 in stage A, 4.9 ± 0.3 in B1, 5.6 ± 0.3 in B2, 6.9 ± 2.0 in C, and 10.4 ± 1.7 in D. Kruskal-Wallis analysis demonstrated significant differences among groups ($P < 0.01$), and Dunn's post-hoc testing revealed significant pairwise differences among the groups, with the highest CIMRI values observed in Stage D (*Fig. 2*).

ROC analysis performed in all 50 dogs yielded an AUC of 0.929 (95% CI: 0.837-1.000, $P < 0.0001$) for discriminating dogs with current or previous CHF (Stages C-D, n=20) from dogs without current or previous CHF (Stages A-B2, n=30). The optimal CIMRI cut-off determined by the Youden index was > 5.8 , yielding a sensitivity of 95.0% (95% CI: 75.1-99.9%) and specificity of 90.0% (95% CI: 73.5-97.9%). The corresponding PPV and NPV were 86.4% (95% CI: 65.1-97.1%) and 96.4% (95% CI: 81.7-99.9%), respectively (*Table 3* and *Fig. 3*).

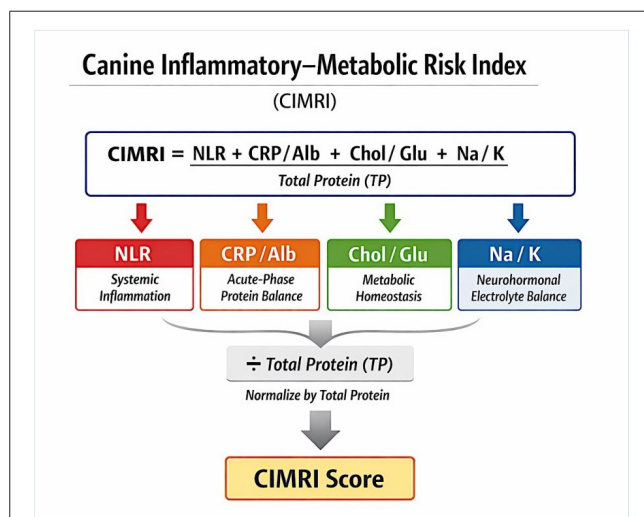


Fig 1. Schematic representation of the CIMRI - Integrated Risk Assessment in MMVD. The index integrates four complementary pathophysiological dimensions-systemic inflammation (neutrophil-to-lymphocyte ratio, NLR), acute-phase protein balance (C-reactive protein to albumin ratio, CRP/Alb), metabolic homeostasis (cholesterol to glucose ratio, Chol/Glu), and neurohormonal-electrolyte regulation (sodium to potassium ratio, Na/K)-and normalizes them by total protein (TP) to generate a single composite score. CIMRI reflects multisystemic disease activity in dogs with MMVD and correlates with echocardiographic measures and clinical disease stage (ACVIM classification)

Table 1. Clinical characteristics and echocardiographic measurements (Mean $\pm$ SD) in dogs grouped according to ACVIM MMVD stages

Parameters	Dogs with MMVD Staged to ACVIM Guideline					P-value
	A n=10	B1 n=10	B2 n=10	C n=10	D n=10	
Ages (years)	5.2 $\pm$ 2.3	6.1 $\pm$ 2.1	5.9 $\pm$ 1.2	7.3 $\pm$ 2.2	7.1 $\pm$ 2.9	= 0.206
BW (kg)	12.1 $\pm$ 3.4	12.3 $\pm$ 3.3	13.4 $\pm$ 4.5	14.2 $\pm$ 3.5	13.2 $\pm$ 3.9	= 0.194
Sexes (M/F)	5/5	3/7	6/4	5/5	7/3	= 0.474
La/Ao	1.2 $\pm$ 0.1	1.3 $\pm$ 0.1	1.8 $\pm$ 0.3	2.0 $\pm$ 0.3	2.1 $\pm$ 0.4	C vs A, and B1; P<0.001 C vs B2; P=0.039 D vs A, and B1; P<0.001 D vs B2; P=0.014
LVDdN	1.3 $\pm$ 0.1	1.3 $\pm$ 0.2	1.5 $\pm$ 0.2	1.5 $\pm$ 0.2	1.6 $\pm$ 0.3	D vs A; P=0.010 D vs B1; P=0.041 C vs A; P=0.093
EF %	61.1 $\pm$ 10.8	59.7 $\pm$ 6.0	65.7 $\pm$ 7.8	54.5 $\pm$ 6.9	54.6 $\pm$ 10.3	= 0.755
FS %	34.2 $\pm$ 6.9	32.2 $\pm$ 5.7	35.2 $\pm$ 6.6	28.8 $\pm$ 9.6	29.0 $\pm$ 9.3	= 0.705
MAPSE cm	1.4 $\pm$ 0.1	1.0 $\pm$ 0.1	0.9 $\pm$ 0.2	1.0 $\pm$ 0.3	1.1 $\pm$ 0.4	= 0.147
MV E/A	1.5 $\pm$ 0.2	1.7 $\pm$ 0.3	2.1 $\pm$ 0.5	2.2 $\pm$ 0.5	2.2 $\pm$ 0.6	= 0.081

ACVIM, American College of Veterinary Internal Medicine; BW, body weight; LA/Ao, left atrial-to-aortic root ratio; LVDdN, normalized left ventricular internal diameter in diastole; EF, ejection fraction; FS, fractional shortening; MAPSE, mitral annular plane systolic excursion; MV E/A, ratio of peak early (E) to late (A) diastolic transmitral flow velocities; M, male; F, female; vs, versus

Table 2. Inflammatory, metabolic, electrolyte, and protein-related parameters (mean $\pm$ SD) across ACVIM MMVD stages in dogs

Parameters	Dogs with MMVD Staged to ACVIM Guideline					P-value
	A n=10	B1 n=10	B2 n=10	C n=10	D n=10	
NLR	3.6 $\pm$ 1.3	3.8 $\pm$ 1.0	3.8 $\pm$ 1.2	6.5 $\pm$ 3.4	10.5 $\pm$ 6.4	D vs A, B1, and B2; P<0.05
CRP/Alb	1.3 $\pm$ 0.9	1.0 $\pm$ 0.3	1.5 $\pm$ 1.6	7.2 $\pm$ 10.4	14.5 $\pm$ 9.1	D vs A, B1, and B2; P<0.05
Chol/Glu	3.1 $\pm$ 1.3	2.8 $\pm$ 0.7	2.9 $\pm$ 0.6	2.1 $\pm$ 0.9	1.8 $\pm$ 1.1	= 0.092
Na/K	26.8 $\pm$ 1.2	25.5 $\pm$ 1.2	25.9 $\pm$ 1.5	26.3 $\pm$ 5.0	30.1 $\pm$ 6.6	= 0.224
TP g/dL	6.4 $\pm$ 0.2	6.5 $\pm$ 0.4	6.3 $\pm$ 0.3	6.4 $\pm$ 0.8	5.8 $\pm$ 0.9	= 0.331

ACVIM, American College of Veterinary Internal Medicine; NLR, neutrophil-to-lymphocyte ratio; CRP/Alb, serum C-reactive protein-to-albumin ratio; Chol/Glu, serum total cholesterol-to-glucose ratio; Na/K, serum sodium-to-potassium ratio; TP, serum total protein concentration; vs, versus

Relationships Between CIMRI and Echocardiographic Parameters

Spearman's rank correlation analysis (n=50) revealed significant positive correlations between CIMRI and LA/Ao ($\rho = 0.631$, 95% CI: 0.371-0.792, $P < 0.001$), LVDdN ($\rho = 0.561$, 95% CI: 0.315-0.719, $P < 0.01$), and MV E/A ($\rho = 0.456$, 95% CI: 0.193-0.676, $P < 0.001$). CIMRI was also significantly negatively correlated with MAPSE ($\rho = -0.336$, 95% CI: -0.569 to -0.066, $P = 0.017$). In contrast, CIMRI was not significantly correlated with EF ($\rho = -0.189$, 95% CI: -0.428 to 0.083, $P = 0.189$) or FS ($\rho = -0.234$, 95% CI: -0.480 to 0.047, $P = 0.102$).

DISCUSSION

In canine MMVD, numerous clinical, hematologic, biochemical, and echocardiographic variables have been

proposed as indicators of disease severity and progression. However, these parameters are typically evaluated in isolation and may therefore provide only a partial representation of the complex multisystemic processes associated with MMVD. In this context, the present study introduces and preliminarily evaluates CIMRI, an exploratory composite biomarker approach integrating systemic inflammation, acute-phase protein balance, metabolic homeostasis, electrolyte regulation, and systemic protein status into a single metric. By combining complementary physiological domains, CIMRI may capture multiple systemic alterations associated with MMVD severity. The principal findings were that CIMRI differed across ACVIM stages, with the highest values observed in Stage D; was associated with selected echocardiographic indices; and showed a high ability to discriminate dogs with current or previous CHF from

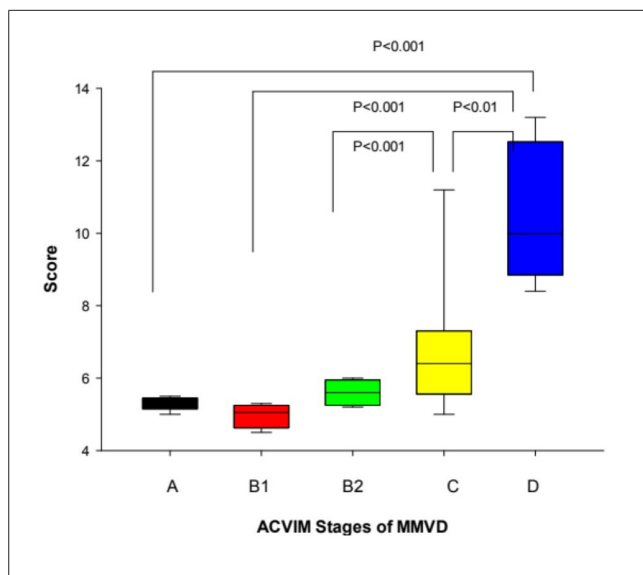


Fig 2. Box-and-whisker plots showing the distribution of Canine Inflammatory-Metabolic Risk Index (CIMRI) scores across ACVIM stages of myxomatous mitral valve disease (MMVD). The boxes represent the interquartile range, the central line indicates the median, and whiskers denote the minimum and maximum values. CIMRI scores differed significantly across ACVIM stages, with the highest values observed in Stage D. Significant pairwise comparisons are indicated ($P<0.01$ and $P<0.001$)

those without CHF in this exploratory cohort. However, these findings should not be interpreted as evidence that CIMRI reflects longitudinal disease progression, independently reflects cardiac remodeling, or has established prognostic validity.

The concept of composite indices has gained increasing recognition in both human and veterinary medicine as a means of integrating multidimensional biological information. In human medicine, indices such as the MISI and IMI have demonstrated associations with adverse outcomes in inflammatory and cardiometabolic

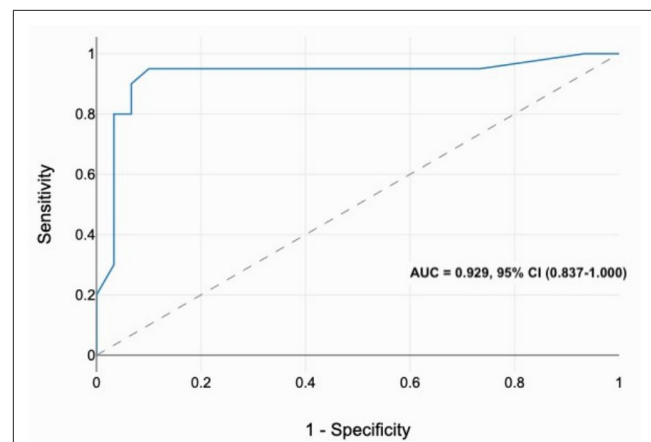


Fig 3. Receiver operating characteristic (ROC) curve analysis of CIMRI for discriminating dogs with current or previous congestive heart failure (CHF; ACVIM Stages C-D) from dogs without current or previous CHF (ACVIM Stages A-B2)

conditions [9]. Similarly, in veterinary medicine, the HALP score has been associated with survival in dogs with CHF secondary to MMVD [8]. In addition, CBC-derived indices, including NLR, monocyte-to-lymphocyte ratio, and platelet-to-lymphocyte ratio, increase with disease severity and reflect underlying inflammatory activation [10,13,16]. Collectively, these studies support the potential value of multidimensional biomarkers in characterizing the systemic nature of cardiovascular disease.

Consistent with this framework, CIMRI values tended to be higher in more advanced ACVIM stages, with the highest values observed in Stage D, and were positively correlated with echocardiographic indicators of cardiac remodeling, particularly LA/Ao and LVIDdN. These findings agree with previous reports linking increasing MMVD severity to systemic inflammatory and biochemical alterations [23,25]. A positive association was also observed between CIMRI and MV E/A. CIMRI was negatively correlated with MAPSE, although this association was weaker than those observed for LA/Ao and LVIDdN. In contrast, correlations between CIMRI and EF and FS were weak and not statistically significant. Collectively, these findings indicate that CIMRI was associated with indices of cardiac chamber enlargement and selected functional echocardiographic variables, although the strength of these associations varied among parameters. Given the cross-sectional design, these associations should not be interpreted as evidence that CIMRI independently reflects or predicts cardiac remodeling.

A notable finding of the present study was that only NLR and CRP/Alb demonstrated stage-dependent variation among the individual CIMRI components, whereas metabolic (Chol/Glu and TP) and electrolyte (Na/K) parameters remained relatively stable. This pattern is

Table 3. ROC analysis of CIMRI for discriminating dogs with current or previous congestive heart failure (CHF; ACVIM Stages C-D) from dogs without current or previous CHF (Stages A-B2)

Parameter	Estimate	95% CI
AUC	0.929	0.837-1.000
P value (AUC = 0.5)	< 0.0001	-
Optimal cut-off	>5.8	-
Sensitivity (%)	95.0	75.1-99.9
Specificity (%)	90.0	73.5-97.9
PPV (%)	86.4	65.1-97.1
NPV (%)	96.4	81.7-99.9
Youden index	0.85	-

ROC analysis included 20 dogs with current or previous CHF (Stages C-D) and 30 dogs without current or previous CHF (Stages A-B2). The optimal cut-off was determined using the Youden index. AUC, area under the receiver operating characteristic curve; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value

biologically plausible because progressive inflammatory activation and acute-phase responses are recognized features of advancing MMVD, particularly in dogs with CHF [6,17,18,23,26]. In contrast, metabolic and electrolyte parameters are often subject to tighter physiological regulation and compensatory mechanisms, which may limit their ability to discriminate disease stages when evaluated individually.

Despite the relatively limited discriminatory capacity of some individual components, CIMRI tended to show higher values in the more advanced ACVIM stages, particularly Stage D. This observation suggests a potential advantage of composite indices, namely the integration of multiple biological signals into a single metric that may capture complementary systemic alterations. As a composite biomarker approach, CIMRI integrates laboratory variables representing several systemic domains potentially associated with MMVD severity; however, its incremental value over individual biomarkers was not evaluated in the present study. Similar observations have been reported in studies evaluating composite inflammatory-metabolic indices in cardiovascular disease [27]. From a clinical perspective, CIMRI may provide complementary biological information alongside conventional echocardiographic assessment, particularly in intermediate disease stages where progression patterns may be variable.

An additional observation was that CIMRI values were numerically lower in stage B1 than in stage A. However, the difference was small and likely reflects biological variability within early disease stages rather than a meaningful pathological distinction. Given the limited number of dogs included in each group, this finding should be interpreted cautiously and confirmed in larger populations.

ROC analysis showed a high discriminatory ability of CIMRI within the present cohort for discriminating dogs with current or previous CHF (Stages C-D) from those without current or previous CHF (Stages A-B2). It is important to note that the ROC endpoint represents CHF status rather than structural cardiac remodeling, since Stage B2 dogs already exhibit cardiac enlargement as defined by the ACVIM staging criteria. Therefore, the ROC findings and the correlations observed between CIMRI and echocardiographic indices of cardiac remodeling address related but distinct aspects of MMVD and should be interpreted separately. The AUC of 0.929, together with a sensitivity of 95.0% and a specificity of 90.0% at the Youden-derived cut-off point of >5.8 , indicates a high level of discrimination within the present cohort.

An important consideration is that CIMRI was intentionally designed as a simple and clinically applicable

composite score based on routinely available laboratory variables. Equal weighting of the individual components was preferred to maintain ease of calculation and facilitate potential use in daily clinical practice. The inclusion of total protein in the denominator was intended to account for variations in protein status that may influence inflammatory and metabolic biomarkers. Because the primary objective of this study was to explore the potential clinical utility of a composite inflammatory-metabolic index rather than to develop a predictive algorithm, advanced weighting approaches such as multivariable regression-based coefficients or machine-learning optimization were not applied. Future studies involving larger populations should evaluate alternative weighting strategies and validate the mathematical structure of CIMRI.

Several limitations should be acknowledged. The study population consisted primarily of small- to medium-sized breeds, which may limit the generalizability of the findings. The relatively small number of dogs within each disease stage should also be considered when interpreting the results, as individual outlier values may have disproportionately influenced some biomarker components, particularly inflammatory variables such as NLR and CRP/Alb. Although total protein was incorporated as a scaling factor, the mathematical implications of this normalization strategy require further investigation. Alternative weighting and normalization approaches may improve the performance and interpretability of the index in future studies. Although CIMRI showed high discriminatory ability for current or previous CHF status within the present cohort, this finding requires validation in larger, independent populations before its clinical applicability can be established. Moreover, the incremental value of CIMRI over its individual components, particularly NLR and CRP/Alb, was not formally evaluated. Therefore, the present findings should not be interpreted as demonstrating superiority of CIMRI over these individual markers. In addition, the equal weighting strategy used in CIMRI was selected pragmatically and may not fully reflect the relative biological contribution of each component. The correlation analyses were exploratory, and no adjustment for multiple comparisons was applied; therefore, particularly the weaker associations should be interpreted cautiously. The cross-sectional design precludes assessment of prognostic value over time, and longitudinal studies are required to determine whether CIMRI is associated with clinical outcomes such as progression to heart failure or survival. Finally, although inclusion of Stage D dogs reflects real-world clinical conditions, ongoing cardiovascular therapy may have influenced some inflammatory, metabolic, and electrolyte variables included in the index.

In conclusion, the findings of this study provide preliminary support for CIMRI as a composite biomarker approach integrating routinely available inflammatory, metabolic, and electrolyte-related variables in dogs with MMVD. CIMRI differed across ACVIM stages, was associated with selected echocardiographic indices, and showed potential for discriminating dogs with current or previous CHF from those without CHF. Although these findings are encouraging, the cross-sectional design does not establish longitudinal disease progression, prognostic value, or an independent relationship with cardiac remodeling. Larger prospective and longitudinal studies in the independent canine populations are required to validate its clinical applicability and prognostic value.

DECLARATIONS

Availability of Data and Materials: The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

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Ethical Statement: This study was reviewed by the Bursa Uludağ University Local Ethics Committee for Animal Experiments (Decision No: B.30.2.ULU.0.8Z.00.00, Decision Date: 22 June 2026).

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

Declaration of Generative Artificial Intelligence (AI): The authors used generative artificial intelligence (AI) solely for language editing. All scientific content, study design, data analysis, interpretation of the results were performed by the authors.

Ethical Compliance Declaration: The authors declare that the study was conducted in accordance with all applicable animal welfare and research ethics principles, institutional and national/international regulations, and the requirements of the relevant ethics committees and funding/supporting institutions, where applicable. The authors accept full responsibility for the ethical integrity of the study.

Author Contributions: Z.Y.: Conceptualization, Methodology, Supervision, Data analysis, Development of the Canine Inflammatory-Metabolic Risk Index (CIMRI), Writing-original draft, Final approval. T.V.: Investigation, Data curation, Formal analysis, Writing-review & editing, Final approval. D.A.: Data curation, Validation, Writing-review & editing, Final approval.

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RESEARCH ARTICLE

Selection and Phenotypic Characterization of Potential Age-Associated Probiotic Candidates from Canine Fecal Microbiota

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The term “microbiota,” formerly known as “microflora,” generally refers to bacterial communities found on mucosal surfaces or other parts of the body, such as the skin. The canine gut microbiota plays a critical role in host health and undergoes dynamic changes across life stages. This study aimed to isolate, screen, and presumptively characterize potential probiotic candidate bacteria from canine fecal samples obtained from puppy, young, and adult dogs. Fecal samples were collected from three healthy dogs in each of three age groups (nine dogs): puppies (<6 months), young dogs (6-18 months), and adults (>18 months). From an initial pool of approximately 1000 bacterial isolates, 90 Gram-positive, catalase-negative, and oxidase-negative isolates were selected after a multi-stage *in vitro* screening for tolerance to pH 2.0, resistance to 3% porcine bile, blood agar hemolysis, and growth under combined stress (pH 4.5 and 2.5% sodium cholate). Following functional and safety screening, 30 representative candidate strains (10 from each age group, selected via balanced randomization among eligible non-redundant isolates) were biochemically characterized using the VITEK® 2 GP system. Presumptively identified taxa belonged predominantly to lactic acid bacteria, including *Lactiplantibacillus* (*Lactobacillus*) *plantarum*, *Enterococcus faecium*, *Pediococcus acidilactici*, *Limosilactobacillus* (*Lactobacillus*) *reuteri*, *Enterococcus faecalis*, and *Pediococcus pentosaceus*, which were detected across all three age cohorts, as well as stage-associated taxa such as *Lactobacillus acidophilus* and *Weissella confusa*. Isolates exhibited distinct substrate utilization profiles, yielding 17, 16, and 10 positive reactions across variable markers in puppy, young, and adult groups, respectively. These findings establish a well-characterized strain collection and demonstrate that canine fecal microbiota harbors viable, stress-tolerant lactic acid bacteria with age-associated phenotypic profiles, providing candidate strains for future molecular confirmation and functional evaluation.

Keywords: Animal probiotics, Canine intestinal microbiota, Lactic acid bacteria, Phenotypic characterization, Presumptive identification

INTRODUCTION

The gastrointestinal (GI) tract of dogs harbors a highly diverse and dynamic microbial ecosystem that plays essential roles in host metabolism, immune modulation, nutrient absorption, and maintenance of intestinal barrier integrity. The gut microbiota is now recognized as a key determinant of overall health in companion animals, and disturbances in microbial composition (dysbiosis) have been associated with a range of pathological conditions including inflammatory bowel disease, acute diarrhea, metabolic disorders, and immune-mediated diseases [1,2].

In healthy dogs, the intestinal microbiota is predominantly composed of bacterial phyla such as *Firmicutes*, *Bacteroidetes*,

Fusobacteria, *Proteobacteria*, and *Actinobacteria*. The balance among these microbial communities is critical for maintaining gut homeostasis, and alterations in their relative abundance have been linked to gastrointestinal and systemic diseases [1]. Consequently, modulation of gut microbiota has emerged as a central strategy in veterinary medicine to improve animal health and performance.

Probiotics, defined as live microorganisms that confer health benefits to the host when administered in adequate amounts, have gained increasing attention as functional feed additives in companion animals [2,3]. Numerous studies have demonstrated that probiotic supplementation in dogs can enhance intestinal microbial balance,



improve immune responses, and reduce the incidence and severity of gastrointestinal disorders ^[4,5]. In addition, certain probiotic strains have shown anti-inflammatory properties and the ability to strengthen intestinal barrier function by modulating tight junction proteins and cytokine expression ^[5].

Host specificity has been identified as a critical factor influencing probiotic efficacy. Early studies demonstrated that lactic acid bacteria (LAB) isolated from canine gastrointestinal tracts exhibit superior adaptation and functional performance compared to non-host-derived strains ^[6]. Subsequent research has further supported the importance of selecting probiotic candidates directly from the target host to ensure better colonization, survival, and biological activity within the gastrointestinal environment ^[7].

Importantly, the canine gut microbiota is not static but undergoes significant changes throughout the life cycle. During early life, puppies exhibit an immature and unstable microbiota characterized by higher variability and increased susceptibility to dysbiosis. As dogs mature, the microbial community becomes more stable and diverse, resembling the adult microbiota typically after several months of age ^[8]. In contrast, aging is associated with shifts in microbial composition, reduced production of beneficial metabolites such as short-chain fatty acids, and increased prevalence of potentially pathogenic taxa ^[9,10].

These age-related changes in gut microbiota have important physiological and clinical implications. Variations in microbial composition have been linked not only to intestinal health but also to systemic functions including metabolism, immune competence, and even cognitive performance in dogs ^[10]. Furthermore, recent studies indicate that the response to probiotic supplementation may also be age-dependent, with older dogs often showing more pronounced improvements in microbial balance and health-related outcomes following probiotic intervention ^[4].

Despite growing evidence highlighting the importance of microbiota, most commercially available probiotic products for dogs are formulated with generic, single-formula compositions that do not account for the life-stage-dependent characteristics of the host. This conventional approach overlooks the distinct ecological and physiological niches present across different ages, juvenile animals undergo rapid immune and microbiota development, while mature and adult dogs exhibit distinct metabolic pressures and microbial stabilization ^[8,9]. Therefore, evaluating host-derived bacterial candidates associated with specific life stages represents a biologically rational strategy to better match host physiology.

Although culture-independent high-throughput sequencing has greatly expanded our understanding of the canine microbiome architecture, it primarily detects relative gene abundance and includes non-culturable or non-viable fractions ^[1]. In contrast, culture-dependent isolation remains indispensable for functional probiotic development, as viable, robust, and metabolically active bacterial strains are strictly required for *in vitro* stress screening, formulation, and eventual biological delivery ^[11,12].

Based on these considerations, we hypothesized that canine fecal microbiota from different life stages harbors distinct cultivable bacterial candidates displaying differential, life-stage-associated phenotypic and stress tolerance characteristics. In this study, we aimed to isolate, screen, and presumptively characterize potential probiotic candidates from healthy dogs across three distinct life stages (puppy, young, and adult). A multi-stage *in vitro* screening cascade-including gastric acid tolerance, bile resistance, safety profiling, and growth performance under combined stress-was employed to establish a characterized collection of 30 candidate strains (10 per age cohort) and to evaluate their stage-associated biochemical profiles.

MATERIALS AND METHODS

Ethical Statement

All procedures involving animals were approved by the Kafkas University Local Ethics Committee on Animal Experiments Local Ethics Committee on Animal Experiments (KAÜ-HADYEK 2026/083).

Sample Collection

Fresh fecal samples were collected from nine healthy shepherd dogs in Kars region, northeastern Türkiye. These dogs, raised in traditional rural settings with consistent environmental exposure and diet, were selected to minimize external variables. A total of nine healthy dogs were enrolled in the study and equally divided into three age groups (three dogs per group): puppies (<6 months), young dogs (6-18 months), and adult dogs (>18 months). Breed and sex were not primary stratification factors, as the study focused on developmental dynamics. All dogs met inclusion criteria, showing no history of gastrointestinal issues, up-to-date on antiparasitic treatments, and no antimicrobial or anti-inflammatory drug use for at least four months prior.

Immediately after defecation, naturally voided fecal samples were collected. To minimize peripheral and soil contamination, samples were carefully taken from the internal core of the fecal mass. Collected fresh fecal samples were transferred into sterile tubes, rapidly frozen in liquid nitrogen, stored at -80°C.

Isolation and enrichment of acid-tolerant bacteria

For the initial recovery and enrichment of acid-tolerant microbial populations, frozen fecal samples were thawed at 4°C, and 1.0 g from each sample was homogenized in 9.0 mL of sterile Maximum Recovery Diluent (MRD; 1:10, w/v). To simulate early gastric stress and preferentially enrich intrinsically acid-tolerant bacteria, the suspensions were inoculated into seven different liquid media: MRS Broth (Sigma-Aldrich, 69966), M17 Broth (Sigma-Aldrich, 56156), BSM Broth (Sigma-Aldrich, 90273) supplemented with BSM supplement (Sigma-Aldrich, 83055), Brain Heart Infusion (BHI) Broth (Sigma-Aldrich, 75917), Universal Pre-Enrichment Broth (Sigma-Aldrich, 91366), Modified Tryptone Soy Broth (Sigma-Aldrich, 76704), and Nutrient Broth (Sigma-Aldrich, 70122). Using multiple formulations was intended to capture phenotypic heterogeneity and to avoid selective recovery bias toward any single bacterial group. All media were adjusted to pH 3.2 with 1.0 M HCl prior to inoculation. Ten milliliters of each broth was inoculated separately with a 100 µL aliquot of the MRD suspension from each of the nine stool samples (nine tubes per medium; 63 enrichment cultures in total). The enrichment tubes were incubated statically at 37°C for 12 h under microaerophilic conditions and then stored at 4°C until plating.

Each enrichment culture was subsequently plated onto both de Man, Rogosa and Sharpe (MRS) Agar (Oxoid, CM0361B) and M17 Agar (Oxoid, CM0785B), resulting in a total of 126 plates. MRS Agar plates were incubated at 37°C for 48 h under microaerophilic conditions, whereas M17 Agar plates were incubated at 37°C for 48 h under aerobic conditions. From each plate, 10±2 colonies were randomly selected and purified by re-streaking on the same medium. The purified isolates were then inoculated onto Triple Sugar Iron (TSI) Agar slants (Oxoid, CM0277B) and incubated aerobically for 24-48 h^[11,12]. Isolates showing an acid butt/acid slant reaction without H₂S production were considered presumptive acid-tolerant bacteria, and 1000 of these were selected and maintained as stock cultures for subsequent analyses. All purified isolates were suspended in Brain Heart Infusion broth (Oxoid, CM1135B) containing 20% (v/v) glycerol and stored at -80°C until further analysis.

Acid Shock and Bile Salt Tolerance Assays

Acute acid resistance under simulated extreme gastric conditions was evaluated by exposing bacterial cell suspensions (adjusted to 0.5 McFarland standard; ~1.5 × 10⁸ CFU/mL in sterile saline) to MRD adjusted to pH 2.0 using 0.1 M HCl. Bacterial suspensions were incubated at 37°C for 15 min under microaerophilic conditions. This exposure served as a targeted acute shock challenge to select highly resilient candidates following

preliminary acid enrichment. Bacterial viability was assessed by subsequent spot-inoculation and growth monitoring on fresh MRS agar plates incubated at 37°C for 48 h under microaerophilic conditions. Bile tolerance was determined by inoculating surviving acid-tolerant cultures into MRD supplemented with 3.0% (w/v) porcine bile extract (Sigma-Aldrich, B8631). Inoculated cultures were incubated at 37°C for 1 h. Tolerance was confirmed by subculturing onto MRS agar, representing a stringent physiological challenge ensuring survival across the small intestinal barrier.

Hemolytic Activity and Antibiotic Resistance

Candidate strains were evaluated for hemolytic activity on Blood Agar containing 5-7% defibrinated sheep blood (Oxoid, SR0051C). Following incubation at 37°C for 48 h under microaerophilic conditions, non-hemolytic strains were selected for further evaluation.

Antibiotic resistance profiles were assessed utilizing disk diffusion assays on Mueller-Hinton agar (Oxoid CM0337B), adhering to Clinical and Laboratory Standards Institute (CLSI) guidelines. This was complemented by growth evaluations in broth media containing clinically relevant antibiotics (clindamycin (Oxoid, 0064B), ciprofloxacin (Oxoid, 0425B), vancomycin (Oxoid, 0058B), azithromycin (Oxoid, 0906B), methicillin (Oxoid, 0043B), penicillin (Oxoid, 0029B), and imipenem (Oxoid, 0455B)). The observed profiles were subsequently analyzed to distinguish likely intrinsic, non-transmissible resistance characteristics (physiological vancomycin resistance in certain lactobacilli) from potential acquired resistance issues.

Antimicrobial Antagonism and Growth Under Combined Stress

For the isolation of presumptive *E. coli*, 100 µL aliquots of appropriate dilutions were spread onto Tryptone Bile X-glucuronide (TBX) Agar (Oxoid, CM0945B). Plates were incubated aerobically at 44°C for 18-24 h. Blue-green colonies, indicative of β-glucuronidase activity, were considered presumptive *E. coli*. From each plate, five typical colonies were randomly selected and purified by re-streaking on the same medium. Purified isolates were screened by Gram staining and the indole test, and isolates identified as Gram-negative, indole-positive rods were retained as presumptive *E. coli*^[13].

For the isolation of sulfite-reducing anaerobes, 1.0 mL aliquots of appropriate dilutions were inoculated by the pour-plate method into Tryptose Sulfite Cycloserine (TSC) Agar (Oxoid, CM0587B) and overlaid with a thin layer of the same medium. Plates were incubated at 37°C for 24-48 h in anaerobic jar with GasPak/AnaeroGen system (Oxoid, AN0025A). Typical colonies, resulting from the reduction

of sulfite to sulfide, were regarded as presumptive sulfite-reducing anaerobes (*Clostridium perfringens* and related species). Representative colonies were picked, purified by re-streaking on the same medium under anaerobic conditions, and examined by Gram staining and microscopy. All purified isolates were suspended in Brain Heart Infusion broth (Oxoid, CM1135B) containing 20% (v/v) glycerol] and stored at -80°C until further analysis.

Antagonistic activities of the candidate probiotic strains against five presumptive isolates of indicator pathogens (*Escherichia coli* and sulfite-reducing anaerobic bacteria from canine fecal samples) were tested using the agar well diffusion method. Cell-free Supernatant, obtained from 24-h cultures via centrifugation and filtration, was added to 6-mm wells on pre-seeded agar plates. After 24 h of incubation at 37°C under microaerophilic conditions, diameters of growth inhibition zones were measured.

Bacterial proliferation kinetics under combined intestinal stress were assessed by inoculating isolates into Selective Enrichment Broth (SEB). SEB was prepared by adjusting the basal medium to pH 3.2 and supplementing it with 2.5% (w/v) sodium cholate (Sigma-Aldrich, C1254). Growth was monitored spectrophotometrically through optical density measurements at 600 nm (OD_{600}) taken at regular intervals over a 24-h period.

Conventional Biochemical Assays and Candidate Allocation

Microbiological verification of surviving candidates was conducted using conventional methods. Catalase activity was assessed by observing effervescence upon the application of 3% H_2O_2 to colonies on glass slides. Commercial strips were utilized for the oxidase test.

The 1000 ± 1 primary isolates that were Gram-positive, oxidase-negative, catalase-negative, and non-hemolytic were retained. Thirty of these candidate isolates per dog were randomly selected using the RAND function in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA), which assigned a random number to each eligible isolate. Isolates were ranked by these values, and the ten with the lowest values were retained. The generated values were copied and pasted as static values to ensure reproducibility of the selection. This yielded 270 isolates in total.

For each animal, 30 ± 1 isolates were selected from those showing the strongest antimicrobial activity against presumptive pathogens isolated from the same fecal sample and the most favorable antimicrobial resistance profiles. This selection cascade resulted in a finalized quota of 90 candidate strains (30 strains per age groups: P-01 to P-10 for puppies, Y-01 to Y-10 for young dogs, and A-01 to A-10 for adults) being subjected to automated VITEK® 2 GP biochemical characterization.

Phenotypic Characterization Using VITEK® 2 System

Phenotypic characterization of the 90 selected candidate strains was conducted utilizing the automated VITEK® 2 system (VITEK 2 Systems software version 9.04.4), specifically with Gram-Positive (GP) identification cards (bioMérieux, Marcy-l'Étoile, France), adhering to manufacturer guidelines. Inoculum turbidity was standardized to 0.50-0.60 McFarland units using a densitometer in 0.45% sterile saline solution. The GP cards assessed 64 miniaturized biochemical substrates, encompassing carbohydrate fermentations, enzymatic hydrolyses, and inhibitory assays. Presumptive taxonomic assignments were generated by comparison with the VITEK GP database, with identification probabilities of 90% or greater being accepted. A total of 90 isolates were grouped according to their phenotypic characteristics. Within each phenotypic group, isolates were ranked according to the inhibitory activity against presumptive pathogenic strains isolated from the same fecal samples and antimicrobial resistance profile, expressed as no or minimum inhibition zone diameter, and the no or smallest zone producing isolates were selected. In this way, 10 strains were selected from each age group (puppy, young dog and adult), yielding 30 candidate probiotic strains in total [14,15].

Statistical Analysis

Data obtained from the multi-stage phenotypic screening, biochemical substrate utilization profiles, and categorical taxa presence/absence distributions across life stages were analyzed using descriptive statistics. Categorical variables and biochemical reaction frequencies are expressed as absolute counts (n), proportions, and percentages (%). No inferential hypothesis testing was applied due to the exploratory nature of the screening design.

RESULTS

Primary Screening and Functional Selection Cascade

From an initial isolation pool of approximately 1000 colonies recovered on selective media across the nine dogs, the multi-stage *in vitro* screening cascade progressively narrowed candidates based on gastrointestinal resilience and safety attributes. Stepwise acid enrichment and acute shock challenge (pH 2.0, 15 min) along with 3.0% bile exposure eliminated sensitive populations. Safety assessment on 5% sheep blood agar identified and excluded all hemolytic isolates, confirming non-hemolytic phenotypes in surviving candidates. A total of 90 isolates satisfied primary criteria, demonstrating Gram-positive staining, non-motile morphology, negative catalase, and negative oxidase reactions, alongside consistent acid production with predominantly negative gas and no generation in TSI slants (A/A profile). Subsequent secondary screening evaluating combined

gastrointestinal stress kinetics (in SEB at pH 4.5 + 2.5% sodium cholate), broad-spectrum antagonistic activity against *Escherichia coli* and fecal sulfite-reducing anaerobes (inhibition zones ranging from 11 to 21 mm), and non-transmissible antibiotic profiles yielded a finalized quota of 30 candidate strains (10 representative isolates per age group: P-01 to P-10 for puppies, Y-01 to Y-10 for young dogs, and A-01 to A-10 for adults) (Fig. 1).

Presumptive Phenotypic Characterization

Biochemical profiling of the 30 candidate strains via the automated VITEK® 2 GP system generated high-confidence presumptive profiles (identification probabilities ≥90%). All 30 isolates consistently exhibited classic LAB baseline attributes (oxidase-negative, catalase-negative, TSI slant/butt acid-positive [A/A], predominantly gas-negative, and H₂S-negative) (Table 1, Table 2, Table 3).

Table 1, Table 2, and Table 3 report the results of the four discriminating biochemical tests (positive/negative) for all 30 strains, which formed the basis of the preliminary grouping. Carbohydrate fermentation profiles across the four key discriminatory substrates (d-mannose [dMAN], d-trehalose [dTRE], d-sorbitol [dSOR4], and cyclodextrin [CDEX]) demonstrated distinct phenotypic heterogeneity among individual strains within each cohort (Table 1, Table 2, Table 3). The frequency of positive reactions was similar in puppy- and young dog-derived strains (17 and 16 of 40 reactions, respectively) and lower in adult-derived strains (10 of 40). D-Sorbitol fermentation was restricted to 4 of the 30 strains, whereas D-trehalose and D-mannose utilization were more widespread.

Distribution of Presumptively Identified Taxa Across Life Stages

Presumptive taxonomic assignment of the 30 candidate strains resolved 13 distinct bacterial species (Table 4). Six

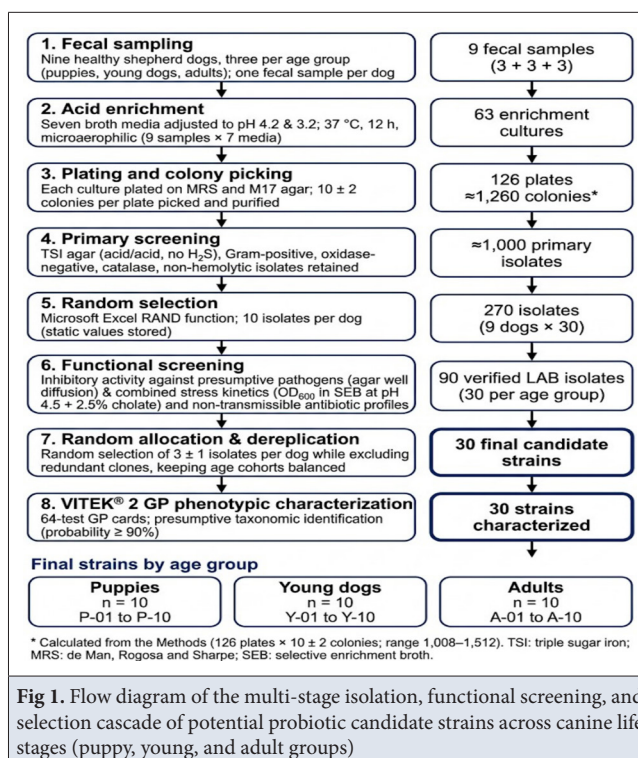


Fig 1. Flow diagram of the multi-stage isolation, functional screening, and selection cascade of potential probiotic candidate strains across canine life stages (puppy, young, and adult groups)

species were consistently recovered across all three age cohorts, representing putative “core” canine probiotic candidates: *Lactiplantibacillus plantarum* (*Lactobacillus plantarum*), *Enterococcus faecium*, *Pediococcus acidilactici*, *Limosilactobacillus reuteri* (*Lactobacillus reuteri*), *Enterococcus faecalis*, and *Pediococcus pentosaceus* (Table 4).

In addition to core taxa, several candidate species displayed stage-associated distribution patterns within the examined collection. *Lactobacillus acidophilus* and *Weissella confusa* were exclusively detected among young dog isolates, whereas *Ligilactobacillus salivarius* (*Lactobacillus salivarius*) was identified in both young and adult cohorts

Table 1. Biochemical and phenotypic characteristics of selected probiotic candidates (Puppy group)

Strain ID	Species	Oxidase	Catalase	TSI (S/B)	Gas	H ₂ S	dMAN	dTRE	dSOR4	CDEX
P-01	<i>Lpb. plantarum</i>	–	–	A/A	–	–	+	+	–	+
P-02	<i>E. faecium</i>	–	–	A/A	–	–	+	–	–	–
P-03	<i>Lcb. rhamnosus</i>	–	–	A/A	–	–	–	+	+	–
P-04	<i>P. acidilactici</i>	–	–	A/A	–	–	+	+	+	–
P-05	<i>Lmb. reuteri</i>	–	–	A/A	–	–	–	–	–	–
P-06	<i>E. faecalis</i>	–	–	A/A	–	–	–	+	–	–
P-07	<i>Lcb. casei</i>	–	–	A/A	–	–	+	–	–	+
P-08	<i>W. cibaria</i>	–	–	A/A	–	–	+	+	–	–
P-09	<i>Lmb. fermentum</i>	–	–	A/A	+	–	–	+	–	+
P-10	<i>P. pentosaceus</i>	–	–	A/A	–	–	+	–	–	–

TSI (S/B): Triple Sugar Iron agar (Slant/Butt); A/A: Acid slant/Acid butt; dMAN: d-mannose; dTRE: d-trehalose; dSOR4: d-sorbitol; CDEX: Cyclodextrin; (+): positive reaction; (–): negative reaction. Identification probabilities generated by VITEK® 2 GP cards were ≥90% for all isolates

Table 2. Biochemical and phenotypic characteristics of selected probiotic candidates (Young group)

Strain ID	Species	Oxidase	Catalase	TSI (S/B)	Gas	H ₂ S	dMAN	dTRE	dSOR4	CDEX
Y-01	<i>E. faecium</i>	–	–	A/A	–	–	+	+	–	–
Y-02	<i>Lpb. plantarum</i>	–	–	A/A	–	–	–	–	–	+
Y-03	<i>L. acidophilus</i>	–	–	A/A	–	–	+	–	–	–
Y-04	<i>P. acidilactici</i>	–	–	A/A	–	–	–	+	–	+
Y-05	<i>E. faecalis</i>	–	–	A/A	–	–	+	–	–	–
Y-06	<i>Lmb. reuteri</i>	–	–	A/A	–	–	–	–	–	+
Y-07	<i>W. confusa</i>	–	–	A/A	–	–	+	+	+	–
Y-08	<i>Lcb. casei</i>	–	–	A/A	–	–	+	+	–	–
Y-09	<i>Lgb. salivarius</i>	–	–	A/A	–	–	–	–	–	–
Y-10	<i>P. pentosaceus</i>	–	–	A/A	–	–	+	+	–	+

Table 3. Biochemical and phenotypic characteristics (Adult group)

Strain ID	Species	Oxidase	Catalase	TSI (S/B)	Gas	H ₂ S	dMAN	dTRE	dSOR4	CDEX
A-01	<i>Lpb. plantarum</i>	–	–	A/A	–	–	–	–	–	–
A-02	<i>E. faecium</i>	–	–	A/A	–	–	+	+	+	–
A-03	<i>Lmb. fermentum</i>	–	–	A/A	+	–	–	–	–	+
A-04	<i>Lcb. rhamnosus</i>	–	–	A/A	–	–	+	–	–	–
A-05	<i>P. acidilactici</i>	–	–	A/A	–	–	–	–	–	+
A-06	<i>W. cibaria</i>	–	–	A/A	–	–	–	–	–	+
A-07	<i>Lmb. reuteri</i>	–	–	A/A	–	–	–	–	–	–
A-08	<i>E. faecalis</i>	–	–	A/A	–	–	–	–	–	–
A-09	<i>Lgb. salivarius</i>	–	–	A/A	–	–	+	+	–	–
A-10	<i>P. pentosaceus</i>	–	–	A/A	–	–	–	–	–	+

Table 4. Distribution of presumptively characterized candidate species across canine life stages

Species	Puppy	Young	Adult
<i>Lpb. plantarum</i>	+	+	+
<i>E. faecium</i>	+	+	+
<i>Lcb. rhamnosus</i>	+	–	+
<i>P. acidilactici</i>	+	+	+
<i>Lmb. reuteri</i>	+	+	+
<i>E. faecalis</i>	+	+	+
<i>Lcb. casei</i>	+	+	–
<i>W. cibaria</i>	+	–	+
<i>Lmb. fermentum</i>	+	–	+
<i>P. pentosaceus</i>	+	+	+
<i>L. acidophilus</i>	–	+	–
<i>W. confusa</i>	–	+	–
<i>Lgb. salivarius</i>	–	+	+

(+): Detected; (–): Not detected in the selected candidate quota

but was absent from puppies (Table 4). Conversely, *Lactocaseibacillus rhamnosus*, *Lactocaseibacillus casei*,

Limosilactobacillus fermentum, and *Weissella cibaria* were recovered in specific combinations across juvenile and mature groups (Table 4).

DISCUSSION

The present study provides a comprehensive culture-based evaluation of probiotic candidate bacteria isolated from canine fecal microbiota across three distinct life stages, revealing both taxonomic and functional differences that are highly relevant for probiotic development. The selection of 30 phenotypically distinct strains from an initial pool of approximately 1000 isolates, combined with their biochemical characterization, highlights the importance of integrating both diversity-based and function-oriented screening strategies in probiotic research.

At the genus level, the isolates obtained in this study were predominantly affiliated with LAB, including *Lactobacillus*, *Enterococcus*, *Pediococcus*, and *Weissella*. This distribution is consistent with previous culture-based studies indicating that LAB constitute the primary group of probiotic candidates isolated from canine feces [6,7]. However, when compared with sequencing-based

microbiome studies, a clear discrepancy emerges. High-throughput analyses have demonstrated that canine fecal microbiota is dominated by phyla such as *Firmicutes*, *Bacteroidetes*, *Fusobacteria*, and *Proteobacteria*, with *Fusobacteria* often representing a major fraction [16]. In contrast, LAB typically represent a relatively minor fraction in sequencing datasets but are preferentially enriched under selective probiotic screening conditions.

This difference reflects the fundamental methodological divergence between culture-based and sequencing-based approaches. While sequencing captures the total microbial community, including non-culturable and functionally irrelevant taxa, selective culture conditions enrich for bacteria with desirable probiotic traits such as acid and bile tolerance. Therefore, the dominance of LAB in the present study confirms the effectiveness of the applied selection strategy in isolating functionally relevant probiotic candidates rather than representing the overall microbiota composition.

Importantly, the biochemical profiling of the selected strains revealed that all isolates shared core LAB characteristics, including oxidase-negative and catalase-negative reactions and a consistent acid-producing phenotype (A/A) in TSI agar. These features are well-established hallmarks of LAB and further support the reliability of the presumptive identification approach. However, despite these shared core traits, substantial differences were observed in carbohydrate utilization patterns, particularly for substrates such as mannose (dMAN), trehalose (dTRE), and sorbitol (dSOR4), which served as key discriminative features among strains.

Carbohydrate fermentation profiles for the four variable substrates (D-mannose, D-trehalose, D-sorbitol and cyclodextrin) differed among strains within each age group (Table 1, Table 2, Table 3). The total numbers of positive reactions were 17 in puppy-derived strains (Table 1), 16 in young dog-derived strains (Table 2) and 10 in adult-derived strains (Table 3). Cyclodextrin (CDEX) fermentation was observed in 4 of 10 adult-derived strains, compared with 3 of 10 puppy-derived strains and 4 of 10 young dog-derived strains. Isolates from young dogs showed 16 positive reactions across the four discriminatory substrates, compared with 17 in puppy-derived isolates and 10 in adult-derived isolates. The distribution of positive reactions among the four substrates (dMAN, dTRE, dSOR4 and CDEX) differed between age groups, as detailed in (Table 1, Table 2, Table 3). As no inferential statistical analysis was performed and the number of strains was small, these observations are descriptive only and should be interpreted with caution [9,17].

The antimicrobial activity observed against *Escherichia coli* and sulfite-reducing anaerobes further confirms the functional relevance of the selected strains. Similar

antagonistic effects have been reported for LAB isolated from canine sources, where probiotic supplementation reduced pathogenic bacterial loads and improved gut health parameters [4,5]. These findings reinforce the importance of combining phenotypic screening with functional assays in probiotic selection.

The findings of this study collectively offer preliminary evidence indicating that the selection of probiotic candidates could benefit from a comprehensive consideration of both host specificity and age-related dynamics. While the concept of host-adapted probiotics has been previously proposed, most commercial products remain age-independent. The present findings extend this concept by demonstrating that both taxonomic composition and phenotypic diversity of probiotic candidates may vary across age groups.

From a practical standpoint, selecting probiotic strains from age matched fecal microbiota may enhance colonization efficiency, ecological compatibility, and functional efficacy. This approach is particularly relevant for young and elderly animals, where microbiota instability increases susceptibility to dysbiosis. Therefore, age-specific probiotic formulations may represent a more effective strategy compared to conventional, generalized products.

The presence of these core taxa is in agreement with previous studies reporting that members of *Lactobacillus* and *Enterococcus* are among the most stable and commonly isolated probiotic-associated genera in dogs [6,7]. These bacteria are known for their resilience to gastrointestinal stress conditions, ability to colonize the intestinal mucosa, and functional roles in maintaining gut homeostasis [1]. Their detection across all life stages in the present study further supports their role as common candidate taxa across canine life stages.

In addition to this shared core, the distribution of some species differed among age groups. *Lactocaseibacillus casei*, *Limosilactobacillus fermentum*, and *Weissella cibaria* were each detected in two of the three age groups. As the number of animals was small and no inferential statistical analysis was performed, these distribution patterns are descriptive only, and their relationship to age-related changes in the gut microbiota cannot be established from the present data [8].

Conversely, species such as *Lactobacillus acidophilus* and *Weissella confusa* were detected only in the young group, indicating a transitional microbiota phase. This observation supports the concept that the gut microbiota of dogs evolves through intermediate stages before reaching a stable adult configuration. Similar age-dependent shifts in microbial composition have been previously reported, where specific taxa appear transiently during microbiota maturation [9,17].

Interestingly, *Ligilactobacillus salivarius* was absent in puppy samples but present in both young and adult groups, suggesting that certain probiotic-associated species may establish later in life and contribute to microbiota stabilization. This finding aligns with previous reports indicating that adult canine microbiota is more stable and less diverse compared to early-life stages, with a selective enrichment of functionally adapted taxa ^[9].

The detection of *Weissella* species in multiple age groups is also noteworthy. Although these bacteria are not typically dominant in sequencing-based canine microbiome studies, their repeated isolation in this study suggests that they may play a more important functional role than previously recognized. Recent studies have highlighted the probiotic potential of *Weissella* spp., including antimicrobial activity and immunomodulatory effects, supporting their inclusion as emerging probiotic candidates ^[17,18].

Although the identification of isolates in the present study was based on phenotypic characterization using the VITEK 2 system, which provides rapid and standardized biochemical profiling, it is important to acknowledge that these results should be considered presumptive. Phenotypic methods may have limited resolution in distinguishing closely related species, particularly within lactic acid bacteria. Therefore, definitive taxonomic identification requires molecular approaches such as 16S rRNA gene sequencing or whole-genome sequencing. In this context, future studies are planned to validate the taxonomic identity of the selected strains using molecular techniques and to further investigate their functional properties at the genomic level. Despite this limitation, the applied selection strategy successfully enriched for physiologically relevant probiotic candidates, and the observed age-associated patterns provide a descriptive baseline at the phenotypic level.

Overall, the distribution patterns observed in this study indicate that the canine gut microbiota consists of both stable core species and taxa that reflect microbiota maturation and adaptation processes. These findings have direct implications for probiotic development. While core species may be suitable for general probiotic formulations, age-related taxa may offer additional benefits when used in targeted formulations designed for specific life stages.

The recovery of *Enterococcus* strains (*E. faecium* and *E. faecalis*) warrants careful safety consideration. While enterococci are natural commensals of the canine gut and several strains are widely used as commercial veterinary probiotics, members of this genus may harbor opportunistic pathogenic traits or transmissible antimicrobial resistance genes (particularly vancomycin resistance) ^[19,20]. All candidate strains were non-hemolytic. Phenotypic

antibiotic testing showed that (the strains lacked inhibition zones for vancomycin), a trait that is generally regarded as intrinsic in many lactobacilli and related lactic acid bacteria and is not considered transferable. However, because only phenotypic methods were used and the species identities are presumptive, it remains to be determined whether the resistance is intrinsic or acquired; genomic analyses are therefore needed to exclude mobile resistance elements and virulence factors before any practical formulation. Although all candidate strains in this study were non-hemolytic and phenotypically susceptible to clinically relevant antibiotics under CLSI criteria, future genomic investigations are mandatory to confirm the absence of mobile resistance elements and virulence factors prior to practical formulation.

This study has some limitations to consider. Firstly, the VITEK® 2 GP system used to identify bacterial strains only provides probable identifications, not definite ones. Future research should use 16S rRNA gene sequencing and whole-genome sequencing for more accurate identification. Secondly, important characteristics like how well the bacteria stick to the gut lining, survive in the digestive system, affect the immune system, or their effectiveness in living organisms were not studied. Lastly, the study only included a small number of dogs (three per life stage), making it an exploratory study. Although factors like environment and diet were controlled by using local shepherd dogs from similar rural settings, larger studies involving multiple centers and more animals are needed to confirm these initial findings.

As a result, this study demonstrates that canine fecal microbiota represents a diverse and functionally rich source of probiotic candidate bacteria, and that both taxonomic composition and metabolic profiles of candidate isolates exhibit distinct stage-associated patterns across life stages. The findings indicate supporting the concept that stage-associated probiotic selection may offer targeted functional traits compared to conventional, generic formulations.

Although the concept of age-adapted probiotics has been previously suggested, the present study contributes experimental evidence supporting this approach and highlights the need for further research in this field. Expanding such studies, particularly through molecular identification and *in vivo* validation, will be critical for developing more targeted, effective, and scientifically validated probiotic products for dogs.

Taken together, the species distribution analysis supports the concept that probiotic selection should consider not only host specificity but also age-dependent microbiota composition. Incorporating both core and age-related strains into probiotic formulations may enhance efficacy

and better align with the physiological and microbial characteristics of the host.

DECLARATIONS

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Declaration of Generative Artificial Intelligence (AI): The authors explicitly declare that the text, tables, and figures within this manuscript were not written, generated, or created by artificial intelligence (AI) or AI-assisted technologies. Generative AI tools were utilized strictly for language editing, grammar corrections, and readability improvements. The final scientific content, data interpretation, and integrity of the work remain entirely the responsibility of the authors.

Ethical Compliance Declaration: The authors declare that the study was conducted in accordance with all applicable animal welfare and research ethics principles, institutional and national/international regulations, and the requirements of the relevant ethics committees and funding/supporting institutions, where applicable. The authors accept full responsibility for the ethical integrity of the study.

Authors Contributions: AGS: Concept Design, Experimental Work, Data Collection, Analysis, Writing - Original Draft, Correspondence; NG: Data Analysis and Interpretations, Writing - Review and Editing; MG: Supervision, Concept Design, Critical Review of the Manuscript. All authors have read and approved the final version of the manuscript and accept responsibility for its scientific integrity.

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RESEARCH ARTICLE

Estimating Goat Milk Production in Türkiye: A Comparative Analysis of Time Series and Machine Learning Approaches for Accurate Forecasting

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Abstract

Goat milk is a valuable food source because of its digestibility, nutritional value and suitability for value-added dairy products. This study forecast Türkiye's annual raw goat milk production for 2025-2029 and compared ARIMA with Decision Tree, MARS, XGBoost and SVM models using FAOSTAT data for 1961-2024. Nested expanding-window cross-validation, in which hyperparameters were tuned within each training window without access to the test year, produced 36 one-year-ahead forecasts for 1989-2024. Although MARS had the lowest errors (RMSE 33.07 versus 34.73 thousand tonnes for ARIMA), no machine-learning model differed significantly from ARIMA in Holm-adjusted Diebold–Mariano tests. The parsimonious ARIMA (2,1,0) model was therefore retained for probabilistic projections. Its point forecast declined from 468.22 thousand tonnes in 2025 to 420.39 thousand tonnes in 2029, whereas the 95% residual-bootstrap interval widened to 204.46-608.23 thousand tonnes, spanning nearly the entire historical range of the series. Sensitivity checks using post-2009 data and a fixed rolling window reproduced these conclusions. The forecasts therefore indicate substantial uncertainty rather than a firm decline and do not by themselves support specific policy measures; productivity, value-added processing and agricultural data systems warrant further investigation.

Keywords: Agricultural planning, ARIMA, Cross-validation, Forecasting, Goat milk, Machine learning, Model comparison, Time series analysis, Türkiye

INTRODUCTION

Goat milk is valued for its easy digestibility and for the production of high-value cheeses ^[1,2]. Its nutritional significance has three aspects: it is an important food source in developing countries affected by hunger and malnutrition, an alternative for consumers with allergies or gastrointestinal disorders in developed countries, and a product sought by health-conscious consumers ^[3]. Goat milk has been reported to support physiological functions in children and the elderly and to be consumed by individuals with milk allergies without adverse symptoms ^[4], and studies have addressed its nutritional properties, health benefits, production methods and comparison with other milk types ^[5].

India, Sudan, Pakistan, Bangladesh and France are the

leading producers of raw goat milk, and Türkiye ranks seventh, with 482.25 thousand tonnes in 2024 ^[6]. In these countries goat farming is closely linked to rural livelihoods. In India, indigenous dual-purpose breeds support small and marginal farmers ^[7,8], and goat milk is popularly believed, without scientific substantiation, to help in the treatment of dengue fever ^[9]. Milk consumption is embedded in traditional dietary and pastoral practices in Sudan ^[10]; rising demand and the contribution of goat farming to household income and employment have increased interest in the activity in Pakistan ^[11,12]; and goat farming is economically important in Bangladesh despite limited production technology and the socio-economic constraints of farmers ^[13,14]. France has the most organized goat milk market in Europe ^[15]; its sector specializes in largely industrial cheese production, and milk accounts for 90-93% of gross income on dairy goat farms ^[16,17].



In Türkiye, goat husbandry has a long history on the Anatolian Plateau, reflecting the country's geography and rural socio-economic structure, and goat products are a major source of animal protein in mountainous regions [18]. Forecasting studies of milk production have relied mainly on time-series models. ARIMA was selected for total milk production in India [19], a hybrid ARIMA-ANN model was the most accurate for buffalo milk production [20], autoregressive models predicted weekly goat milk yield at farm level [21], and ARIMA was more accurate than artificial neural networks (ANN) for goat and total milk production in Karnataka [22]. Goat milk yield and composition also vary across lactation [23], and trend analyses in Türkiye have reported upward trends in several animal products [24]. Machine-learning applications in the goat-milk sector, by contrast, have mostly addressed antioxidant peptides [25], adulteration detection [26], bulk-tank milk quality [27] and adaptive responses of goats to environmental conditions [28] rather than production forecasting.

Two gaps therefore remain. Empirically, the earlier national-level forecasts of Kaygısız and Sezgin [29] relied on data ending in 2016 and could not incorporate the 2021 peak and the subsequent decline. Methodologically, although algorithms such as XGBoost have been applied to Turkish agricultural data [30,31], comparative forecasting studies have generally ranked models by point-error measures such as RMSE [32,33]; such rankings do not show whether differences in accuracy exceed sampling variability, which can be assessed formally with the Diebold-Mariano test [34,35].

The scientific question of this study was whether flexible machine-learning algorithms provide a statistically detectable forecasting advantage over a parsimonious linear benchmark in a short annual production series. The null hypothesis of equal one-step-ahead predictive accuracy between each machine-learning model and ARIMA was tested. The aims were to compare four machine-learning techniques with ARIMA using nested rolling-origin cross-validation on updated FAOSTAT data (1961-2024) and to forecast Türkiye's annual raw goat milk production for 2025-2029 with quantified uncertainty.

MATERIAL AND METHODS

Ethical Approval

Ethics committee approval was not required for this study.

Data Source and Study Design

Annual raw goat milk production data for Türkiye (1961-2024, tonnes) were obtained from the FAOSTAT database [6].

Four machine-learning algorithms (Decision Tree, Multivariate Adaptive Regression Splines, Support Vector Regression and Extreme Gradient Boosting) were compared

with an ARIMA benchmark using nested expanding-window cross-validation, and the selected specification was used for probabilistic forecasts for 2025-2029.

Data Preparation and Time-Series Diagnostics

Stationarity of the untransformed series was assessed with the Augmented Dickey-Fuller (ADF), Phillips-Perron (PP) and KPSS tests [36-38]; ADF and PP test a unit-root null hypothesis and KPSS a stationarity null. Because structural change can reduce the power of conventional tests, the Zivot-Andrews one-break test and the Lee-Strazicich minimum-LM tests with one and two endogenous breaks were also applied [39-41]. Break tests were applied to the level series, and conventional tests to the level and first difference. The break tests were used diagnostically only: the candidate break year was not used to restrict the cross-validation training windows, the machine-learning features or the final ARIMA estimation, all of which used the complete 1961-2024 series.

For the machine-learning models, the response was the annual first difference in production (Δy_t) and the predictors were its first and second lags (Δy_{t-1} and Δy_{t-2}), so every feature was observable at the forecast origin. Full-sample OLS residuals were not used as predictors. For MARS and SVM, the Yeo-Johnson transformation [42] and z-score normalization was applied to the two predictors to reduce skewness and place them on a comparable scale, and were fitted only on the data in each training sample; the tree-based models used the untransformed predictors. Because the response was not transformed, no back-transformation was required when forecasts were converted to production levels.

Forecasting Models

General background on the machine-learning methods and on resampling-based control of overfitting is given in [43].

Decision Tree Algorithms (DT): Decision trees partition the predictor space into homogeneous regions and can be used for classification and regression [44-46]; the CART algorithm was used [47].

Multivariate Adaptive Regression Splines (MARS): MARS represents nonlinear relationships by adding hinge functions and then removing terms that do not improve the fit [43,48]. Because of the small number of annual observations, *prod_degree* was fixed at 1 (main effects only) in the primary model; a sensitivity run allowed *prod_degree* = 2.

XGBoost Algorithm (XGB): XGBoost builds an ensemble of decision trees sequentially, each new tree being fitted to the errors of the current ensemble [49,50].

Support Vector Machines (SVM): Support Vector Regression with a radial basis function (RBF) kernel was used [51-53];

it fits a function whose deviations from the observed values do not exceed a tolerance (ϵ) while penalizing model complexity.

ARIMA (AutoRegressive Integrated Moving Average): ARIMA was used as the univariate benchmark ^[19,54].

Hyperparameter Optimization and Nested Expanding-Window Validation

A nested expanding-window procedure separated hyperparameter tuning from model evaluation. After lag construction, the data set contained 61 observations (1964-2024). The first outer training window included 25 observations, and each model was tested on the following year, giving 36 one-step-ahead forecasts for 1989-2024. Within each outer training sample, an inner expanding-window resampling initially using 65% of the training observations (minimum inner initial window 12 observations; at least 5 inner assessments) selected hyperparameters without access to the outer assessment year. Up to 30 randomly drawn candidates were evaluated for the general machine-learning grids, and 14 candidates for the primary MARS model. Preprocessing parameters were learned inside each resample. ARIMA was re-estimated at each outer forecast origin; d was chosen by the sequential KPSS rule (maximum $d = 1$), and p and q by an exhaustive AICc search over non-seasonal models. Model comparisons used only the pooled predictions from the outer test years ^[55-57]. Validation was based on one-step-ahead ($h = 1$) forecasts, whereas the final projections covered $h = 1-5$.

To limit overfitting in this small sample ^[43], the hyperparameter search ranges were restricted and kept identical in every training window (maximum tree depth 5 for the Decision Tree and 4 for XGBoost), interaction terms were excluded from the primary MARS model, the predictor set was limited to two lagged differences, and hyperparameter selection was separated from performance evaluation by the nested design.

For each tunable model, candidates were drawn from the search ranges in [Table 1](#) using random grids (values drawn

from uniform or log-uniform distributions, oversampled and de-duplicated, then randomly subsampled to the target grid size); the small discrete MARS grid was evaluated exhaustively. Within each outer training sample, the configuration with the lowest pooled inner RMSE (computed on the differenced scale; ties broken by pooled inner MAE) was selected. [Table 1](#) reports the search range for each hyperparameter and the value selected when each model was refit to the full 1964-2024 sample.

Structural-Break Sensitivity Checks

Two checks assessed the diagnostic-only treatment of the candidate 2009 break. First, ARIMA was re-estimated using only post-break observations (2009-2024; 16 observations), and its order and 2025-2029 forecasts were compared with those of the full-sample model. A single refit was used because lag construction leaves only 13 post-break observations, too few for nested cross-validation; its intervals were obtained with the same 5,000-path i.i.d. residual bootstrap, without the residual-diagnostic selection rule. Second, the complete model comparison, with identical hyperparameter search, was repeated using a fixed 25-observation rolling training window for the machine-learning models, producing 36 one-step-ahead forecasts (1989-2024) and the same Holm-adjusted Diebold-Mariano tests. ARIMA was re-estimated from all observations available before each forecast origin under both schemes, so its errors were identical in the two analyses.

Forecast Accuracy Metrics and Statistical Comparison

Forecast accuracy was measured with RMSE, MAE, MAPE and bias on the original production scale. Forecasts of annual changes were converted to production levels by adding them to the last observed value at each forecast origin. All measures were computed from the 36 pooled outer-fold errors, because each outer test fold contained a single observation ^[33,55].

Error Metrics

- Forecast Error: the difference between the observed and predicted production level at horizon i from origin T . Written as: $\epsilon(T+i) = y(T+i) - \hat{y}(T+i)$.

Table 1. Hyperparameter search ranges and final selected values for the four tunable machine-learning models

Model	Hyperparameter(s) and Search Range	Final Selected Value(s), Full-Sample Refit
Decision Tree	cost_complexity: $10^{\wedge}U(-6, -1)$; tree_depth: {1..5}; min_n: {2..12}	cp=3.94e-05; depth=2; min_n=6
MARS	num_terms: {2..15} (14 candidates); prod_degree: fixed at 1 for the primary model (1:2 evaluated only in the separate sensitivity run)	num_terms=3; prod_degree=1
SVM (RBF)	cost: $2^{\wedge}U(-5, 7)$; rbf_sigma: $10^{\wedge}U(-5, 0)$; margin (ϵ): $10^{\wedge}U(-3, \log_{10} 0.5)$	cost=65.661; sigma=0.00829; margin=0.347
XGBoost	trees: {10, 20, ..., 500}; tree_depth: {1..4}; learn_rate: $10^{\wedge}U(-3, \log_{10} 0.3)$; loss_reduction (γ): $10^{\wedge}U(-6, 1)$; min_n: {2..10}; sample_size: $U(0.60, 1.00)$	trees=340; depth=2; min_n=6; rate=0.0036; gamma=0.0577; sample=0.946

Search ranges follow standard notation $U(a, b)$ for a uniform draw on $[a, b]$ and $10^{\wedge}U(a, b)/2^{\wedge}U(a, b)$ for a log-uniform draw exponentiated on base 10 or 2, respectively; braces $\{ \}$ denote a discrete uniform draw over the listed integer set

- RMSE (Root Mean Squared Error): $RMSE = \sqrt{[(1/n) \times \sum \epsilon(T+i)^2]}$, with $n = 36$ pooled outer-fold predictions.
- MAE (Mean Absolute Error): $MAE = (1/n) \times \sum |\epsilon(T+i)|$
- MAPE (Mean Absolute Percentage Error): $MAPE = 100 \times (1/n) \times \sum |\epsilon(T+i)/y(T+i)|$, where $y(T+i)$ is the observed production level ^[55].

Pairwise forecast accuracy was assessed using the Harvey-Leybourne-Newbold small-sample modification of the Diebold-Mariano test with a one-step horizon ($h = 1$) and squared-error loss ^[34,35]. The tests used the aligned production-level errors from the 36 outer folds. Because ARIMA was compared with four alternative models, Holm-adjusted P-values were additionally reported to control family-wise error. As a robustness check, the tests were repeated under absolute-error loss.

Final Model Estimation and Forecast Uncertainty

After model comparison, ARIMA was re-estimated on all observations up to 2024. Residual autocorrelation was assessed with a Ljung-Box test (fitdf-corrected for the estimated AR and MA orders) and conditional heteroscedasticity with a Ljung-Box test on the squared residuals (McLeod-Li test; 10 lags). Under a pre-specified rule, 95% forecast intervals were obtained from a 5.000-path i.i.d. residual bootstrap if neither test was significant at the 5% level, and from the analytic Gaussian approximation otherwise. Residual normality

was examined with the Shapiro-Wilk and Jarque-Bera tests, and analytic Gaussian intervals were computed for comparison (*Table 3*).

The four machine-learning models were also refit to the complete 1964-2024 sample with the hyperparameters in *Table 1*; only ARIMA was used for the 2025-2029 forecasts.

Software

Analyses were conducted in R 4.5.3 under Debian GNU/Linux 13. The principal package versions were tidyverse 2.0.0, tidymodels 1.4.1, forecast 9.0.2, earth 5.3.5, kernlab 0.9-33, xgboost 3.4.2.1, rpart 4.1.27, urca 1.3-4, and future 1.70.0. The structural-break sensitivity checks were run in a separate script in the same R session.

RESULTS

Model Evaluation and Selection

Türkiye's position among the world's raw goat milk producers in 2024 is shown in *Fig. 1*. Raw goat milk production in Türkiye declined in the early 1960s, remained relatively stable from the mid-1960s to the late 1970s and decreased from the 1980s to its lowest value in 2009 (192.21 thousand tonnes). It then increased until 2021 (622.79 thousand tonnes) and has declined since (*Fig. 2-A*).

The conventional tests indicated that the series was integrated of order one: ADF and PP did not reject a unit root at the level but rejected it for the first difference, whereas KPSS rejected level stationarity but not first-

Table 2. Pooled outer rolling-origin forecast performance and selected final specifications

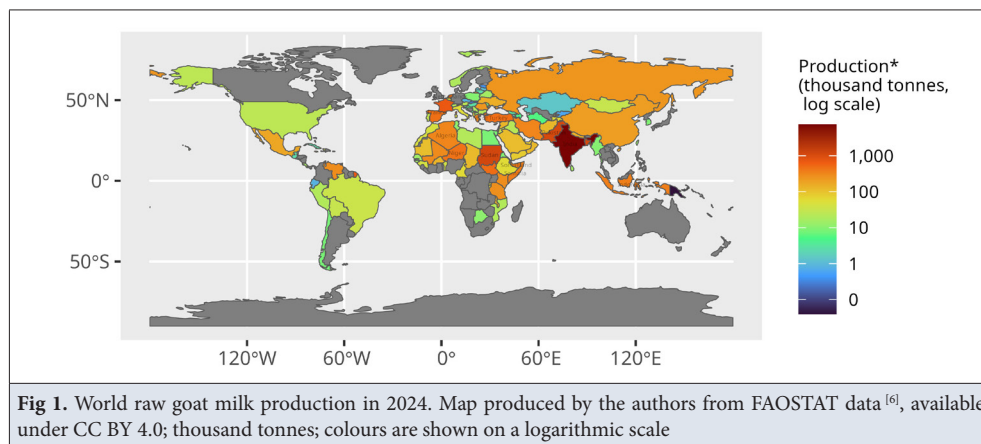
Model	Selected Final Specification	RMSE	MAE	MAPE (%)	Bias
MARS	num_terms=3; prod_degree=1	33.07	21.25	6.05	4.53
SVM	cost=65.661; sigma=0.00829; margin=0.347	34.11	22.51	6.37	4.55
XGBoost	trees=340; depth=2; min_n=6; rate=0.0036; gamma=0.0577; sample=0.946	34.36	22.76	6.44	6.67
ARIMA	ARIMA (2,1,0) final refit	34.73	22.88	6.61	4.75
Decision Tree	cp=3.94e-05; depth=2; min_n=6	36.92	24.84	7.11	4.05

RMSE, MAE, and bias are given in thousand tonnes; MAPE is given as a percentage. The figures are based on 36 one-year-ahead forecasts covering 1989-2024. Errors were calculated after the forecasts had been converted back to production levels. The specifications in the table come from models refitted to the full sample and therefore may differ from those selected in individual folds. Bias was calculated as actual production minus forecast production. A positive value means that production was underestimated on average

Table 3. Analytic Gaussian versus i.i.d. residual-bootstrap 95% interval widths for the final ARIMA (2,1,0) forecasts

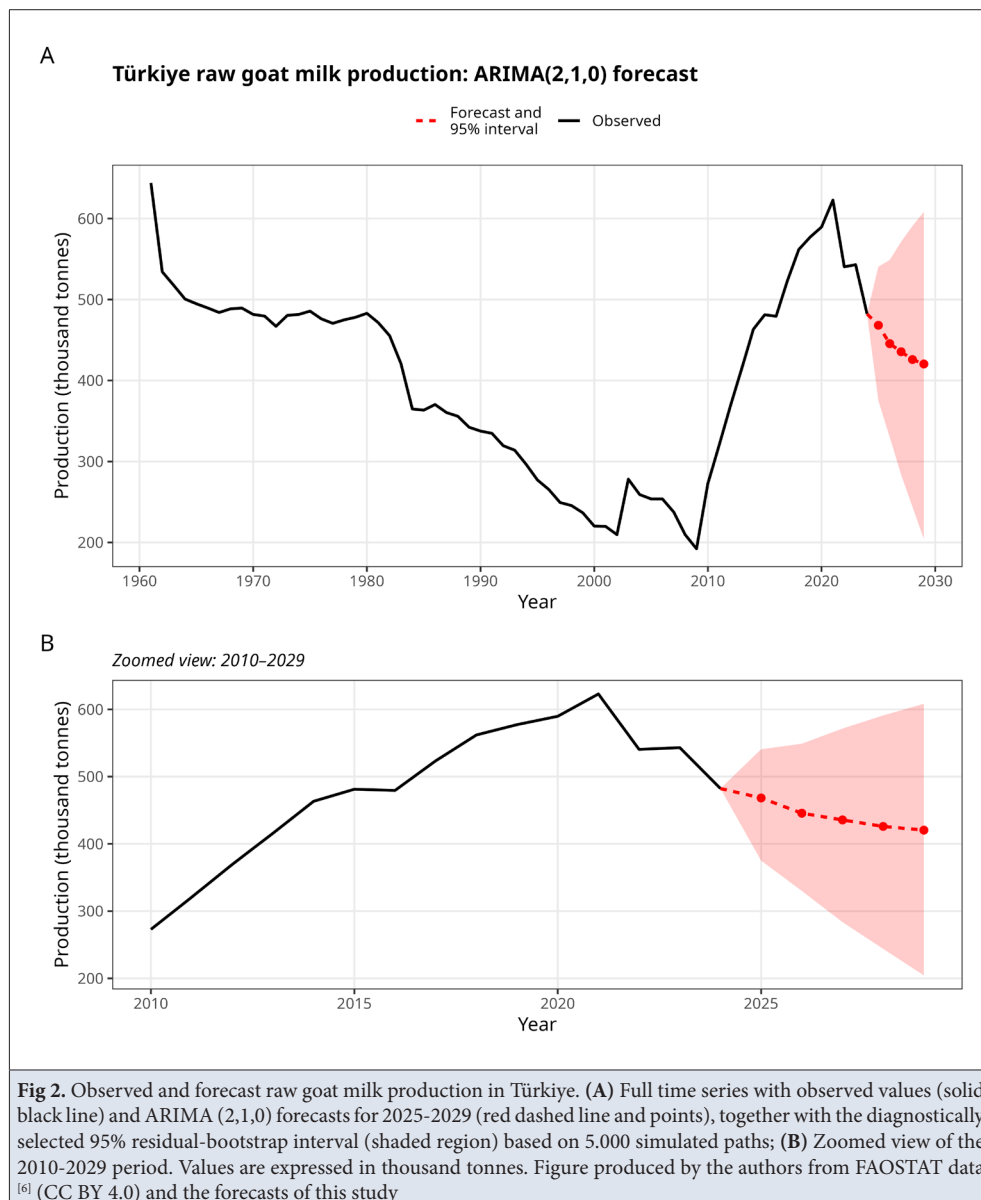
Year	Analytic Gaussian 95% Width	i.i.d. Residual-bootstrap 95% Width
2025	110.51	165.53
2026	176.42	218.79
2027	251.41	287.70
2028	319.93	346.91
2029	385.80	403.77

Interval widths (Upper 95 minus Lower 95) are in thousand tonnes, computed from the analytic Gaussian and i.i.d. residual-bootstrap forecasts produced for the same final ARIMA (2,1,0) specification



difference stationarity (*Table 4*). Zivot-Andrews rejected the unit root only in the intercept-break model, with a break in 2009; the trend-break and combined-break

models and all Lee-Strazicich specifications did not reject it (*Table 4*). First differencing was therefore retained for the primary models.



Pooled performance of the 36 one-step-ahead outer forecasts (1989-2024) is given in [Table 2](#).

MARS had the lowest RMSE, MAE and MAPE, with an RMSE 4.79% lower than that of ARIMA; SVM, XGBoost and ARIMA had similar errors, and the Decision Tree had the highest ([Table 2](#)). None of the four Diebold-Mariano comparisons with ARIMA was significant, before or after Holm adjustment ([Table 5](#)), and the conclusion was unchanged under absolute-error loss (DM statistics -0.983 to 0.941 ; raw $P \geq 0.332$; all Holm-adjusted $P = 1.000$). All models had a positive mean bias ([Table 2](#)). For every model, the two largest absolute errors occurred in 2010 and 2022, the years following the 2009 trough and the

2021 peak: production was underpredicted in 2010 (by 87.67-98.51 thousand tonnes) and overpredicted in 2022 (by 89.63-112.75 thousand tonnes). The largest single error accounted for 20.1-25.9% of each model's pooled squared error.

The unrestricted MARS variant (prod_degree = 1:2) gave the same predictions as the primary model in 33 of 36 folds, but its pooled RMSE was more than twice as high ([Table 6](#)). This difference arose mainly from the 2012 test year, in which the lagged-difference input fell outside the training range: the unrestricted variant predicted -19.01 thousand tonnes, whereas the primary model predicted 316.48 thousand tonnes (actual: 369.43 thousand tonnes).

Table 4. Unit-root and structural-break test results for the annual goat milk production series, 1961-2024

Test	Series and Specification	Statistic	5% Critical Value	Break Year(s)	Decision at 5%
ADF	Level; constant and trend	-1.750	-3.450	–	Unit root not rejected
PP	Level; constant and trend	-1.706	-3.481	–	Unit root not rejected
KPSS	Level; constant and trend	0.156	0.146	–	Stationarity rejected
ADF	First difference; constant	-3.059	-2.890	–	Unit root rejected
PP	First difference; constant	-6.769	-2.908	–	Unit root rejected
KPSS	First difference; constant	0.288	0.463	–	Stationarity not rejected
Zivot-Andrews	Level; intercept break	-5.918	-4.800	2009	Unit root rejected
Zivot-Andrews	Level; trend break	-3.636	-4.420	2004	Unit root not rejected
Zivot-Andrews	Level; intercept and trend break	-3.591	-5.080	2003	Unit root not rejected
LS, one break	Level; Model A (level break)	-1.807	-3.566	2003	Unit root not rejected
LS, one break	Level; Model C (level and trend break)	-3.812	-4.463	2010	Unit root not rejected
LS, two breaks	Level; Model A (level breaks)	-2.023	-3.842	2003; 2010	Unit root not rejected
LS, two breaks	Level; Model C (level and trend breaks)	-4.914	-5.730	2006; 2016	Unit root not rejected

ADF, augmented Dickey-Fuller (lag length selected by BIC, maximum 4); PP, Phillips-Perron and KPSS (bandwidth 10); LS, Lee-Strazicich minimum LM test (lag selection general-to-specific, maximum 4). ADF, PP, Zivot-Andrews, and LS test the null of a unit root; KPSS tests the null of stationarity. The break tests were used diagnostically only (see Materials and Methods)

Table 5. Primary (expanding-window) Diebold-Mariano tests against the ARIMA benchmark, all four comparisons

Comparison (ARIMA vs.)	DM Statistic	Raw P-value	Holm-adjusted P
MARS	0.943	0.352	1.000
SVM	0.299	0.767	1.000
XGBoost	0.176	0.861	1.000
Decision Tree	-0.947	0.350	1.000

Figures are taken directly from the pooled outer-fold predictions of the primary analysis

Table 6. MARS interaction-term (prod_degree) sensitivity check

Measure	MARS (prod_degree=1, primary)	MARS (prod_degree=1:2, unrestricted)
Pooled outer-fold RMSE (thousand tonnes)	33.07	72.14
Folds with a different prediction than the other variant (of 36)	–	3
Worst single-fold outcome (2012 test year)	316.48 (valid; error +52.95)	-19.01 (invalid: negative production)

RMSE and forecast values are in thousand tonnes

The difference between the two variants was not significant (DM = -0.999, raw P=0.324, Holm P=0.324).

Re-estimation on the post-break observations (2009-2024) selected the same ARIMA (2,1,0) order. Its forecasts were close to the full-sample forecasts in 2025 but lower thereafter (376.19 versus 420.39 thousand tonnes in 2029), and its interval was wider at the five-year horizon (Table 7).

With a fixed 25-observation rolling window, the pooled RMSE and MAPE of all four machine-learning models were higher than with the expanding window; the increase was largest for the Decision Tree and SVM and smallest for XGBoost and MARS (Table 8). MARS again had the lowest pooled error, and none of the Holm-adjusted Diebold-Mariano comparisons with ARIMA was significant (Table 9).

Final Production Forecasts

The full-sample ARIMA (2,1,0) model (AIC = 1474.20, AICc = 1474.61, BIC = 1480.63) showed no residual autocorrelation (Ljung-Box $Q^* = 6.517$, df = 8, P=0.590)

and no squared-residual dependence (McLeod-Li, 10 lags, statistic = 2.637, P=0.989); residual-bootstrap intervals were therefore used. Residual normality was rejected (Shapiro-Wilk $W = 0.871$, P<0.001; Jarque-Bera = 54.50, df = 2, P<0.001), mainly because of excess kurtosis (7.48; skewness -0.31), and three of the 64 standardized residuals exceeded 3 in absolute value. The bootstrap intervals were wider than the analytic Gaussian intervals at every horizon (Table 3).

The ARIMA (2,1,0) point forecast declined from 468.22 thousand tonnes in 2025 to 420.39 thousand tonnes in 2029, while the 95% residual-bootstrap interval widened from 375.08-540.60 to 204.46-608.23 thousand tonnes (Table 7; Fig. 2). The 2029 interval includes values above the 2024 production level (482.25 thousand tonnes) and spans most of the range observed in the historical series (Fig. 2-A).

DISCUSSION

MARS ranked first on all three point-error measures, but its RMSE advantage over ARIMA (4.79%) was not statistically significant, and none of the four machine-learning models differed significantly from ARIMA

Table 7. Full-sample versus post-break-only ARIMA (2,1,0) forecasts, 2025-2029

Year	Full-sample ARIMA (2,1,0) Forecast [95% CI]	Post-break-only ARIMA (2,1,0) Forecast [95% CI]
2025	468.22 [375.08–540.60]	469.99 [375.52–528.11]
2026	445.53 [330.08–548.87]	432.10 [304.95–527.43]
2027	435.54 [283.64–571.34]	416.43 [226.80–556.53]
2028	425.91 [243.94–590.85]	391.00 [149.95–577.54]
2029	420.39 [204.46–608.23]	376.19 [70.47–616.03]

Figures are taken directly from the structural-break sensitivity checks described in Materials and Methods; forecast values are in thousand tonnes

Table 8. Pooled outer-fold performance under the expanding-window (primary) and fixed rolling-window training schemes

Model	Expanding RMSE	Rolling RMSE	Expanding MAPE (%)	Rolling MAPE (%)
MARS	33.07	34.62	6.05	6.26
ARIMA	34.73	34.73*	6.61	6.61*
XGBoost	34.36	34.82	6.44	6.49
SVM	34.11	38.48	6.37	7.05
Decision Tree	36.92	41.74	7.11	7.72

RMSE is in thousand tonnes and MAPE in percent. *ARIMA's rolling-window and expanding-window metrics are identical by construction (see Methods). Expanding-window values are those reported in Table 2

Table 9. Rolling-window Diebold-Mariano tests against the ARIMA benchmark

Comparison (ARIMA vs.)	DM Statistic	Raw P-value	Holm-adjusted P
MARS	0.048	0.962	1.000
XGBoost	-0.046	0.963	1.000
SVM	-1.430	0.161	0.646
Decision Tree	-1.128	0.267	0.800

Figures are taken directly from the rolling-window structural-break sensitivity check described in Materials and Methods

after Holm adjustment. These results should be read as a ranking without statistical evidence of a difference, not as evidence of equivalence: a non-significant Diebold–Mariano test indicates only that the 36 pooled one-step-ahead errors did not provide sufficient evidence of a difference, and with so few errors the test has limited power to detect differences of the size observed, even with the modified Diebold-Mariano statistic recommended by Harvey et al.^[35]. ARIMA was therefore retained for the final projections neither because the test favoured it nor because it ranked first, but for reasons that do not depend on the test result: it is the most parsimonious specification, with directly interpretable parameters; it yields coherent multi-step prediction intervals directly^[55], whereas the machine-learning models were set up here only as one-step-ahead forecasters of annual changes and do not provide comparable probabilistic multi-step forecasts; and its point-forecast accuracy was close to that of the best-ranked model. The choice is therefore a practical one. It does not imply that ARIMA was the most accurate point forecaster, that all models performed equally, or that differencing removed all nonlinear behaviour or structural change from the series; a direct comparison of the five-year trajectories implied by MARS and ARIMA was not possible within the present one-step framework and is noted as a limitation below.

ARIMA has also performed well in earlier forecasts of aggregate milk production^[19], and Kumar et al.^[22] reported ARIMA to be more accurate than ANN for goat and total milk production, whereas an ARIMA-ANN hybrid was the most accurate model for buffalo milk production in India^[20]. In Turkish livestock series, ARIMA also provided the most successful forecasts of goat red-meat production in a comparison with exponential smoothing and STLF methods^[32]. At a much finer temporal scale, autoregressive models based on current and previous yields predicted weekly goat milk yield well in a farm-level study^[21], although that setting is not directly comparable to annual national aggregates. In our analysis, no model was clearly superior, and the machine-learning models were retained as out-of-sample comparators rather than used to generate the 2025–2029 projections. Evidence from other domains is mixed: in seasonal rainfall forecasting in India, XGBoost was more accurate than ARIMA and several other machine-learning models^[50], whereas in an early comparison of support vector machines, recurrent neural networks and ARIMA models, ARIMA performed best on linearly generated data, support vector machines gained an advantage only on a strongly nonlinear chaotic benchmark, and the two performed equally on a real industrial series^[53]. The absence of a detectable advantage for the machine-learning models here is consistent with, but does not demonstrate, a largely linear dynamic in

the differenced production series. Updating the series to 2024 matters because the 2021 peak and the subsequent decline are now part of the observed data: the ARIMA forecast for 2026 (445.53 thousand tonnes) is lower than the 495.54 thousand tonnes projected by Kaygısız and Sezgin^[29] from data ending in 2016. Because the current 95% interval for 2026 (330.08–548.87 thousand tonnes) includes their estimate, this difference is not sufficient to conclude that the earlier forecast was inaccurate. Using breed-level Turkish Statistical Institute data for 1991–2019, Serin and Siren^[24] found that trend tests disagreed on the direction of Anatolian Black goat milk production, with some indicating an upward and others a downward trend; because their series ended in 2019 and differed in source and coverage from the national FAOSTAT totals used here, it did not include the 2021 peak and subsequent decline, and the two analyses are not directly comparable. Finally, Şahin Demirel and Erik^[31] used XGBoost to examine the effect of climatic variables on small-ruminant milk yield at the provincial level; multivariate applications of this kind complement the univariate national-level comparison presented here.

Goat milk production rose after the 2009 trough, peaked in 2021, and has declined since then^[6]. Goat milk supply in Türkiye rests largely on the indigenous Hair goat, which accounts for most of the national herd, whereas specialised dairy genotypes such as Maltız and Saanen are limited in number and concentrated in the Aegean and Marmara regions^[18]. Structural factors of this kind are not observed by a univariate model, and their contribution to the turning points in the series could not be assessed here. The ARIMA point forecasts continue this decline gradually, from 468.22 thousand tonnes in 2025 to 420.39 thousand tonnes in 2029, but the residual-bootstrap intervals widen considerably as expected for a differenced ($d = 1$) ARIMA model whose prediction intervals continue to grow with the forecast horizon^[55], and, by 2029, are compatible with further decline, stabilization, or recovery; the point forecasts are therefore best read as one plausible path rather than a firm prediction. Because the final-model residuals were heavy-tailed, the residual-bootstrap intervals, which do not assume normality, were wider than the analytic Gaussian intervals at every horizon (*Table 3*), indicating that a Gaussian approximation would have understated forecast uncertainty. All models also had a positive average bias, that is, they underpredicted production on average (*Table 2*). Because every model forecasts annual changes from the most recently observed level, it can respond to a turning point only after that turning point has been observed; consistent with this, the two largest errors of every model occurred in 2010 and 2022, immediately after the 2009 trough and the 2021 peak.

Methodologically, the study combines nested rolling-origin validation, which separates hyperparameter tuning from performance evaluation ^[44] and follows recommendations to use rolling origins, recalibration at each origin and multiple test periods when evaluating forecasts on a single series ^[56,57], with Holm-adjusted Diebold-Mariano tests, which assess whether ranking differences exceed what would be expected from sampling variability ^[34]. Earlier comparative forecasting studies of Turkish livestock and crop series identified the best-performing method for each series ^[32] or ranked models by test-set RMSE from a single training-testing split ^[33]; the present results show that a first-place ranking need not be statistically distinguishable from a parsimonious benchmark. While machine-learning algorithms continue to be applied in regional yield modelling and climate-livestock studies ^[31], and, in the goat sector, mostly to tasks other than production forecasting, such as adulteration detection ^[26], milk-quality prediction ^[27] and modelling adaptive responses to climatic conditions ^[28], their added value for short univariate macro-level series should be demonstrated empirically rather than assumed.

The MARS interaction-term sensitivity check provides empirical support for the a priori decision to fix `prod_degree` at 1 in the primary MARS specification. Allowing interaction terms changed the prediction in only 3 of 36 folds, so the practical cost of this restriction was minimal in most years; however, because MARS represents interactions as products of spline basis functions ^[48], an input outside the training range enters the prediction through a product term; in the one fold where the lagged input fell outside the range observed in training, permitting interactions led the model to extrapolate to a physically impossible negative production forecast, more than doubling the pooled RMSE. This single fold dominated the mean-squared-error comparison of the two variants, yet the Diebold-Mariano test did not identify the difference as significant. It also supports preferring the simpler, more stable specification when the training sample is this small ^[44]. The Decision Tree ranked last on all three point-error measures; because a regression tree predicts a constant value within each terminal node ^[44], the selected depth-2 tree could return at most four distinct predicted changes, limiting its ability to follow the series.

The Zivot-Andrews test, which estimates the break date from the data ^[39], indicated a candidate intercept break around 2009, whereas the Lee-Strazicich tests, which allow for breaks under both the null and the alternative hypotheses so that a rejection unambiguously implies trend stationarity ^[40] and spurious rejections are avoided ^[41], did not reject the unit root under any specification (Table 4). Because this candidate break was used only diagnostically in the primary analysis rather than to

restrict model estimation or validation, two sensitivity checks examined whether this choice mattered in practice. Re-estimating ARIMA from post-break data alone reproduced the same model order and a broadly similar, if somewhat lower and less certain, five-year forecast path, suggesting that the diagnostic-only treatment of the break did not conceal a materially different post-break dynamic. Repeating the full model comparison with a fixed rolling training window, rather than the expanding window used throughout ^[56], did not improve the machine-learning models' relative standing against ARIMA - if anything, every machine-learning model performed somewhat worse with less historical data to draw on, and none was significantly different from ARIMA under the same Holm-adjusted Diebold-Mariano procedure. Taken together, these checks support treating the reported model comparison and forecast trajectory as reasonably robust to the candidate structural break, while the wider intervals obtained under the post-break-only re-estimation reinforce, rather than contradict, the emphasis already placed on forecast uncertainty. These checks do not, however, model the break explicitly (for example, through break dummies or regime-specific parameters). The post-break analysis, based on a single refit, addresses the stability of the model order and forecast path rather than comparative accuracy, and the rolling-window check restricted the training history only for the machine-learning models, so it does not show whether a rolling-window ARIMA would perform differently.

A key limitation of this study is its univariate approach, which relies solely on historical production data. In addition, the machine-learning models were given only two lagged differences as predictors, a design similar to the three-lag autoregressive set-up used with log-differenced annual FAOSTAT data for cherry production in Türkiye ^[33]; this safeguards against overfitting with 61 observations, but it effectively reduces these algorithms to flexible second-order autoregressive functions and prevents them from exploiting their main strength, the combination of many predictors. With 61 observations after lag construction, flexible algorithms such as XGBoost and RBF-SVR also carry a risk of overfitting and of unstable, high-variance predictions ^[43]; the design restrictions described in the Methods limit but do not eliminate this risk, and with only 36 pooled errors small-sample instability cannot be excluded as an explanation of the non-significant Diebold-Mariano results. This design constraint, and not only the small sample, is a plausible explanation for the absence of significant differences from ARIMA. Future work could incorporate exogenous variables such as feed prices, climate conditions, and macroeconomic indicators, support policies and animal-health events, the latter two having been proposed as

factors behind trends in Turkish livestock production ^[24], using model classes designed for such inputs, for example ARIMAX- or SARIMAX-type dynamic regression models, structural and Bayesian structural time-series models, or panel-data models across provinces; in a multivariate comparison for raw milk supply in Türkiye, SARIMAX gave the most accurate forecasts, followed by XGBoost ^[30]. Hybrid approaches, such as modelling ARIMA residuals with a machine-learning model, analogous to the ARIMA-ANN hybrid that was most accurate for buffalo milk in India ^[20], were also not evaluated here.

A further limitation concerns the forecast horizon used for model evaluation: model comparison and selection, including the Diebold-Mariano tests reported above, were based exclusively on one-step-ahead ($h=1$) rolling-origin errors, whereas the final projections extend to a five-year horizon ($h=1$ through $h=5$). The relative ranking of ARIMA and the machine-learning alternatives at longer horizons was therefore not assessed within the same cross-validated framework, and the reported comparative accuracy should be read as evidence about one-year-ahead performance rather than about the full five-year forecast path; future work should evaluate multi-step accuracy directly. Finally, the sampling uncertainty of the error metrics themselves was not quantified (for example, through bootstrap confidence intervals for RMSE differences), although the Diebold-Mariano conclusions did not change when absolute-error loss was used instead of squared-error loss.

Because this study forecasts aggregate production without identifying the causal drivers of the projected trajectory, its results cannot determine which interventions would reverse or mitigate a decline. The width of the prediction intervals also argues for flexible, adaptive planning rather than commitments tied to a single point forecast. The following areas are therefore suggested for further policy investigation; they are consistent with, but not derived from, the forecasts, and their relevance does not depend on which forecast scenario materializes:

- Farm productivity and herd quality: whether support for small-ruminant farming that focuses on animal health, farm management, and productivity, including, where appropriate for local climate and production systems, the use of higher-yielding breeds (within Türkiye, the Kilis and Shami breeds are already recognised for higher milk yield than the dominant Hair goat ^[18]), can stabilize supply warrants dedicated evaluation.
- Value-added processing: if the supply of raw goat milk falls or remains uncertain, processing a larger share into higher-value products such as cheese, yoghurt, and ice cream ^[5] could raise income from the

existing supply. In European dairy goat sectors, milk is largely processed on-farm for national markets, with some protected-designation cheeses exported ^[17], and in some markets the commercial range of goat milk products, including fermented drinks and organic products, has been expanding ^[4]. Feasibility nevertheless requires separate market and cost analysis, particularly because marketing margins along the Turkish goat-product value chain have been described as irregular ^[18].

- Data infrastructure: annual aggregates ^[6] cannot capture within-year variation such as the lactation-stage changes in milk yield and composition reported for goats ^[23]; more frequent, granular, and multivariate production data would allow future studies to model the drivers of production directly and could improve the precision of forecasts.

In conclusion, none of the four machine-learning models provided a statistically detectable one-step-ahead accuracy advantage over a parsimonious ARIMA benchmark for Türkiye's annual goat milk production, and the ARIMA projections for 2025-2029 carry uncertainty wide enough to encompass further decline, stabilization, or recovery. Forecasts of this kind are most useful as a transparent, regularly updated reference for decision support, interpreted together with their uncertainty rather than as point targets.

DECLARATIONS

Availability of Data and Materials: The production data analyzed in this study are publicly available from the FAOSTAT database ^[6] under the Creative Commons Attribution 4.0 International license (CC BY 4.0); *Fig. 1* and *Fig. 2* were produced by the authors from these data. The analysis code (main analysis script and structural-break sensitivity module) and outputs are available from the corresponding author (ODB) on reasonable request.

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Ethical Compliance Declaration: The authors declare that this study involves the analysis of pre-existing, fully anonymized secondary data. No animal subjects were directly involved during this phase of the research, and all data were handled strictly in accordance with fundamental principles of data privacy and academic integrity.

Authors' Contributions: KK: Revision, editing, and finalization of the manuscript. All authors participated in the analysis process, ensuring the accuracy and validity of the results. ODB: Revision, editing, and finalization of the manuscript. HD, ST: Conceptualization, design, planning, investigation, data analysis, and writing the original draft. AKG, ZÇK: Revision, editing, and finalization of the manuscript. BEAU: Data analysis, revision and editing of the manuscript. They also reviewed and approved the final manuscript.

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RESEARCH ARTICLE

A sRNA Subset Present in *Brucella melitensis* 16M OMVs Regulates Intracellular Survival of *Brucella melitensis*

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Abstract

Intercellular communication mediated by extracellular vesicles (EVs) is a key component of host-pathogen interactions. Emerging evidence suggests that Outer Membrane Vesicles (OMVs) enter host cells via multiple pathways and release cargo, including proteins and small RNAs (sRNAs), that regulate gene expression and function in recipient cells. In this study, we used *Brucella melitensis* 16M and its OMVs to construct an OMV-specific sRNA dataset and investigate their unique role in *B. melitensis* 16M intracellular survival. A total of 1,783,364 unique sRNA sequences were identified, and the ten most abundant sequences were determined by sequencing sRNAs extracted and purified from *B. melitensis* 16M and its OMVs. Bioinformatic analysis showed that eight sRNAs within OMVs exhibited stable secondary structures, and six were predicted to interact with mouse mRNAs. Notably, sRNA t0000835 was predicted to target the G protein-coupled receptor 45 (GPR45) gene and suppress its expression by binding to Argonaute 2 (AGO2). Transfection of sRNA t0000835 markedly enhanced intracellular survival of *B. melitensis* 16M. Collectively, these findings support the hypothesis that sRNAs t0000835 of *B. melitensis* 16M OMVs modulates host gene expression to promote the intracellular viability of *B. melitensis* 16M.

Keywords: *B. melitensis* 16M, OMVs, Regulatory mechanisms, sRNA

INTRODUCTION

Brucella is a Gram-negative, facultative intracellular bacterium with a short-rod-shaped morphology ^[1]. It belongs to the α -Proteobacteria class ^[2] and comprises multiple species that infect a wide range of mammalian hosts ^[3,4]. Traditionally, the genus has been divided into six distinct species based on host preference: *B. suis*, *B. melitensis*, *B. abortus*, *B. ovis*, *B. neotomae*, and *B. canis* ^[5]. More recently, additional species from humans (*B. inopinata*), aquatic mammals (*B. pinnipedialis* and *B. ceti*), and a common vole (*B. microti*) have been identified, bringing the total number to ten recognized species in the genus ^[6].

In animal, *Brucella* infection typically causes abortion, infertility, and lameness. As a facultative intracellular pathogen, it survives and replicates within macrophages ^[7,8]. This capacity depends on the specialized secretion system of the bacterium, particularly the Type IV Secretion

System (T4SS) ^[9], which translocates a diverse repertoire of effector proteins into host cells. These effector proteins alter host signaling pathways, cytoskeletal organization, and immune response mechanisms ^[10], thereby creating an intracellular microenvironment that facilitates *Brucella*'s survival and replication.

Extracellular vesicles (EVs) have attracted significant interest in recent years as important mediators of secretory functions and intercellular communication. Specifically, they function in intraspecies cellular information transduction and also act as a crucial nexus in host-pathogen interactions ^[11, 12], which are fundamentally shaped by their distinctive biological and structural properties. EVs are nanoscale, membrane-bound particles with a spherical morphology, typically 10-1000 nm in diameter ^[13]. These vesicles are delimited by a lipid bilayer and encapsulate a diverse array of cellular constituents, including proteins, lipids, nucleic acids, and other biomolecules ^[11,14]. The lipid bilayer protects



intravesicular RNA and proteins from extracellular degradation, thereby significantly enhancing cargo stability. EVs can enter target cells through multiple pathways and molecular components, including proteins, small RNAs (sRNAs), tRNA fragments, and microRNAs (miRNAs), that regulate host gene expression and cellular functions [14].

OMVs (Outer Membrane Vesicles) are spherical structures (approximately 10 to 300 nm in diameter) derived from the outer membrane of Gram-negative bacteria [15,16]. They encapsulate periplasmic and cytoplasmic components, such as sRNAs, tRNA fragments, DNA, lipids, and proteins [14]. OMVs mediate the delivery of sRNAs and tRNA fragments, typically 18-50 nucleotides in length, into mammalian host cells [12]. These genetic elements, derived from transcriptional processes, contain complementary binding sites for specific host miRNAs. They regulate gene expression through two principal mechanisms: 1) base-pairing with either the translation initiation region or coding sequences of target mRNAs and 2) functioning as competitive endogenous RNAs that sequester host miRNAs, thereby acting as sRNA sponges [17-19]. tRNAs (70-100 nucleotides) adopt evolutionarily conserved sequences that assemble into distinctive L-shaped tertiary structures with characteristic cloverleaf secondary configurations [20]. Although systematic investigations remain limited, accumulating evidence indicates that tRNA-derived fragments exert bidirectional regulatory effects on protein translation [21], including mediating gene silencing in eukaryotic cells through an Argonaute 2 (AGO2)-dependent, miRNA-like mechanisms [22]. However, few studies have described the role of OMVs in the delivery of sRNA, tRNA fragments, and miRNAs to target cells or have elucidated the mechanisms by which they mediate intercellular signaling [23].

Current evidence supports the working hypothesis that *Brucella* employs OMV biogenesis as a T4SS-independent non-canonical secretory mechanism for intracellular adaptation. This vesicle-mediated pathway potentially constitutes an evolutionary innovation that enables bacterial persistence under periplasmic stress, particularly during nutrient deprivation, phagosomal acidification, and exposure to host-derived reactive oxygen/nitrogen species (RONS). These microenvironmental pressures are key modulators governing *Brucella*'s parasitic strategy within host cells.

The experimental foundation for this hypothesis was derived from established protocols for the isolation/purification of OMVs from *B. melitensis* 16M strains, coupled with preliminary characterization of their stress-responsive release dynamics and functional proteomic profiles. To mechanistically validate this paradigm, this study employed a dual approach. First, we systematically

constructed a comprehensive OMV-associated sRNA dataset using high-throughput sequencing to characterize vesicular RNA cargo. Second, we evaluated the functional roles of selected sRNA effectors in modulating intracellular survival. This integrated strategy aimed to decode the novel regulatory mechanisms by which *B. melitensis* 16M employs vesicles to mediate host-pathogen co-adaptation.

MATERIAL AND METHODS

Ethical Statement

This study did not involve any procedures requiring ethical approval.

Isolation and Purification of OMVs from *B. melitensis* 16M

Approximately 1×10^6 *Brucella* cells were inoculated onto *Brucella* agar (BD Difco) and statically incubated at 37°C with 5% CO₂ (vol/vol) for 4 days. Single-colony strains were transferred to *Brucella* broth (BD Difco) and cultured at 37°C with shaking (180 × g) until the logarithmic growth phase (OD_{600 nm} = 0.4). OMVs were purified as described previously by Gamazo et al. [24]. Briefly, bacterial cells were harvested using a rubber policeman and suspended in 250 mL sterile phosphate-buffered saline (PBS; 1M, pH7.3). The suspension was centrifuged at 10 000 × g for 30 min, and the supernatant was filtered through a 0.22 µm membrane (Millipore Corp.). Sterility test was performed by culturing an aliquot onto a Tryptic Soy Agar (TSA) plate followed by incubation for 72 h at 37°C; the filtrate was stored at 4°C during the viability check. The filtered supernatant was ultracentrifuged at 100.000 × g for 2 h at 4°C. The resulting pellet was washed twice with 25 mL sterile PBS, and the OMVs were resuspended in 1 mL sterile PBS. Total protein concentration was determined using the PIERCE-BCA (PIERCE) reagent, according to the manufacturer's recommendations. The OMVs samples were divided into 0.5 mL aliquots and stored at -20°C until use. All procedures involving live *Brucella* were performed in Biosafety Level 3 (BSL3) facility.

RNA Isolation and Purification from *B. melitensis* 16M

Approximately 5×10^9 CFU of *Brucella* were resuspended in 1 mL of TRIzol (Invitrogen) and RNA purification was conducted using the MiniBEST Universal RNA Extraction kit (Takara, Beijing, China). RNA quantification and purity were determined using an OPTIZEN NanoQ microvolume spectrophotometer (KLAB CO, LTD). Samples consistently showed A260/280 ratios higher than 2.0, and A260/230 ratios higher than 1.8.

RNA Isolation from *Brucella melitensis* 16M OMVs

Enrichment, purification and identification of OMV RNA was performed using a modified lysis and organic

extraction protocol. Briefly, 200 μ L of OMV suspension was mixed with an equal volume of 7% sodium dodecyl sulfate (SDS) lysis buffer and 10 μ L protein digestion solution, vortexed for 10 s, and incubated at 65°C for 10 min. The sample was transferred to the adsorbent column, mixed with 0.5 mL anhydrous ethanol and centrifuged at $12.000 \times g$ for 10 min at 4°C. The supernatant was collected and transferred to an 1.5 mL RNase-free centrifuge tube, added chloroform and vortexed for 10 s, and incubated at room temperature for 8 min. After centrifugation ($12.000 \times g$, 4°C for 10 min), the supernatant was collected and mixed with pre-cooled isopropanol to precipitate RNA. The pellet was washed with 1 mL pre-cooled 75% ethanol, air-dried at room temperature for 5 min and resuspended in 30 μ L RNase-free water. The extracted RNA was stored at -80°C for subsequent analysis.

B. melitensis 16M and sRNA Sequencing of Its OMVs

RNA samples isolated and purified from *B. melitensis* 16M and OMVs, were treated with DNase and quality was assessed using a bioanalyzer. For each sample, 1 μ g of DNase-treated total RNA was used to prepare cDNA libraries using the TruSeq Small RNA Library Preparation Kit (Illumina). Libraries were sequenced as 50 bp single-end reads using an Illumina Genome Analyzer. Reads were trimmed and aligned to the reference genome (NC_003317.1, NC_003318.1) using the CLC Genomics Workbench v24.0.2. The sRNA-Seq analysis was performed with the following modifications to standard parameters. First, an additional 50 bases upstream and downstream of annotated genes were included to capture sRNAs aligned to intergenic regions. To minimize nonspecific alignment of yeast sequences in the culture medium, the maximum number of mismatches was set to zero, and read mapping was limited to four locations. Uniquely mapped reads were compiled, and sequence frequencies were generated using the CLC small RNA analysis tool for normalization, statistical calculations, and visualization in the R version 4.2.1 (R Core Team, Vienna, Austria; <https://www.r-project.org/>) [25]. Quality control included trimming low-quality bases from read ends, removing adapter sequences, discarding reads shorter than 18 bp, and retaining sequences between 18–30 bp in length based on known sRNA characteristics.

Bioinformatic Prediction of sRNA Target

Secondary structures of the ten most abundant OMV-associated sRNAs were predicted using the RNAfold web server [26]. Complementary sequences of the ten most abundant OMVs sRNAs were aligned with the NCBI mouse reference genome using BLASTN 2.2.31 [27] to identify perfect matches with the mouse mRNA. Target prediction scans were performed using the miRanda algorithm for subsequent analyses.

RNA-AGO2 Molecular Docking

The 3D structures of the AGO2 protein and OMV sRNA were predicted using the Phyre2 server and RNAComposer software, respectively. Structural optimization was performed using ChemBio3D Ultra 7.0 and AutoDock Tools 1.5.6, including energy minimization, water removal, and addition of nonpolar hydrogens. Protein–RNA docking was performed using the HDock server [28] with a hybrid docking strategy. Protein and RNA molecules were set as rigid bodies, and 100 conformations were generated and scored using the ITScorePP function. A more negative docking score indicated stronger binding affinity. The top-ranked conformation was further optimized using the minimization module using MOE 2019.1 with the Amber10: ETH force field and the water solvation model with the steepest descent and conjugate gradient methods. The final optimized structure was visualized using PyMOL version 2.1 (Schrödinger, New York, NY, USA; <https://pymol.org/>).

RNA Pull-down

Cells (6×10^6) from a 100 mm dish were washed with PBS, trypsinized, and pelleted at $3000 \times g$ for 2 min. The pellet was lysed in buffer A for 15 min on ice, mixed with buffer A containing 2.5% NP40, and centrifuged at $4000 \times g$ for 5 min at 4°C to collect the cytoplasmic supernatant. Biotin-labeled RNA probes were synthesized by in vitro transcription using a Promega kit and incubated with cytoplasmic extracts to form RNA-protein complexes. Complexes were captured using streptavidin magnetic beads, eluted, and analyzed by western blotting to identify specific RNA-binding proteins.

Cellular Infection Assay

RAW264.7 cells (Cell Resource Center, Beijing, China) were seeded in 24-well plates at 4×10^6 cells per well and cultured for 24 h at 37°C with 5% CO₂. Cell infection was performed as previously described [29]. Cells were infected with *B. melitensis* 16M at a multiplicity of infection of 100:1 with gentle mixing, and a mock-infected control was included. After 4 h of incubation, cells were washed three times with sterile PBS and incubated for 1 h in DMEM containing 50 μ g/mL gentamicin to eliminate extracellular bacteria. The medium was then replaced with DMEM supplemented with 25 μ g/mL gentamicin and 10% fetal bovine serum, and cells were cultured for another 12 h.

CFU Assay

Intracellular bacterial counts were determined at 0, 4, 8, 12, and 24 h post-infection. Cells were washed three times with PBS and lysed with 200 μ L 0.1% Triton X-100 for 10 min at room temperature to release intracellular bacteria. Serial 10-fold dilutions of the lysate were prepared, and 100 μ L of each dilution was plated onto TSA agar in triplicate.

Plates were incubated at 37°C for 72 h and colonies were counted. Only plates containing 30-300 colonies were included in the analysis. Results were expressed as log CFU per well.

Statistical Analysis

All experiments were independently repeated at least three times. Statistical analyses were performed using GraphPad Prism version 9.00 (GraphPad Software, San Diego, CA, USA; <https://www.graphpad.com/scientific-software/prism/>). Data from multiple groups were analyzed using one-way analysis of variance (ANOVA) or Dunnett's multiple comparison test. The results are presented as mean $\pm$ standard deviation ($\bar{x} \pm SD$), where “*” indicates a significant difference ($P < 0.05$), “**” indicates a highly significant difference ($P < 0.01$), and “ns” indicates no significant difference.

RESULTS

OMVs Contain Differentially Packaged sRNAs

From RNA-Seq analysis of the *B. melitensis* 16M and OMVs conducted to determine whether sRNAs were packaged in OMVs, sRNAs spanning a length range of 18-30 nucleotides were selected for analysis (Fig. 1). Sequences with identical nucleotides were collapsed into unique entries (Table 1), which were then used to evaluate the distribution and relative abundance of shared and specific sRNA sequences in the samples (Fig. 1-A, B). Reads were aligned to the reference genome using the Bowtie algorithm to profile the genomic distribution (Fig. 1-C, D). In total, 1,783,364 unique sRNA sequences were identified in the OMVs of *B. melitensis* 16M (Fig. 2-A). The ten most abundant OMV-associated RNAs mapped to the *B. melitensis* 16M genome. Notably, eight of these ten sRNAs were significantly enriched in OMVs compared to whole-cell samples from *B. melitensis* 16M (Fig. 2-B).

sRNAs in OMVs of Target Mouse mRNAs

When we assessed whether the ten most abundant sRNAs in OMVs form stable secondary structures and interact with host mRNAs, eight sRNAs were predicted to form stable stem-loop structures (Fig. 3). Sequence alignment against of the eight most abundant OMVs sRNAs against the mouse reference mRNA sequences

using BLASTN 2.2.31, followed by miRanda analysis identified six sRNAs with perfect matches to the mouse mRNA (Table 2).

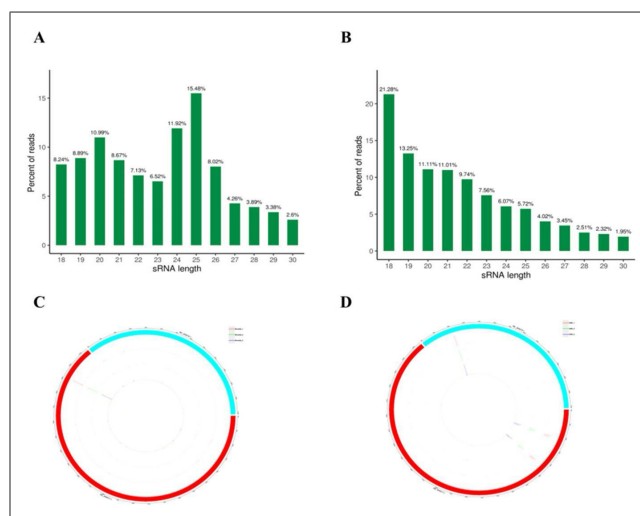


Fig 1. Analysis of repeat merging and shared sRNA sequences across samples. A- Read length distribution of *B. melitensis* 16M whole-cell sRNAs; B- Read length distribution of *B. melitensis* 16M OMV-derived sRNAs; C- Chromosomal distribution density of *B. melitensis* 16M genomic sequences; D- Chromosomal distribution density of *B. melitensis* 16M OMV-associated sequences

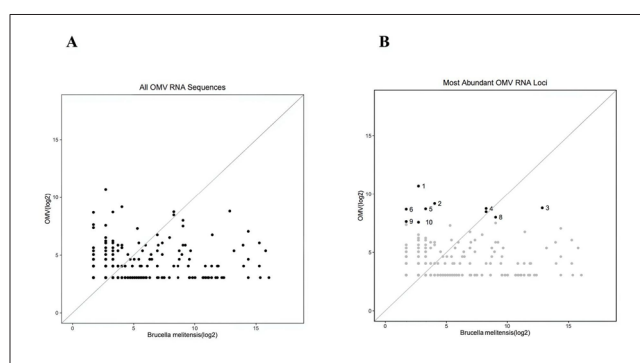


Fig 2. OMVs contain differentially packaged sRNAs. A- Relative abundance of 1,783,364 unique OMV sRNA reads versus whole-cell sRNA reads in *B. melitensis* 16M. Sequences to the left of the solid diagonal line are more abundant in OMVs than in whole cells, whereas those to the right are less abundant in OMVs. The sRNA sequence lengths range from 18 to 30 nt. B- Relative abundance of OMV-associated sRNAs mapped to genomic loci in *B. melitensis* 16M. Loci to the left of the solid diagonal line are more abundant in OMVs than in whole bacteria, while those to the right are less abundant in OMVs. Overall, the high-abundance genomic loci are enriched in OMVs compared to *B. melitensis* 16M whole cells. Results were presented as mean $\pm$ SD of three individual experiments

Table 1. Statistical result of sequence profiles and shared sequences across samples

Type	Unique sRNA	Percent of Unique	Total sRNA	Percent of Total
Total_sRNAs	5.775.639	100%	42.548.889	100%
OMVs unique	1.783.364	30.88%	9.107.598	21.41%
Brucella 16M unique	3.977.013	68.86%	16.775.066	39.42%
OMVs & <i>B. melitensis</i> 16 M common	15.262	0.26%	16.666.225	39.17%

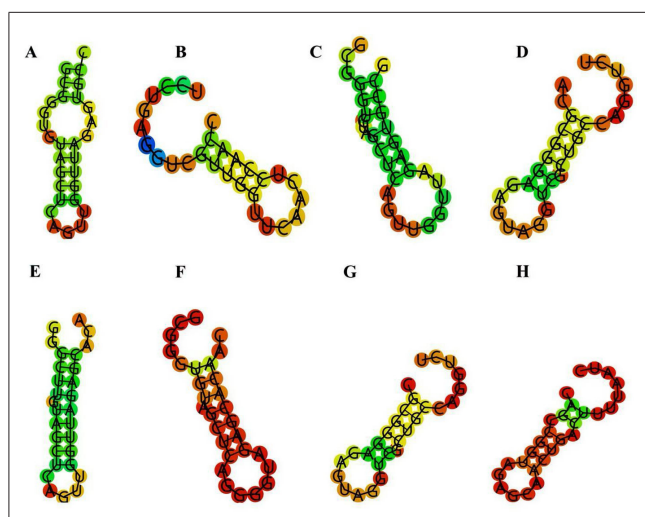


Fig 3. Secondary structure prediction of the 8 most abundant sRNAs in *B. melitensis* 16M OMVs. The secondary structures were predicted using the RNAfold WebServer with default parameters. The figures show the minimum free energy structures of each sRNA. The negative free energy values (ranging from -23.5 kcal/mol to -11.2 kcal/mol) indicate that all these sRNAs can form stable secondary structures

OMV sRNA Interact with AGO2

Molecular docking simulations demonstrated that all six high-abundance sRNAs from *B. melitensis* 16M OMVs could bind to AGO2 (Fig. 4). Among this, sRNA t0000835 exhibited the strongest binding affinity and the most stable interaction with AGO2. Consistent with this, RT-qPCR analysis revealed that sRNA t0000835 significantly suppressed transcription of the G protein-coupled receptor 45 gene (GPR45) (Table 3) (Fig. 5-A). The binding mode of the sRNA-AGO2 complex was visualized using PyMOL version 2.1 (Fig. 5-B).

Molecular docking analysis further revealed that the sRNA t0000835-AGO2 complex was stabilized through multiple interactions between key amino acid residues in AGO2 and specific nucleotides in sRNA t0000835 (Fig. 4), mediated by various intermolecular interactions, including electrostatic and hydrogen bonds, which significantly enhance the stability of the sgRNA-protein complex. Contact mapping between Cα atoms of AGO2 and O5' atoms of the RNA strand further supported

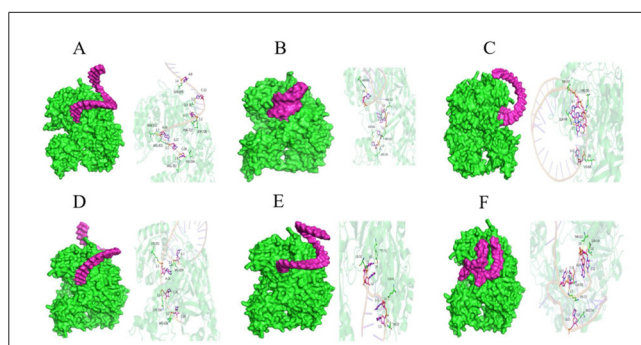


Fig 4. Analysis of sRNA-AGO2 binding in *B. melitensis* 16M. The 3D structures of AGO2, predicted using Phyre2 .0 (<https://www.sbg.bio.ic.ac.uk/phyre2/>), and sRNA, modeled using RNAComposer1.0 (<https://rnacomposer.cs.put.poznan.pl/>), were optimized by energy minimization and non-polar hydrogen addition. Rigid-body molecular docking was performed with HDock server 2017 (<http://hdock.phys.hust.edu.cn/>) under full-surface contact mode with 100 conformations generated and ITScorePP scoring applied. The most stable docking conformation was re-optimized using MOE 2019.1 (<https://www.chemcomp.com>) with the Amber10:ETH force field, and finally visualized with PyMOL 2.1 (<https://pymol.org/>)

these interactions (Fig. 5-C). RNA pull-down assays confirmed that biotinylated sRNA t0000835 specifically binds to AGO2 in vitro (Fig. 5-D). These complementary approaches provided robust evidence for the formation of a stable sRNA t0000835-AGO2 complex.

OMVs and sRNA t0000835 Promote Intracellular Replication of *B. melitensis* 16M

Functional assessment of OMVs during infection showed that OMV treatment significantly enhanced intracellular bacterial proliferation, particularly during the early stages (0.5-8 h) (Fig. 6). This suggests that OMVs may facilitate initial intracellular colonization and replication prior to full activation of the type IV secretion system (T4SS). Notably, intracellular survival assays using RAW264.7 revealed that cells expressing sRNA t0000835 exhibited significantly enhanced intracellular *Brucella* survival compared to the controls (Fig. 7), supporting the hypothesis that sRNA modulates host pathways to create a favorable intracellular niche for bacterial survival.

Table 2. sRNAs in OMVs predicted to target mouse mRNAs

OMVs sRNA	OMVs Counts ^a	Log2 Ratio ^b	Stable Secondary Structure	Targeted mRNA	Function
t0000206	1657	7.99	YES	Lancl1	lanC-like protein 1
t0000788	447	-4.05	YES	Lancl1	lanC-like protein 1
t0000833	430	0.49	YES	Mdfic2	Uncharacterized protein
t0000835	422	5.43	YES	Gpr45	G-protein coupled receptor 45
t0000964	356	0.21	YES	Mdfic2	Uncharacterized protein
t0001585	198	5.93	YES	Sil1	Nucleotide exchange factor SIL1

^a OMV counts show the total number of reads for each locus in OMV-derived sRNAs; ^b The log2 ratio is the number of total reads in OMVs divided by the number of total reads in *Brucella* 16M whole cells for each locus

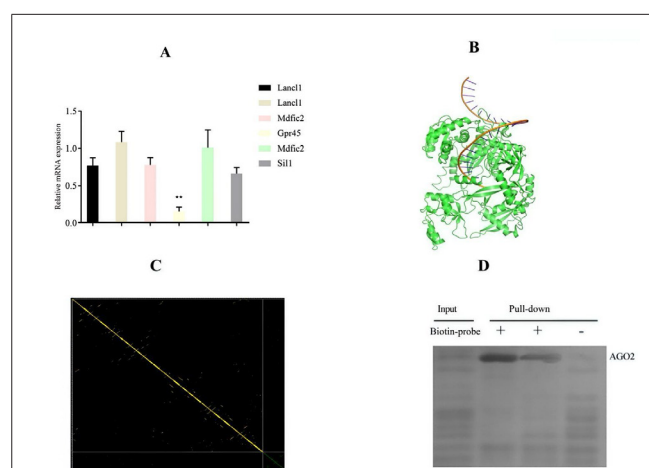


Fig 5. *B. melitensis* 16M sRNA binding AGO2 in vitro. **A-** Real-time fluorescence quantitative PCR detection of the effect of *B. melitensis* 16M OMVs sRNA on the mRNA of host target cells (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$); **B-** sRNA t0000835-AGO2 protein molecular docking complex; **C-** Residue-level resolution contact map of sRNA t0000835-AGO2 protein docking complex; **D-** RNA pull-down. Lane 1: Input group, representing the total protein sample of cells. Lane 2 and Lane 3: After adding the biotin-labeled RNA probe, the AGO2 protein forms an RNA-AGO2 protein complex with the biotin-labeled RNA. Lane 4: No biotin-labeled RNA probe was added, serving as a negative control. Results were presented as mean $\pm$ SD of three individual experiments

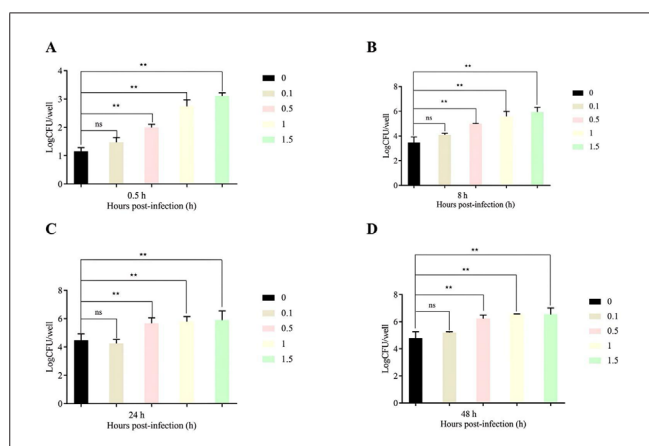


Fig 6. OMVs enhance the intracellular survival of *Brucella*. RAW264.7 cells were pre-incubated with 0, 0.1, 0.5, 1, or 1.5 μ g of OMVs purified from *B. melitensis* 16M for 1 h before infection with *Brucella* at MOI=100:1. At 0.5, 8, 24 and 48 h post-infection, cells were lysed with 0.1% Triton-100, serially diluted, and plated to enumerate intracellular *Brucella*. Statistical analysis was performed using GraphPad Prism version 9.00 (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$). Results were presented as mean $\pm$ SD of three individual experiments

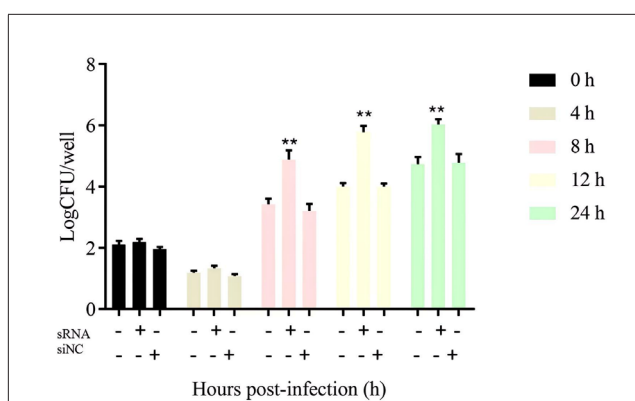


Fig 7. Transfection with sRNA t0000835 can increase the intracellular survival count of *Brucella*. Compared with cells transfected with negative control small interfering RNA (siNC), the intracellular survival count of *Brucella* in RAW264.7 cells transfected with sRNA t0000835 is significantly increased (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$). Results were presented as mean $\pm$ SD of three individual experiments

DISCUSSION

In the present study, we described an example of trans-kingdom regulatory activities of OMV-derived sRNAs in *Brucella* infection. sRNA t0000835, encapsulated in *B. melitensis* 16M OMVs, represents a novel mechanism of pathogen-host communication, which modulates host gene expression to promote intracellular survival of bacteria. This finding reveals a T4SS-independent regulatory strategy used by *Brucella* during early stages of infection.

We demonstrated that OMVs produced by *Brucella* delivered functional sRNAs to host macrophages. However, the relative abundance of sRNAs in OMVs did not fully mirror their predicted targeting efficiency, probably owing to variable sRNA stability or AGO2-binding affinity among sequences, which led to differential functional availability in host cells. sRNAs in bacterial OMVs have been previously characterized [12,30]; however, a direct role of OMV-derived sRNAs in promoting intracellular survival of bacteria by targeting GPCR signaling pathways has not been demonstrated.

The presence of sRNAs in OMVs has been recently reported in various gram-negative bacteria including

Table 3. All primers were listed for qPCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Length (bp)
Lancl1	TAAACGCGCCGCATCTGTT	GAGCACACGGAGATGATGGA	87
Mdfic2	CACAATCGCATGTCTCGCTG	GCCACAATGCAGCTACCAAG	156
Gpr45	GTGCCAACTCAGGGTCTCCA	TGTCCTGTTTCTGTGGTAGCC	86
Sil1	TCCCACCACTCAGGTTTCT	CCTGCTCTGTTTCCACCT	106
GAPDH	GATGCCCCCATGTTTGTGAT	GGCATGGACTGTGGTCATGAG	162

Pseudomonas aeruginosa [12], *Helicobacter pylori* [31], and *Escherichia coli* [32]. However, the functional significance of these sRNAs in modulating host cell biology to benefit intracellular survival of bacteria has remained largely unexplored. For example, sRNA52320 has been reported to reduce IL-8 secretion [12]; however, this study has focused on an extracellular pathogen in airway epithelial cells, without addressing the query whether intracellular pathogens, like *Brucella*, use analogous sRNA-based mechanisms to establish a replicative niche. Differential packaging of tRNA fragments into *E. coli* OMVs has been noticed [32]; however, the biological consequences of this selective packaging during infection have not been reported. Our study fills this gap by demonstrating that sRNA t0000835, a tRNA-derived fragment, directly suppresses GPR45 expression via AGO2 binding, thereby enhancing intracellular survival of *Brucella*.

Notably, sRNA t0000835 targeted GPR45, a GPCR family member. GPCRs are the largest family of cell surface receptors and play central roles in regulating immune responses including phagocytosis and cytokine secretion [33]. Although bacterial effectors modulate GPCR signaling [34], our study provides the first evidence that a bacterial OMV-derived sRNA directly suppresses GPCR expression at the transcriptional level by recruiting AGO2. Given that GPR45 has been implicated in intracellular calcium homeostasis and MAPK signaling pathways [35], its down regulation may create a relatively highly permissive intracellular environment for *Brucella* by attenuating bactericidal responses.

Remarkably, sRNA t0000835 appeared to specifically target GPR45, rather than globally suppressing host gene expression, probably because the secondary structure of sRNA t0000835 enabled sequence-specific binding to the 3'UTR of *GPR45* mRNA via AGO2-mediated base pairing. This specificity may be biologically advantageous for *Brucella* as global suppression of host gene expression may trigger stress responses or be metabolically costly. Consistent with this hypothesis, bacterial sRNAs often target key nodes in host signaling networks to fine-tune immune responses without causing catastrophic cellular damage [36,37].

The ability of *Brucella* to use OMV-delivered sRNAs to enhance intracellular survival has important implications for understanding its pathogenesis. Brucellosis is characterized by chronic, persistent infections that are difficult to eradicate. Our findings suggest that OMV sRNAs may contribute to this persistence by subverting host defense mechanisms from the earliest stage of infection, even before T4SS-mediated effector delivery becomes fully operational. Therefore, targeting OMV biogenesis or sRNA packaging could represent a novel therapeutic strategy to attenuate *Brucella* virulence,

particularly given the increasing concerns regarding antibiotic resistance in chronic brucellosis.

OMV-mediated delivery of functional sRNAs to host cells may be a common strategy among intracellular pathogens that need to establish a replicative niche. Other clinically relevant intracellular bacteria, such as *Salmonella*, *Legionella*, and *Mycobacterium*, may also use OMV-based sRNA-related mechanisms during the early “stealth” phase of infection before their dedicated secretion systems are fully activated. Beyond the host-pathogen interface, *Brucella* OMV sRNAs may play roles in microbe-microbe competition within the host or environment.

This study has some limitations. First, the precise mechanism, by which sRNA t0000835 is selectively packaged into *Brucella* OMVs has not been elucidated. Second, whether sRNA t0000835 targets additional host mRNAs besides GPR45 awaits further investigation. Third, the temporal dynamics of sRNA delivery relative to T4SS activation during infection need to be characterized. Future studies considering genetic deletion of sRNA t0000835 in *B. melitensis* 16M will be essential to confirm its role in virulence *in vivo*. An in-depth understanding of OMV sRNA biology may open new avenues for therapeutic intervention against chronic brucellosis and other intracellular bacterial infections.

In summary, sRNA t0000835 significantly enhances intracellular survival of *B. melitensis* 16M through an AGO2-dependent mechanism that represses GPR45 expression. This study provides new experimental evidence for understanding how bacterial sRNAs promote their own survival by modulating host gene expression.

DECLARATIONS

Availability of Data and Materials: The raw sRNA sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under the BioProject accession number PRJNA1452185 (BioSample: SAMN57209791, SAMN57209792). Since the data are currently private and not yet published, a reviewer link is provided for temporary access: <https://dataview.ncbi.nlm.nih.gov/object/PRJNA1452185?reviewer=75hfrmg97ccubuv73jk4dkfcdt>.

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RESEARCH ARTICLE

Molecular Analysis of *Campylobacter* spp. Isolates from Sheep and Goats and Examination of Their Antibiotic Resistance

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Abstract

This study aimed to isolate and identify *C. jejuni*, *C. coli*, and *C. fetus* subsp. *fetus* from rectal swabs of diarrheic sheep and goats, characterize antimicrobial resistance and virulence genes, and determine genetic diversity using PCR-RFLP. Rectal swabs (n=374) were analyzed via selective culture and species-specific PCR. Resistance profiles, virulence genes, and *flaA* gene genetic diversity (via PCR-RFLP with *DdeI*) were evaluated. *Campylobacter* spp. was detected in 30.74% (115/374) of samples (87.82% *C. jejuni*, 12.17% *C. coli*; not determined *C. fetus* subsp. *fetus*). High susceptibility was observed to gentamicin (97.39%) and amoxicillin-clavulanic acid (93.04%). The highest resistance was against cephalothin (84.34%), reflecting intrinsic resistance. Acquired resistance to ciprofloxacin was significant (56.52%). Resistance genes 23S rRNA (100%), *gyrA* (96.92%), and *tet(O)* (89.58%) predominated. Main virulence genes were *cadF* (94.78%), *dnaJ* (92.17%), and *cdtB* (91.30%); *ggt* not determined. PCR-RFLP revealed 13 band patterns. In conclusion, *C. jejuni* was predominant in diarrheic sheep and goats. Isolates were highly susceptible to gentamicin and amoxicillin-clavulanic acid, representing efficacious antimicrobial options, though regional clinical guidelines and potential off-label use should be considered.

Keywords: Antimicrobial resistance, *Campylobacter* spp., *C. jejuni*, PCR-RFLP, Virulence gene

INTRODUCTION

Small ruminant breeding, primarily for meat, milk, and wool, is a key source of livelihood in the Siirt region and across South-eastern Anatolia, where these animals are particularly well adapted to the local climate, geography, and marginal lands [1-3]. Thermophilic *Campylobacter* spp. are the leading cause of foodborne gastroenteritis in humans, while *Campylobacter fetus* subsp. *fetus* colonises the gastrointestinal tract of small ruminants and can translocate to the urogenital system, causing abortions, underscoring the zoonotic potential of these bacteria [4].

Thermophilic *Campylobacter* spp., which are common inhabitants of the normal intestinal microbiota of domestic animals, can be transmitted to humans through the consumption of contaminated water, unpasteurized milk, or undercooked meat, particularly poultry [5]. Since *C. jejuni* and *C. coli* are frequently present in the intestinal tract of small ruminants, they can contaminate carcasses and other tissues during the slaughtering process, leading to environmental spread and public health risks [4,5]. In

addition, faecal-oral transmission, aborted fetuses, and placenta are also important sources of transmission [4]. Apart from these, workers in animal shelters and animal product processing facilities, wild animals, and rodents also play a role in contamination and transmission, with flies serving as mechanical vectors [6,7].

Thermophilic *Campylobacter* spp, known to cause gastroenteritis in both human and animals, may continue to spread and infect the intestines without causing any clinical symptoms [8,9]. They may also cause death in infected animals due to abortion, weak lamb births, metritis, and diarrhoea or gastroenteritis in lambs [10]. In humans, they can cause diarrhoea, fever, and weight loss, as well as systemic complications including meningitis and myocarditis. Furthermore, they can cause significant neurological symptoms that may lead to serious conditions such as Miller Fisher syndrome and Guillain-Barré syndrome [8,11].

Treatment is rarely administered for uncomplicated *Campylobacter* infections [8]. However, antibiotics such as fluoroquinolones, tetracyclines, and macrolides may



be indicated for severe or systemic cases in both humans and small ruminants [6,12,13]. In certain regions, the historically widespread or uncontrolled use of these antibiotics has contributed to the emergence of increasing resistance mechanisms in *Campylobacter* spp [12]. This rising resistance, particularly in *C. jejuni* and *C. coli* isolates, can negatively affect the clinical efficacy of these conventional therapeutics against infection, a concern that persists globally despite varying regional antimicrobial stewardship regulations [8,12].

At the molecular level, resistance to fluoroquinolones is primarily mediated by point mutations in the *gyrA* gene, which encodes the DNA gyrase subunit A [14]. Tetracycline resistance is frequently associated with the plasmid-mediated or chromosomal *tet(O)* gene, which protects the bacterial ribosome [15]. For beta-lactams and aminoglycosides, resistance is commonly driven by the *bla*_{OXA-61} (encoding OXA-61 beta-lactamase) and *aphA-3-1* (encoding aminoglycoside phosphotransferase) genes, respectively [15]. Macrolide resistance, a major clinical concern, is predominantly linked to specific mutations in the 23S rRNA gene, often identified via markers such as *ERY2074* and *ERY2075* [16]. Furthermore, multidrug-resistant (MDR) phenotypes can be significantly amplified by the energy-dependent *CmeABC* efflux pump system, encoded by the *cmeA*, *cmeB*, and *cmeC* genes, which actively extrudes various antimicrobials out of the bacterial cell [15,16]. In addition to antimicrobial resistance (AMR) mechanisms, the pathogenicity of *Campylobacter* species is strongly governed by virulence factors responsible for motility, adherence, invasion, and toxin production [8,15]. Therefore, profiling both resistance determinants and virulence-associated genes is vital to clarifying the molecular epidemiology and pathogenic potential of small ruminants isolates [15].

Although various molecular tools are available for the characterization of *C. jejuni* and *C. coli*, their discriminatory power and epidemiological utility vary significantly [17]. Among these, *flaA* gene-based molecular techniques are widely preferred in epidemiological surveillance due to their rapidity, relative simplicity, and cost-effectiveness, while still offering acceptable discriminatory power [17,18]. Specifically, *flaA* gene-based Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) has become a well-established and widely utilized method for genotyping *Campylobacter* isolates [18]. Furthermore, while sequence analysis of short variable regions (SVRs) of *flaA* and *flaB* genes is another common alternative, particularly for short-term and localized studies, PCR-RFLP remains a highly practical and reliable approach for screening the genetic relatedness of large sample sets without requiring complex sequencing infrastructure [17].

The aim present study was to identify *C. jejuni*, *C. coli*, and *C. fetus* subsp. *fetus* strains from rectal swab samples of sheep and goats raised in the Siirt region, and to determine the antimicrobial susceptibility of these isolates. The presence of genes causing AMR in isolates was investigated to determine the genetic mechanism of the resistance profile. In addition, the presence of virulence-associated genes in the obtained isolates was investigated, and the genotypic characterization of the strains was performed using the *flaA*-based RFLP method.

MATERIAL AND METHODS

Ethical Statement

This study was approved by Siirt University Animal Experiments Local Ethics Committee (Approval No: 2023/07/48).

Study Area and Sampling Strategy

This active surveillance study was conducted between 2024 and 2025 across traditional family farms operating in the Siirt city center and its surrounding rural districts. A convenience sampling strategy based on passive epidemiological surveillance was employed, aiming to capture all accessible clinical cases during the study timeframe. The inclusion criteria strictly required small ruminants (sheep and goats) presenting with active diarrhoeic symptoms.

A total of 374 rectal swab samples were collected from affected animals, comprising 271 sheep (Hamdani breed) and 103 goats (Hair goat breed). The higher proportion of sheep samples naturally reflects the demographic distribution of livestock in the Siirt region, where sheep farming predominantly exceeds goat production. The sampled animals ranged in age from 1 to 5 years and represented both sexes.

Sample Collection and Transport

For sample collection, rectal swabs were collected from each animal using a sterile cotton-tipped swab rotated gently against the mucosal wall. Rectal swab samples were transported in tubes containing charcoal-supplemented Cary-Blair transport medium (Cary-Blair, 104054, Türkiye) and delivered to the Laboratory of the Department of Microbiology at Siirt University Faculty of Veterinary Medicine.

Isolation and Identification of *Campylobacter* spp.

Swab samples were inoculated in modified charcoal cefoperazone deoxycholate (mCCDA) agar (Biolife, NL2403, Italy) containing 5-10% sheep blood and *Campylobacter* mCCDA selective supplement (Oxoid, SR0069E, UK). Petridishes were incubated in microaerophilic environment

at 41.5°C for 36-48 h. Pure cultures were obtained from the colonies formed in the medium after incubation. Following the incubation period, a single presumptive *Campylobacter* colony was selected and subcultured onto blood agar plates in accordance with ISO 10272-1:2017^[19]. The plates were then incubated under microaerophilic conditions at 42°C for 48 h. After incubation, the colony morphology was re-examined to verify the purity of the culture. The colonies obtained were stained using the Gram's method and evaluated for their microscopic morphology, growth on MacConkey agar, motility, oxidase, catalase, and indole reactions^[14].

The suspected isolates were confirmed by PCR using species-specific primers ([Table 1](#)).

DNA Extraction

Genomic DNA, which will be used for the PCR confirmation of suspected isolates, was obtained by the boiling method^[17].

PCR Amplification

A commercial mastermix (EcoTaq, 2X PCR Mastermix, ET5, Türkiye) was used to prepare the PCR mixture, following the manufacturer's recommendations. The PCR protocol was optimized^[20] and the total volume of the reaction mixture was adjusted to 25 µL: 12.5 µL master mix, 5 µL genomic DNA, 1.5 µL (10 µM) forward primer, 1.5 µL reverse primer, and 4.5 µL PCR broth. During the amplification process, the binding temperature was optimized according to the recommendations of the company from which the primers were synthesized (Probesynthesis Biyoteknoloji, Ankara, Türkiye). Thermal cycling profiles consisted of 35 cycles, including an initial denaturation step lasting 10 min at 94°C, followed by 1 min of denaturation at 94°C, 1 min of annealing at temperatures specifically adjusted for each primer pair, and 1 min of extension at 72°C; and finally, a final extension step lasting 10 min at 72°C. Amplicons produced by PCR were electrophoresised on agarose gel and analyzed using a gel imaging system (Gen-Box ImagER, Ankara, Türkiye).

Antimicrobial Susceptibility Test

The susceptibility of the isolates towards various antimicrobial agents was determined using the disk diffusion method.

Ciprofloxacin (5 µg, Bioanalyse, Türkiye), nalidixic acid (30 µg, Bioanalyse, Türkiye), cefalotin (30 µg, Bioanalyse, Türkiye), amoxicillin-clavulanic acid (20/10 µg, Bioanalyse, Türkiye), chloramphenicol (30 µg, Bioanalyse, Türkiye), erythromycin (15 µg, Liofilmchem, Italy), tetracycline (30 µg, Bioanalyse, Türkiye), gentamicin (10 µg, Bioanalyse, Türkiye), aztreonam (30 µg, Bioanalyse, Türkiye), and streptomycin (10 µg, Bioanalyse, Türkiye) antibiotic discs were used in the test.

The results were evaluated primarily in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2007)^[21] and the *Campylobacter*-specific interpretive criteria established by Miflin et al.^[22] and Luangtongkum et al.^[23]. For antimicrobial agents lacking defined specific breakpoints for *Campylobacter*, the interpretive criteria for *Enterobacteriaceae* specified by the CLSI were applied. The clinical breakpoints applied are summarized in [Table 2](#).

As a result of the test, the isolates were assessed as susceptible, intermediate, or resistant to the antimicrobial discs used. In addition, the isolates resistant to three or more antimicrobial groups were assessed as MDR^[24].

Identification of Antimicrobial Resistance Genes

The genetic mechanism of resistance was investigated by PCR using gene-specific primers in isolates phenotypically resistant to quinolone, beta-lactam, erythromycin, tetracycline, and aminoglycoside antibiotics. [Table 3](#) shows the primer sequences used for the identification of AMR genes by PCR.

Identification of Virulence-Associated Genes

The presence of virulence-associated genes in the isolates was investigated using PCR with gene-specific primers. [Table 4](#) shows the primer sequences used for the identification of virulence genes by PCR.

Table 1. Primer sequences used for the PCR identification of the suspected isolates

Bacteria	Target Gene	Oligonucleotide (5'-3')	Amplicon Size (bp)	Ref.
<i>Campylobacter</i> spp.	23S rRNA	F: TATACCGGTAAGGAGTGCTGGAG R: ATCAATTAACCTTCGAGCACC	816	[20]
<i>C. jejuni</i>	<i>hipO</i>	F: ACTTCTTTATTGCTTGCTGC R: GCCACAACAAGTAAAGAAGC	323	[20]
<i>C. coli</i>	<i>glyA</i>	F: GTAAAACCAAAGCTTATCGTG R: TCCAGCAATGTGTGCAATG	126	[20]
<i>C. fetus</i> subsp. <i>fetus</i>	<i>sapB2</i>	F: GCAAATATAATGTAAGCGGAGA R: TGCAGCGGCCCCACCTAT	435	[20]

Table 2. Breakpoints for *Campylobacter* spp.

Antibiotic	Potency	R	I	S	Ref.
Amoxicillin-clavulanic acid	20/10 µg	≤18	19-23	≥24	[21]
Aztreonam	30 µg	≤15	16-21	≥22	[21]
Cloramphenicol	30 µg	≤11	12-22	≥23	[22]
Ciprofloxacin	5 µg	≤13	14-17	≥18	[22]
Eritromycin	15 µg	≤15	16-18	≥19	[22]
Gentamicin	10 µg	≤12	13-14	≥15	[23]
Cephalotin	30 µg	≤14	15-17	≥18	[21]
Nalidixic acid	30 µg	≤13	14-18	≥19	[23]
Streptomycin	10 µg	≤10	11-12	≥15	[21]
Tetracyclin	30 µg	≤14	15-18	≥19	[23]

Table 3. Primer sequences used for the identification of antimicrobial resistance genes by PCR

Target Gene	Oligonucleotide (5'-3')	Amplicon Size (bp)	Ref.
Quinolone Resistance Genes (<i>C. jejuni</i>)	CampyMAMAgryA-F F: TTTT'TAGCAAAGAT'TCTGAT	265	[25]
	CampyMAMAgryA-R R: CAAAGCATCATAAACTGCAA		
	CampyMAMAgryA1-F F: TTTT'TAGCAAAGAT'TCTGAT	368	[25]
	GZgyrA4 R: CAGTATAACGCATCGCAGCG		
Quinolone Resistance Genes (<i>C. coli</i>)	GZgyrACcoli3F-F F: TATGAGCGTTAT'TATCGGTC	192	[25]
	CampyMAMAgryA8-R R: TAAGGCATCGTAAACAGCCA		
	GZgyrACcoli3F-F F: TATGAGCGTTAT'TATCGGTC	505	[25]
	GZgyrACcoli4R-R R: GTCCATCTACAAGCTCGTTA		
Beta-lactam Resistance Gene	<i>bla</i> _{OXA-61} F: AGAGTATAATACAAGCG R: TAGTGAGTTGTCAAGCC	372	[26]
Erythromycin Resistance Gene	23S rRNA-F F: TTAGCTAATGTTGCCCGTACCG R: AGCCAACCTTGTAAAGCCTCCG	697	[27]
	ERY2075-R R: TAGTAAAGGTCCACGGGGTTCGC	485	
	ERY2074-R R: AGTAAAGGTCCACGGGGTCTGG	485	
Tetracycline Resistance Gene	<i>tet</i> (O) F: GCGTTTTGTTTATGTGCG R: ATGGACAACCCGACAGAAG	559	[26]
Aminoglycoside Resistance Gene	<i>aphA-3-1</i> F: TGCCTAAAAGATACGGAAG R: CAATCAGGCTTGATCCCC	701	[26]
<i>Cme</i> ABC Efflux System	<i>cmeA</i> F: TAGCGGCGTAATAGTAAATAAAC R: ATAAAGAAATCTGCGTAAATAGGA	435	[28]
	<i>cmeB</i> F: AGGCGGTTTGTAAATGTATGTT R: TGTGCCGCTGGGAAAAAG	444	[28]
	<i>cmeC</i> F: CAAGTTGGCGCTGTAGGTGAA R: CCCCAATGAAAAATAGGCAGAGTA	431	[28]

Genotyping

The *flaA* gene region PCR products were analyzed by RFLP. Reactions were carried out using the *DdeI* restriction enzyme (New England BioLabs, SR0175S, Türkiye) in a thermal cycler at 37°C for 15 min. Fragments were visualized on a 2.5% agarose gel (GelRed stained, UV light) using a gel imaging system (Gene-Box FX, ER Biotech, Türkiye). The obtained images were analyzed

with PyElph software (v.1.4). The resulting band profiles underwent phylogenetic analysis using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method.

Statistical Analysis

Minitab (v21) software was used for the statistical analysis of the data. Pearson's Chi-square (x2) method and Fischer's Exact test were used to compare the AMR of the obtained genotypes. Statistical significance level was accepted as P<0.05.

Table 4. Primer sequences used to investigate the presence of virulence-associated genes

Target Gene		Oligonucleotide (5'-3')	Amplicon Size (bp)	Ref.
Toxin Genes	<i>cdtA</i>	F: GGAAATTGGATTGGGGCTATACT R: ATCACAAGGATAATGGACAAT	165	[29]
	<i>cdtB</i>	F: GTTAAAATCCCCTGCTATCAACCA R: GTTGGCACTTGGAATTGCAAGGC	495	[29]
	<i>cdtC</i>	F: TGGATGATAGCAGGGGATTTAAC R: TTGCACATAACCAAAAGGAAG	555	[29]
Adhesion-Colonization-Invasion Genes	<i>flaA</i>	F: GATTTCTGATTAAACACAAATGGTGC R: CTGTAGTAATCTTAAAACATTTTG	1725	[30]
	<i>ggt</i>	F: GAGTGCTATGCTTGATCGCT R: TAGGTGGCGACATGGAAATG	419	[31]
	<i>cadF</i>	F: TTGAAGGTAATTTAGATATG R: CTAATACCTAAAGTTGAAAC	400	[32]
	<i>ciaB</i>	F: TGCGAGATTTTTTCGAGAATG R: TGCCCGCCTTAGAAGTTACA	527	[33]
	<i>pldA</i>	F: AAGAGTGAGGCGAAATTTCCA R: GCAAGATGGCAGGATTATCA	385	[33]
	<i>dnaJ</i>	F: ATTGATTTTGCTGCGGGTAG R: ATCCGCAAAAGCTTCAAAAA	177	[33]

RESULTS

Isolation and Identification

A total of 115 (30.74%) *Campylobacter* spp. were isolated and identified from the rectal swab samples. Through PCR in which species-specific primers were used, 87.36% (n=83) of the 95 *Campylobacter* spp. isolates obtained from sheep rectal swab samples were identified as *C. jejuni* and 12.63% (n=12) as *C. coli*. Of the 20 *Campylobacter* spp. isolates obtained from goat rectal swab samples, 90% (n=18) were identified as *C. jejuni* and 10% (n=2) as *C. coli*. In the samples examined in the study, *C. fetus* subsp. *fetus* was not identified by PCR.

Antimicrobial Susceptibility

Test results showed that 97.39% (n=112) of the 115 *Campylobacter* spp. isolates were susceptible to gentamicin, and 93.04% (n=107) were fully susceptible to amoxicillin-clavulanic acid. Susceptibility rates were 91.30% for chloramphenicol, 79.13% for erythromycin, and 72.17% and 54.78% for streptomycin and aztreonam, respectively. The highest resistance was detected for cephalothin (84.34%) and ciprofloxacin (56.52%). MDR was determined in 41.73% (n=48) of the isolates. Resistance profiles at the species level (*C. jejuni* and *C. coli*) are shown in Table 5.

Table 5. Antimicrobial resistance distribution of *C. jejuni* and *C. coli* isolates

Bacterial Distribution	Antibiotic Resistance Rates (%)									
	AMC	ATM	C	CIP	ERY	GEN	KF	NA	S	TE
<i>C. jejuni</i> (n=83) Sheep	0% (n=0)	33.73% (n=28)	6.02% (n=5)	61.44% (n=51)	22.89% (n=19)	2.40% (n=2)	90.36% (n=75)	16.86% (n=14)	14.45% (n=12)	38.55% (n=32)
<i>C. jejuni</i> (n=18) Goat	5.55% (n=1)	44.44% (n=8)	50% (n=9)	50% (n=9)	38.88% (n=7)	5.55% (n=1)	61.11% (n=11)	5.55% (n=1)	44.44% (n=8)	50% (n=9)
Total (%)	0.99% (n=1)	35.64% (n=36)	13.86% (n=14)	59.40% (n=60)	25.74% (n=26)	2.97% (n=3)	85.14% (n=86)	14.85% (n=15)	19.80% (n=20)	40.59% (n=41)
<i>C. coli</i> (n=12) Sheep	8.33% (n=1)	50% (n=6)	16.66% (n=2)	8.33% (n=1)	25% (n=3)	0% (n=0)	83.33% (n=10)	16.66% (n=2)	16.66% (n=2)	58.33% (n=7)
<i>C. coli</i> (n=2) Goat	0% (n=0)	0% (n=0)	0% (n=0)	50% (n=1)	50% (n=1)	0% (n=0)	100% (n=2)	0% (n=0)	0% (n=0)	0% (n=0)
Total (%)	7.14% (n=1)	42.85% (n=6)	14.28% (n=2)	14.28% (n=2)	28.57% (n=4)	0% (n=0)	85.71% (n=12)	14.28% (n=2)	14.28% (n=2)	50% (n=7)

AMC: Amoxicillin-clavulanic acid; ATM: Aztreonam; C: Chloramphenicol; CIP: Ciprofloxacin; ERY: Erythromycin; GEN: Gentamicin; KF: Cefalotin; NA: Nalidixic acid; S: Streptomycin; TE: Tetracycline

Antimicrobial Resistance Genes

The *tet(O)* gene was detected in 89.58% (43/48) of the tetracycline-resistant isolates, the *bla*_{OXA-61} gene in 88.34% (91/103) of the beta-lactam-resistant isolates, the *aphA*-3-1 gene in 4.34% (1/23) of the aminoglycoside-resistant isolates. Resistance-associated point mutations in the 23S rRNA target region were detected in 100% (24/24) of the phenotypically erythromycin-resistant isolates. Specifically, among these 24 erythromycin-resistant isolates, the *ERY2075* mutation was identified in 37.5% (9/24) of cases, and the *ERY2074* mutation in 54.16% (13/24). Among the 48 *Campylobacter* spp. isolates with MDR, *cmeA* was detected in 45.83% (n=22), *cmeB* in 95.83% (n=46), *cmeC* in 52.08% (n=25), and all three resistance genes in 31.25% (*cmeABC*) (Fig. 1). Furthermore, the presence of the *Thr*-86-*Ile* point mutation was determined in 98.33% (n=59) of 60 *C. jejuni* isolates- phenotypically determined to be quinolone-resistant and in which the *gyrA* gene was detected-, and in 80% (n=4) of 5 *C. coli* isolates.

Virulence Genes

The *cdtA* virulence gene was detected in 20.86% of *Campylobacter* spp. isolates (n=115), *cdtB* virulence gene in 91.30%, and *cdtC* virulence gene in 21.73%. In addition,

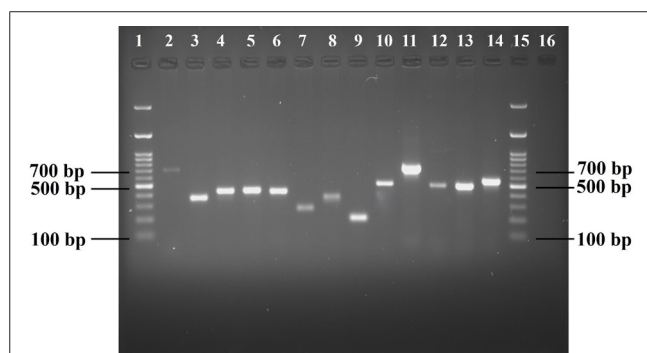


Fig 1. Agarose gel image of antimicrobial resistance-related genes obtained by PCR in the isolates [1,15: 100 bp DNA Marker; 2: *aphA*-3-1 positive isolate (701 bp); 3: *bla*_{OXA-61} positive isolate (372 bp); 4: *cmeA* positive isolate (435 bp); 5: *cmeB* positive isolate (444 bp); 6: *cmeC* positive isolate (431 bp); 7: *C. jejuni* quinolone resistance gene positive isolate (265 bp); 8: *C. jejuni* quinolone resistance gene positive isolate (368 bp); 9: *C. coli* quinolone resistance gene positive isolate (192 bp); 10: *C. coli* quinolone resistance gene positive isolate (505 bp); 11: 23S rRNA gene positive isolate (697 bp); 12: *ERY2074* gene positive isolate (485 bp); 13: *ERY2075* gene positive isolate (485 bp); 14: *tet(O)* gene positive isolate (559 bp)]

the presence of the *cadF* gene was detected in 94.78% of the isolates, *dnaJ* gene in 92.17%, *ciaB* gene in 88.69%, *flaA* gene in 79.13%, and *pldA* gene in 15.65%, while *ggt* gene was not detected (Table 6; Fig. 2).

Genotyping

The restriction fragments of the 1.7 kb *flaA* gene PCR products ranged from 100 to 1000 base pairs. It was observed that 91 different *flaA* gene-positive isolates had 13 different RFLP patterns (Fig. 3). Accordingly, a total of 13 (Cj1 to Cj13) different band patterns were observed in *C. jejuni* isolates. Five of these band patterns (Cc1 to Cc5) were also observed in *C. coli* isolates. The phylogenetic analysis of the band profiles is presented in Fig. 4.

Statistical Analysis

According to the results of the Chi-square test, the prevalence of *Campylobacter* spp. was found to be significantly higher in sheep than in goats ($P < 0.05$).

The results revealed no relationship between *flaA* genotypes and resistance to cephalothin, streptomycin, chloramphenicol, erythromycin, nalidixic acid, and amoxicillin-clavulanic acid antimicrobials ($P > 0.05$). However, tetracycline and ciprofloxacin AMR were higher in isolates with the Cj2 and Cj7 genotypes compared to isolates with the other genotypes ($P < 0.001$). In isolates with Cj3 and Cj6 genotypes, aztreonam AMR was higher

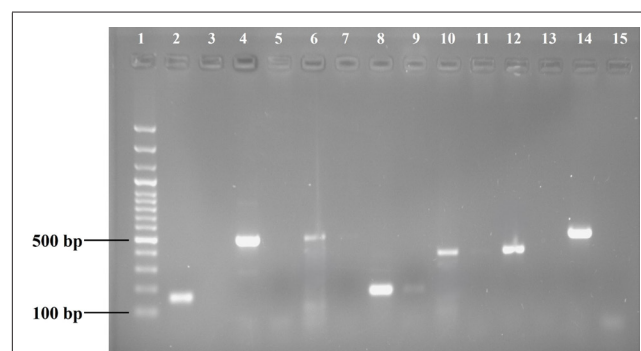


Fig 2. Agarose gel image of amplicons obtained from the screening of virulence-related genes in the isolates by PCR [1: 100 bp DNA Marker; 2: *cdtA* positive isolate (165 bp); 3: *cdtA* negative isolate; 4: *cdtB* positive isolate (495 bp); 5: *cdtB* negative isolate; 6: *cdtC* positive isolate (555 bp); 7: *cdtC* negative isolate; 8: *dnaJ* positive isolate (177 bp); 9: *dnaJ* negative isolate; 10: *pldA* positive isolate (385 bp); 11: *pldA* negative isolate; 12: *cadF* positive isolate (400 bp); 13: *cadF* negative isolate; 14: *ciaB* positive isolate (527 bp); 15: *ciaB* negative isolate]

Table 6. Distribution of virulence-associated genes in *C. jejuni* and *C. coli* isolates

Bacteria	Number of Isolates	Ratio of Virulence Genes (%)								
		<i>cadF</i>	<i>cdtA</i>	<i>cdtB</i>	<i>cdtC</i>	<i>ciaB</i>	<i>dnaJ</i>	<i>flaA</i>	<i>ggt</i>	<i>pldA</i>
<i>C. jejuni</i>	101	98 (97.02)	22 (21.78)	97 (96.03)	25 (24.75)	96 (95.04)	96 (95.04)	84 (83.16)	0 (0)	18 (17.82)
<i>C. coli</i>	14	11 (78.57)	2 (14.28)	8 (57.14)	0 (0)	6 (42.85)	10 (71.42)	7 (50)	0 (0)	0 (0)
Total	115	109 (94.78)	24 (20.86)	105 (91.30)	25 (21.73)	102 (88.69)	106 (92.17)	91 (79.13)	0 (0)	18 (15.65)

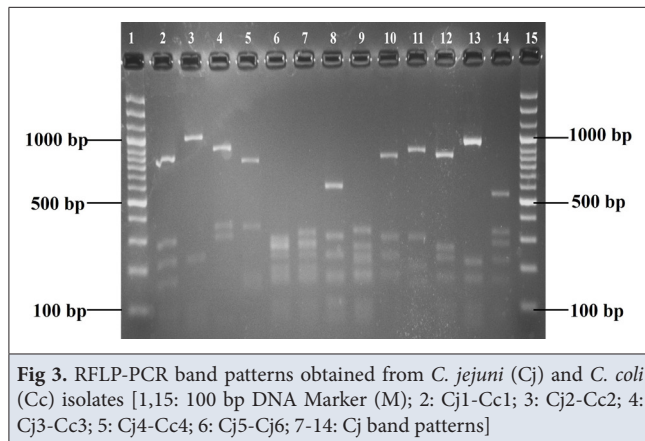


Fig 3. RFLP-PCR band patterns obtained from *C. jejuni* (Cj) and *C. coli* (Cc) isolates [1,15: 100 bp DNA Marker (M); 2: Cj1-Cc1; 3: Cj2-Cc2; 4: Cj3-Cc3; 5: Cj4-Cc4; 6: Cj5-Cc6; 7-14: Cj band patterns]

than in isolates with the other genotypes ($P < 0.05$; Table 7). Isolates were not included in the analysis for gentamicin due to the absence of AMR.

When AMR was evaluated based on the presence of virulence genes, only isolates that were *pldA* positive were found to have higher resistance to nalidixic acid than negative isolates ($P < 0.05$). No difference was observed in AMR for the *cdtA* and *cdtC* virulence genes. Because *cdtB*, *ciaB*, *cadF*, and *dnaJ* virulence genes were positive in all isolates, they were not included in the analysis (Table 8).

DISCUSSION

The study revealed that *Campylobacter* spp. was isolated from 30.74% (115/374) rectal swab samples taken from sheep and goats in the Siirt region. Currently, there is limited data in the literature regarding the investigation

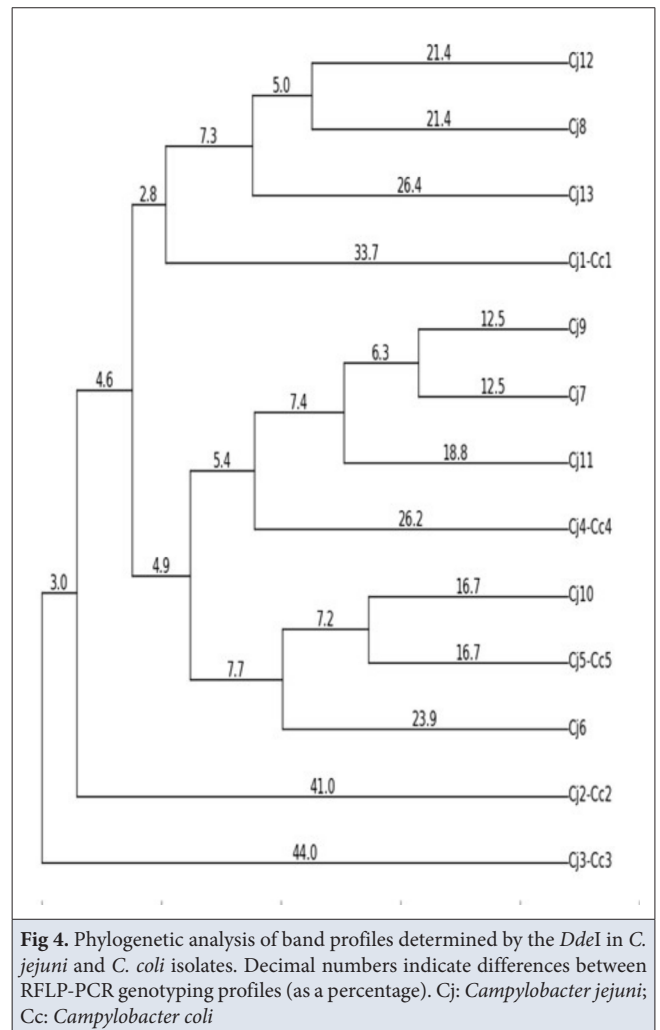


Fig 4. Phylogenetic analysis of band profiles determined by the *DdeI* in *C. jejuni* and *C. coli* isolates. Decimal numbers indicate differences between RFLP-PCR genotyping profiles (as a percentage). Cj: *Campylobacter jejuni*; Cc: *Campylobacter coli*

Table 7. Relationship between *flaA* genotypes and antibiotic resistance status

Genotype	AMC	ATM	C	CIP	ERY	KF	NA	S	TE
Cj1 (n=10)	0 (0%)	0 ^b (0%)	0 (0%)	7 ^a (70%)	0 (0%)	10 (100%)	2 (20%)	0 (0%)	0 ^b (0%)
Cj2 (n=9)	1 (11.11%)	0 ^b (0%)	0 (0%)	8 ^a (88.88%)	2 (22.22%)	8 (88%)	0 (0%)	0 (0%)	8 ^a (0%)
Cj3 (n=7)	0 (0%)	7 ^a (100%)	0 (0%)	5 ^a (71.42%)	1 (14.28%)	7 (100%)	1 (14.28%)	2 (28.57%)	2 ^b (28.57%)
Cj4 (n=2)	0 (0%)	2 ^b (100%)	0 (0%)	0 ^b (0%)	0 (0%)	2 (100%)	0 (0%)	0 (0%)	2 ^b (100%)
Cj5 (n=15)	0 (0%)	1 ^b (6.66%)	1 (6.66%)	9 ^b (60%)	3 (20%)	13 (86.66%)	1 (6.66%)	3 (20%)	6 ^b (40%)
Cj6 (n=11)	0 (0%)	8 ^a (72.72%)	1 (9.09%)	3 (27.27%)	2 (18.18%)	10 (90.90%)	3 (27.27%)	6 (54.54%)	4 (36.36%)
Cj7 (n=13)	0 (0%)	3 ^b (23.07%)	1 (7.69%)	13 ^a (100%)	1 (7.69%)	13 (100%)	1 (7.69%)	3 (23.07%)	12 ^a (92.30%)
Cj8 (n=2)	0 (0%)	1 (50%)	0 (0%)	1 ^b (50%)	1 (50%)	2 (100%)	0 (0%)	1 (50%)	0 ^b (0%)
Cj9 (n=1)	0 (0%)	0 ^b (0%)	0 (0%)	1 ^b (100%)	0 (0%)	1 (100%)	1 (100%)	0 (0%)	0 ^b (0%)
Cj10 (n=9)	0 (0%)	5 ^{ab} (55.55%)	0 (0%)	4 ^b (44.44%)	6 (66.66%)	8 (88.88%)	3 (33.33%)	0 (0%)	0 ^b (0%)
Cj11 (n=2)	0 (0%)	0 ^b (0%)	0 (0%)	2 ^b (100%)	1 (50%)	2 (100%)	0 (0%)	1 (50%)	1 ^b (50%)
Cj12 (n=2)	0 (0%)	1 ^b (50%)	0 (0%)	2 ^b (100%)	0 (0%)	2 (100%)	1 (50%)	0 (0%)	0 ^b (0%)
Cj13 (n=1)	0 (100%)	1 ^b (100%)	0 (0%)	1 ^b (100%)	0 (0%)	1 (100%)	1 (100%)	0 (0%)	1 ^b (100%)
P-value	>0.05	0.000***	>0.05	0.011*	>0.05	>0.05	>0.05	>0.05	0.000***

* $P < 0.05$, *** $P < 0.001$. ^{ab}Different letters in the same column indicate significant differences in antibiotic resistance among the corresponding genotypes; AMC: Amoxicillin-clavulanic acid; ATM: Aztreonam; C: Chloramphenicol; CIP: Ciprofloxacin; ERY: Erythromycin; GEN: Gentamicin; KF: Cefalotin; NA: Nalidixic acid; S: Streptomycin; TE: Tetracycline

Table 8. Relationship between the presence of virulence genes and antibiotic resistance status

Virulence Genes	AMC	ATM	C	CIP	ERY	KF	NA	S	TE
<i>cdtA</i> Positive (n=17)	1 (5.88%)	7 (41.17%)	0 (0%)	11 (64.70%)	4 (23.52%)	16 (94.11%)	5 (29.41%)	1 (5.88%)	4 (23.52%)
<i>cdtA</i> Negative (n=67)	0 (0%)	22 (32.83%)	3 (4.47%)	45 (67.16%)	13 (19.40%)	63 (94.02%)	9 (13.43%)	15 (22.38%)	32 (47.76%)
P-value	p>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
<i>cdtC</i> Positive (n=12)	1 (8.33%)	5 (41.66%)	0 (0%)	9 (75%)	4 (33.33%)	11 (91.66%)	4 (33.33%)	4 (33.33%)	4 (33.33%)
<i>cdtC</i> Negative (n=72)	0 (0%)	24 (33.33%)	3 (4.16%)	47 (65.27%)	13 (18.05%)	68 (94.44%)	10 (13.88%)	12 (16.66%)	32 (44.44%)
P-value	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
<i>pldA</i> Positive (n=12)	1 (8.33%)	7 (58.33%)	1 (8.33%)	9 (75%)	3 (25%)	11 (91.66%)	5 (41.66%)	2 (16.66%)	4 (33.33%)
<i>pldA</i> Negative (n=72)	0 (0%)	22 (30.55%)	2 (2.77%)	47 (65.27%)	14 (19.44%)	68 (94.44%)	9 (12.5%)	14 (19.44%)	32 (44.44%)
P-value	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	0.012*	>0.05	>0.05

*P<0.05; AMC: Amoxicillin-clavulanic acid; ATM: Aztreonam; C: Chloramphenicol; CIP: Ciprofloxacin; ERY: Erythromycin; GEN: Gentamicin; KF: Cefalotin; NA: Nalidixic acid; S: Streptomycin; TE: Tetracycline

of *Campylobacter* spp. in sheep and goat fecal samples in Türkiye, as studies have mostly focused on foods offered for consumption^[16,17]. However, there are various studies conducted internationally for the isolation and identification of *Campylobacter* spp. When these studies were examined, it was determined that the prevalence of *Campylobacter* spp. varied between 11.3% and 98.8%^[8,34-37]. When studies conducted at the species level were evaluated, it was reported that the prevalence of *C. jejuni* and *C. coli* ranged from 5.7% to 60.34% and 15.15% to 69.6%, respectively^[34,35,38]. In the current study, 87.82% (n=101) of 115 (30.74%) *Campylobacter* spp. isolates obtained were identified as *C. jejuni*, 12.17% (n=14) as *C. coli*, and no positive results were obtained for *C. fetus* subsp. *fetus*. The differences observed in the study results were thought to be related to geographical conditions, animal husbandry conditions, sample size, and sampling methods.

Historically, fluoroquinolones, macrolides, tetracyclines, and aminoglycosides have been among the primary therapeutic options for treating campylobacteriosis in the veterinary field^[16]. While their current use is subject to varying regional regulations and antimicrobial stewardship programs, the development of resistant strains and their potential transmission to humans through contaminated food remains a significant public health concern^[15,39].

In the present study, ciprofloxacin resistance exhibited significant geographic variation across studies. The resistance rate observed in our study was lower than those reported in African and Middle Eastern settings^[5,38,40,41], yet markedly higher than data reported from European livestock^[42].

The observed variability in results is attributed to methodological differences and regional practices. On one hand, the exceptionally high resistance rates reported in certain studies^[40,41] must be interpreted with caution due to limited sample sizes that could lead to sampling bias.

In this context, our larger pool of isolates provides a more robust and representative baseline dataset. On the other hand, the discrepancy between findings in Europe^[42] and our results highlights differences in antimicrobial stewardship practices within the veterinary field. While strict European restrictions on fluoroquinolone use in livestock effectively limit the development of resistance, higher resistance levels in other regions point to ongoing selective pressure resulting from unregulated antibiotic use in animal production.

Conversely, a high level of cephalothin resistance was observed in our study, aligning with previous reports^[5,40]. This finding is expected, as *Campylobacter* species exhibit well-documented intrinsic resistance to first-generation cephalosporins^[43]. Therefore, this particular resistance profile reflects the innate microbiological characteristics of the genus rather than acquired resistance driven by selective pressure.

It was thought that the differences in the AMR rates could be due to uncontrolled antibiotic use, differences in the number of isolates obtained, regional conditions, and differences in the methods used in the studies. Furthermore, the evaluation of the obtained data reveals the importance of the One Health perspective, which acknowledges the interconnectedness of animal health, human health, and the shared environment^[43]. Small ruminants serve as important reservoirs for *Campylobacter* species, and the high rates of AMR detected in this study have significant implications for public health and food safety. Zoonotic transmission can occur through direct contact with infected animals, environmental contamination, or, most importantly, through the consumption of undercooked meat and unpasteurized dairy products. When MDR *Campylobacter* strains enter the food chain, they can lead to treatment failures, limiting the available empirical treatment options for human campylobacteriosis^[15]. Therefore, the implementation of coordinated surveillance

systems between the veterinary and public health sectors is vital to reduce the spread of these resistant zoonotic pathogens from farm to table.

Regarding molecular resistance determinants, this study revealed distinct gene distribution profiles. The detection of the 23S rRNA gene (which confers macrolide resistance) in every sample aligns with the observations of Gharbi et al.^[15]. However, variations in the frequencies of specific point mutations (*ERY2075* and *ERY2074*) point to local genomic micro-evolution among *Campylobacter* strains, likely shaped by regional macrolide administration protocols in veterinary medicine. The detection rate of the beta-lactamase determinant (*bla_{OXA-61}*) among the analyzed isolates was found to be higher compared to reports from Egypt, Indonesia, and Türkiye^[15,44-46]. In contrast, the frequency of the aminoglycoside resistance gene (*aphA-3-I*) showed a lower profile compared to African isolates^[44], whereas the tetracycline resistance determinant [*tet(O)*] exceeded previously reported levels^[15,38,46]. These differences across studies likely reflect varying exposure profiles to specific antimicrobial classes within regional animal production systems. Methodologically, while the limited sample sizes in some studies^[44,45] introduce potential selection biases, the expanded pool of isolates evaluated in this study offers greater epidemiological reliability. Furthermore, the widespread presence of multidrug resistance determinants particularly active efflux systems such as *cmeB* supports current evidence highlighting the importance of efflux-mediated processes in the survival mechanisms of *Campylobacter* under persistent drug pressure^[47]. When combined with antibiotic heterotolerance and phenotypic persistence^[48], these dynamic survival strategies significantly complicate standard therapeutic interventions. To reconcile these regional differences and elucidate the dynamics of resistance transmission across ecosystems, future research should be supported by methods such as Whole Genome Sequencing (WGS), which enables high-resolution profiling of novel genomic variants and high-risk lineages^[49].

Virulence genes play a crucial role in the colonization, adhesion, and invasion of bacterial pathogens^[15]. Virulence genes, which cause an increase in the severity of infections, are also of great importance in the pathogenesis of the disease^[50]. Analysis of virulence-associated determinants revealed diverse pathogenic profiles among the evaluated isolates. Regarding adhesion and colonization factors, the high detection frequency of the *cadF* gene aligns closely with observations by Lúcio et al.^[51], though it presents a minor divergence from the complete detection reported by Khoshbakht et al.^[52] and the lower frequency described by Hizlısoy et al.^[53]. Similarly, the motility-associated *flaA* gene was present in the majority of isolates, surpassing the prevalence reported by Hizlısoy et al.^[53] while remaining

slightly below the figures presented by Hafez^[38]. Because adherence and motility are foundational for mucosal colonization, these high prevalence rates underscore a conserved pathogenic potential across the analyzed strains.

Conversely, distinct patterns emerged concerning cytolethal distending toxin (*cdt*) cluster genes (*cdtA*, *cdtB*, and *cdtC*). The detection frequencies for *cdtA* and *cdtC* in the present investigation were comparatively lower than those reported in earlier studies^[51-53]. For *cdtB*, however, the frequency exceeded data from South America^[51], despite remaining below full or near-full prevalence documented in other regional reports^[52,53]. These variations in toxin gene profiles likely reflect genomic instability, insertion sequences, or point mutations within the *cdt* operon across geographically distinct lineages, which may alter PCR binding efficiency or lead to gene loss.

Remarkably, the stress response and invasion-related determinants *dnaJ* and *ciaB* were prominently represented in the analyzed isolates, substantially surpassing the prevalence levels documented by Hafez^[38] and Lúcio et al.^[51]. The high conservation of *dnaJ* and *ciaB* suggests an enhanced capacity for intracellular survival and thermal adaptation within the host environment. Methodologically, the complete absence of the *ggt* gene in our dataset indicates lineage-specific genetic divergence, as *ggt* is known to be heterogeneously distributed and primarily associated with specific strain colonization dynamics. Overall, these inter-study discrepancies highlight the genetic plasticity of *Campylobacter* virulence factors, where regional selective pressures and host-adaptation mechanisms shape the pathogenic repertoire.

The *DdeI* is known as the most widely preferred restriction enzyme worldwide for the differentiation of *Campylobacter* isolates within a specific ecological niche^[54]. Within the scope of the study, RFLP analysis performed on *flaA*-positive isolates using the *DdeI* resulted in the observation of 13 different band patterns. When the related studies were reviewed, Aslantaş^[17] reported that they observed 11 different band profiles in 55 *flaA* gene-positive *C. jejuni* isolates and 2 different band profiles in 24 *C. coli* isolates in their study conducted in Hatay and Adana. Yadav et al.^[54] identified 15 different band patterns in 43 *C. jejuni* isolates obtained from cloacal swab samples of poultry in India.

The differences between the studies are thought to be caused by the number of isolates obtained, the diversity of variations in the bacteria, and the different resistance profiles. Thus, in this study, we observed that AMR is not randomly distributed, but rather closely associated with specific genotypes and virulence genes. In particular, tetracycline and ciprofloxacin resistance was significantly clustered in Cj2 and Cj7 genotypes ($P < 0.001$), while

aztreonam resistance was higher in Cj3 and Cj6 genotypes ($P<0.05$). This diverse distribution demonstrates that specific genetic profiles of *Campylobacter* spp. may be associated with resistance to critically important antibiotics. Furthermore, we identified a potential link between virulence and resistance: *pldA*-positive isolates showed significantly higher resistance to nalidixic acid ($P<0.05$). This association suggests that antibiotic selection pressure in small ruminants may inadvertently co-select for strains carrying specific virulence markers. In conclusion, these observed resistance-virulence patterns indicate that the transmission of these resistant profiles complicate clinical treatment. Therefore, monitoring these specific molecular genotypes is vital for regional disease control and public health.

A notable limitation of this study relates to the selective culture parameters employed for isolation. Sample incubation was conducted under microaerophilic conditions at 41.5-42°C, a temperature regime that selectively favors the growth of thermophilic species such as *C. jejuni* and *C. coli*. However, non-thermophilic species-most notably *C. fetus*, which requires lower incubation temperatures (37°C) for optimal growth-may have been inhibited or underdetected during primary culture. Therefore, the absence of *C. fetus* subsp. *fetus* isolates in our findings should be interpreted with caution and should not be construed as definitive proof of its absence in the regional small ruminant population.

Additionally, another key limitation is that sampling was restricted exclusively to diarrheic animals without the inclusion of a non-diarrheic healthy control group. Because *Campylobacter* species can naturally colonize the gastrointestinal tract of small ruminants as commensal organisms, a definitive causal relationship between the isolated strains and the observed clinical diarrhoea cannot be firmly established. Furthermore, potential co-infections with other enteric pathogens (e.g., viral, parasitic, or other bacterial agents) were not systematically evaluated. Accordingly, our findings reflect the prevalence, antimicrobial resistance, and virulence profiles of *Campylobacter* strains recovered from diarrheic cases rather than proving direct etiology.

Consequently, the current study suggests that *C. jejuni* and *C. coli* are frequently recovered from diarrheic sheep and goats in the Siirt region. While high *in vitro* susceptibility to gentamicin, streptomycin, amoxicillin-clavulanic acid, and erythromycin identifies these antimicrobials as potential candidates for veterinary use, further *in vivo* clinical studies are required to confirm their therapeutic efficacy under field conditions. Notably, higher resistance tetracycline and ciprofloxacin was observed among isolates with Cj2 and Cj7 genotypes, whereas aztreonam resistance prominent in Cj3 and Cj6 genotypes. Additionally, isolates

carrying the *pldA* gene combined with nalidixic acid resistance reflect specific genotype-phenotype patterns that warrant continued surveillance. Although these findings provide valuable baseline data for regional small ruminant management in Southeastern Anatolia, caution should be exercised before generalizing these results to other regions.

Furthermore, given that human isolates were not evaluated, small ruminants should be regarded as potential reservoirs rather than confirmed sources of direct zoonotic transmission. Future research utilizing comprehensive 'One Health' surveillance frameworks and comparative genomic tools will be essential to clarify transmission dynamics and safeguard public health.

DECLARATION

Availability of Data and Materials: The data used in this study are available from the corresponding author (MY) on reasonable request.

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Ethical Compliance Declaration: The authors declare that the study was conducted in accordance with all applicable animal welfare and research ethics principles, institutional and national/international regulations, and the requirements of the relevant ethics committees and funding/supporting institutions, where applicable. The authors accept full responsibility for the ethical integrity of the study.

Authors' Contributions: MY and ÖG contributed to the project idea, design and execution of the study. MY contributed to the acquisition of data. MY analysed the data. MY and ÖG drafted and wrote the manuscript. MY, ÖG and AOT reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

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RESEARCH ARTICLE

Effects of Dietary Methylsulfonylmethane (MSM) Supplementation on Apparent Nutrient Digestibility, Blood Parameters and Faecal Fermentation in Golden Retriever Dogs

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Abstract

Methylsulfonylmethane (MSM) is a naturally occurring organosulfur compound whose effects in dogs are poorly characterised. This study evaluated dietary MSM on nutrient digestibility, blood parameters and faecal fermentation in Golden Retriever dogs. Twenty-four healthy adults (2-8 years; 30.7±3.3 kg) were randomly allocated to three groups of eight: a basal diet without MSM (Control) and the same diet top-dressed with 4 g (MSM4; 127 mg/kg) or 8 g (MSM8; 259 mg/kg) MSM per head daily for 30 days. Digestibility (acid-insoluble ash), blood (days 0 and 30) and faecal short-chain fatty acids (SCFA) and ammonia were assessed. Apparent digestibility of dry matter, organic matter, crude protein and crude fat did not differ among groups ($P>0.05$). Serum glucose and creatinine differed among groups ($P<0.01$); triglyceride showed only a linear trend (overall $P=0.064$; linear $P=0.026$). The relative monocyte proportion rose and the neutrophil proportion fell with dose. Faecal consistency and pH were unchanged, but faecal acetate was lower in MSM8 than in the control ($P=0.021$), total SCFA declined linearly ($P=0.011$) and faecal ammonia-N was higher in MSM8 ($P=0.014$). In this small 30-day study, MSM at 4 or 8 g/head/day was not associated with detectable differences in digestibility or faecal consistency; some clinical-pathology and faecal metabolite measures differed or showed dose trends, but their biological significance is uncertain and the fermentation findings require confirmation.

Keywords: Methylsulfonylmethane, Dog, Nutrient digestibility, Blood parameters, Faecal fermentation, Short-chain fatty acids

INTRODUCTION

Functional feed ingredients are increasingly incorporated into premium pet foods to support gastrointestinal function, antioxidant status and overall wellbeing of companion animals [1,2]. Among these, methylsulfonylmethane (MSM; dimethyl sulfone, $(CH_3)_2SO_2$) has drawn particular attention. MSM is a naturally occurring organosulfur compound that contains approximately 34% sulfur by weight and represents a stable oxidised metabolite of dimethyl sulfoxide within the biological sulfur cycle [3,4]. It is readily absorbed, widely distributed across tissues and is able to cross the blood-brain barrier, and it has been granted “Generally Recognized as Safe” status [3,5]. As an organic sulfur source, MSM is considered more

bioavailable and metabolically advantageous than inorganic sulfur salts, which are often poorly utilised by animals [4,6].

A range of biological activities has been ascribed to MSM, including antioxidant and anti-inflammatory effects mediated through enhancement of glutathione-dependent defences and inhibition of nuclear factor- κ B signalling [7-9]. In production animals, dietary MSM has improved growth performance, antioxidant capacity and immune indices in broilers and Pekin ducks, and has increased ileal protein digestibility in poultry [10,11]. In companion animals, most available evidence concerns integumentary and microbial endpoints: MSM improved hair quality and modulated the faecal microbiota in Ragdoll kittens and in



poodles, without adverse effects on growth, feed intake or faecal output ^[1,12]. In laying hens, and particularly under the oxidative and metabolic stress of feed restriction, dietary MSM has improved performance and egg quality, reduced serum and yolk malondialdehyde, and altered serum biochemical variables such as glucose and alkaline phosphatase ^[13]. Reviews of MSM in animal nutrition have pointed to its potential to support intestinal health and modulate immune and metabolic responses ^[4,14].

Despite this interest, the effects of MSM on nutrient digestibility, serum biochemistry and haematology in dogs remain largely unexplored, and dose-response information is scarce. The canine evidence to date rests on a single trial in poodles ^[1], whereas the digestibility, blood and antioxidant findings summarised above derive from poultry, rodents and humans and cannot be assumed to transfer to dogs. Because dogs are frequently supplemented with MSM for joint and skin health, characterising its influence on digestive efficiency and routine clinical-pathology variables is important both for efficacy and for safety. The two doses were chosen to represent a practical supplementary level (4 g/head/day, about 130 mg/kg) and a two-fold multiple of it (8 g/head/day, about 260 mg/kg), both far below the no-observed-adverse-effect level of 1.5 g/kg/day established in rats ^[15]. The primary objective was to determine whether these doses altered the apparent total-tract digestibility of dry matter, organic matter, crude protein and crude fat; the hypothesis was that digestibility would be maintained. Secondary objectives were to document serum biochemical and haematological variables and faecal consistency and pH under the same regimen, with the expectation of no clinically relevant change. Faecal short-chain fatty acids and ammonia were measured as exploratory indicators of hindgut fermentation without a directional hypothesis.

MATERIAL AND METHODS

Ethical Statement

The animal experiments were approved by the Selçuk University Faculty of Veterinary Medicine Experimental Animal Production and Research Centre Ethics Committee (SÜVDAMEK; decision no. 2025/165; date 30/10/2025). Every procedure was carried out in accordance with the relevant laws and standards, and the study was conducted in accordance with the 3Rs principle (Replacement, Reduction, Refinement) of animal welfare.

Animals and Housing

The study was carried out at the Dog Unit of the Prof. Dr. Hümeýra Özgen Research and Application Farm, Faculty of Veterinary Medicine, Selçuk University (Konya, Türkiye), where the dogs are bred and maintained for scientific studies. Twenty-four clinically healthy adult

Golden Retriever dogs of mixed gender (2-8 years; body weight 30.7 ± 3.3 kg, range 24.5-37.4 kg; body condition score 4-7 on a nine-point scale) were housed individually in pens comprising a closed indoor (190 × 190 cm) and an open outdoor (510 × 230 cm) concrete-floored area, with free access to clean water throughout the study. Eligibility required a normal clinical examination by a veterinarian before enrolment; no dog was excluded, all 24 completed the trial and every dog contributed to every outcome, so there was no attrition and no missing animal-level value. The study is reported in line with the ARRIVE 2.0 guidelines ^[16].

Diets and Feeding

Dogs were allocated to three groups of eight by drawing lots (simple randomisation) by an investigator not involved in feeding or sampling; the resulting groups were then checked for balance in body weight (29.9 ± 2.8 , 31.4 ± 3.7 and 30.9 ± 3.7 kg; $P=0.68$), sex and pen location. The groups received the basal diet without MSM (control), or the same diet top-dressed with 4 g (MSM4) or 8 g (MSM8) MSM per head daily. The supplement was pharmaceutical-grade methylsulfonylmethane (purity 99%; ZAG Kimya, Türkiye; product code ZK.101678.1000) supplied as a pure crystalline powder without carrier or excipient; the achieved doses were 127 (range 107-148) and 259 (216-300) mg/kg body weight per day. Daily portions were weighed into coded containers each week and mixed onto the daily ration immediately before feeding. The MSM was added on top of the full basal allowance and did not displace any food, so intake of the basal diet and of its energy and nutrients was identical across groups. Dogs were fed once daily at the same time, and the daily allowance was calculated to meet the maintenance energy requirement of kennel-housed adult dogs with low activity (metabolisable energy = body weight × 0.75 × 105 kcal; mean 1370 kcal/day) ^[17-19]. Consumption was checked at every feeding and any refusal was to be weighed back; the ration was consumed completely by every dog on every day, so no refusals or spillage were recorded. Because MSM was top-dressed as a visible powder and no placebo powder was given to the control group, the feeding staff could not be blinded to the presence of a supplement. Faecal scoring, sample processing and all laboratory analyses, however, were performed by personnel unaware of group allocation, and samples were identified by dog number only. The ingredient and analysed chemical composition of the basal diet are presented in [Table 1](#). The feeding trial lasted 30 days, including an initial adaptation period, and serum and haematological samples were obtained on days 0 and 30.

Chemical Analysis

Experimental feed and faeces were analysed for dry matter, crude ash, crude protein (CP), acid-hydrolysed ether extract (EE) and crude fibre (CF) according to the

Table 1. Ingredient and chemical composition of the basal diet

Item	Value
Ingredient, %	
Meat-and-bone meal	13.0
Poultry meal	14.0
Maize	25.3
Brewer's yeast	5.0
Rice	23.45
Wheat middlings	9.0
Vitamin-mineral premix	0.25
Poultry fat	10.0
Analysed composition (dry-matter basis unless stated; single batch)	
Dry matter, % as-fed	88.0
Crude ash, % DM	11.6
Crude fat, % DM	7.2
Crude fibre, % DM	5.8
Crude protein, % DM	22.9
Nitrogen-free extract, % DM (calculated)	52.5
Metabolisable energy, kcal/kg DM (calculated)*	3002

* Metabolisable energy calculated on a dry-matter basis. DM: dry matter

official methods of AOAC [20]; starch was determined by the same reference. Nitrogen-free extract (NFE) and the metabolisable energy (ME) of the diet were then calculated from the analytical results using the following equations [17]:

$$NFE (\%) = 100 - (CP\% + EE\% + CF\% + moisture\% + crude\ ash\%)$$

$$ME (kcal/kg) = [(5.7 \times CP \times 10) + (9.4 \times EE \times 10) + (4.1 \times (NFE \times 10 + CF \times 10))] \times (91.2 - (1.43 \times CF)) / 100 - (1.04 \times CP \times 10)$$

Digestibility Trial

Apparent total-tract digestibility was determined using acid-insoluble ash (AIA) as an internal indigestible marker [21,22]. All faeces voided by each dog over the final five days of the trial were collected quantitatively, weighed immediately after defecation and stored at -20°C in individually labelled bags. The faeces of each dog were then thawed, pooled, homogenised, dried at 55°C to constant weight, ground to pass a 1-mm screen and stored at -20°C until analysis. For AIA determination, samples were ashed at 600°C , boiled in 2 N hydrochloric acid, filtered, re-ashed and weighed [20]. Digestibility was calculated as follows:

$$\text{Dry-matter digestibility } (\%) = 100 - 100 \times (AIA \text{ diet} / AIA \text{ faeces})$$

$$\text{Nutrient digestibility } (\%) = 100 - 100 \times [(AIA \text{ diet} \times \text{nutrient faeces}) / (AIA \text{ faeces} \times \text{Nutrient diet})]$$

Organic matter was calculated as $100 - \text{crude ash}$, and apparent digestibility coefficients were computed for dry matter (DMD), organic matter (OMD), crude protein (CPD) and crude fat (CFD).

Blood Sampling and Analyses

Blood was collected from each dog on the first (day 0) and last (day 30) day of the trial, in the morning after a 12-h fast, from the cephalic or saphenous vein under aseptic conditions. Blood for serum was drawn into plain (additive-free) tubes and, in a separate draw, into $K_3\text{EDTA}$ tubes for haematology; the serum tube was always filled first to avoid anticoagulant carry-over. Serum was obtained by centrifugation (3000 rpm, 10 min) after 30 min at room temperature and stored at -20°C until analysis [23]. Serum from both sampling days was analysed in a single batch by a commercial veterinary diagnostic laboratory (Institute MG, Konya, Türkiye) with an automated chemistry analyser operated according to the laboratory's standard procedures; the storage interval between the day-30 sampling and analysis was two months. The serum variables measured were blood urea nitrogen (BUN), creatinine, glucose, total cholesterol, triglyceride, total protein, albumin, aspartateaminotransferase (AST), alanineaminotransferase (ALT), gamma-glutamyltransferase (GGT), amylase, calcium, phosphorus and magnesium. A complete blood count, including erythrocyte and leukocyte indices and platelet variables, was performed with an automated haematology analyser. Reference intervals were those supplied by the laboratory, taken from the MSD Veterinary Manual (2024 edition), and were used only for descriptive interpretation [24].

Faecal Consistency Score and Faecal pH

During the collection period, faecal consistency was independently scored immediately after defecation by four trained observers blinded to treatment using a five-point scale [25], defined as: 1 = liquid formless; 2 = soft, poorly formed; 3 = soft, formed and moist, leaving a mark on the ground; 4 = well-formed and firm, not leaving a mark; 5 = hard and dry. On this scale, scores of 3-4 are generally regarded as desirable, whereas values near 2.5 lie at the soft end of the range. The mean of the observers' scores over the scoring days was used for each dog. Inter-observer agreement, assessed on 12 dogs scored on three consecutive days by all four observers (36 samples), was good: the intraclass correlation coefficient was 0.73 for a single observer (ICC(2,1)) and 0.92 for the mean of the four observers (ICC(2,4)). Faecal pH was measured in fresh faeces collected during the same period using a calibrated pH meter, and the mean of duplicate readings per sample was recorded.

Faecal Short-Chain Fatty Acids and Ammonia

During the last four days of the trial a fresh faecal sample was collected from each dog immediately after

defecation on each day, always in the morning within the same time window after the previous day's meal. The four daily samples of each dog were pooled into one composite per dog, which was analysed in duplicate, so that one dog-level value entered the analysis and the dog was the experimental unit for all faecal variables. Two grams of faeces were mixed with 5 mL of 2 M hydrochloric acid and stored at -20°C until analysis [26]. After thawing, the acidified suspension was clarified by centrifugation and the supernatant was diluted 1:10 (v/v) with deionised water before analysis (dilution factor $D = 10$). Acetate, propionate and butyrate were quantified by gas chromatography (6890N, Agilent Technologies, USA) equipped with a split injector, a flame-ionisation detector and a capillary column ($30\text{ m} \times 320\text{ }\mu\text{m} \times 1.00\text{ }\mu\text{m}$; Agilent J&W). The oven programme was 80°C for 1 min, $80\text{--}120^{\circ}\text{C}$ at $20^{\circ}\text{C}/\text{min}$, then 230°C for 3 min; injector temperature 250°C , injection volume $1\text{ }\mu\text{L}$, split ratio 10:1; detector temperature 250°C ; carrier gas hydrogen (purity $\geq 99.998\%$) at $30\text{ mL}/\text{min}$ with air at $300\text{ mL}/\text{min}$ [27]. A certified short-chain fatty acid mixture (acetic, propionic, isobutyric, butyric, isovaleric, valeric and n-heptanoic acids; 99.5% purity; Chem Service, West Chester, USA) was used as the quantitative external standard [28]. Each extract was injected in duplicate and the mean of the two injections was used for the individual dog; the between-injection coefficient of variation was below 5%. Valeric, isocaproic and hexanoic acids were above the limit of detection in only 1, 1 and 4 of the 24 dogs, respectively, and were not quantified; no substitution of zeros was needed for the three major acids, which were detected in every sample. Ammonia nitrogen ($\text{NH}_3\text{-N}$) was determined in the same diluted extracts, in duplicate, by the phenol-hypochlorite method of Weatherburn [29] (UV Mini 1240, Shimadzu, Japan; absorbance at 625 nm) against an external ammonium standard curve [30]. Concentrations were converted from the extract to a faecal dry-matter basis for each dog, as in previous canine studies [26,31], with the equation $C\text{ (}\mu\text{mol/g DM)} = C_{\text{extract}}\text{ (mmol/L)} \times V_{\text{extract}}\text{ (mL)} \times D/m_{\text{DM}}\text{ (g)}$. V_{extract} is the 5 mL of acid plus the water in the 2 g faecal aliquot, calculated from the individual faecal dry-matter content (mean 6.02 mL). $D = 10$ is the dilution factor. m_{DM} is the dry mass of the aliquot ($2\text{ g} \times \text{dry-matter fraction}$; mean 0.98 g). SCFA and ammonia-N are reported as $\mu\text{mol/g DM}$ ($1\text{ }\mu\text{mol NH}_3\text{-N} = 14.007\text{ }\mu\text{g N}$). Total SCFA was calculated as the sum of acetate, propionate and butyrate; the proportion of each acid in this total and the acetate-to-propionate (A:P) ratio were then derived.

Statistical Analysis

The primary outcomes, fixed before the trial, were the apparent digestibility coefficients of dry matter, organic matter, crude protein and crude fat. Serum biochemical and

haematological variables and faecal consistency and pH were secondary outcomes, and faecal SCFA and ammonia were exploratory. With eight dogs per group the study had 80% power ($\alpha = 0.05$) to detect a large effect only (Cohen's $f = 0.68$ among three groups, or $d = 1.5$ between two groups), equivalent to an 11.6-percentage-point difference in dry-matter digestibility or 3.7 points in crude-fat digestibility at the observed pooled standard deviations. Smaller true differences could not have been detected reliably. Data were analysed with SPSS (v26.0; IBM Corp., Armonk, NY, USA). Normality was assessed with the Shapiro-Wilk test together with visual inspection of Q-Q plots and skewness/kurtosis values, and homogeneity of variance with Levene's test. For variables measured on both days (serum biochemistry and haematology), among-group differences at day 30 were assessed by one-way analysis of covariance (ANCOVA) using the corresponding day-0 value as a covariate, thereby adjusting for baseline; equality of groups at baseline was confirmed by one-way ANOVA. The homogeneity-of-regression-slopes assumption (group $\times$ baseline interaction) was tested for every variable and held in all cases except BUN, eosinophil percentage, platelet count and plateletcrit, none of which showed a treatment effect; for these four the ANCOVA result is reported descriptively. Baseline-adjusted (least-squares) means with 95% confidence intervals were derived from these models and are reported in the text and in Fig. 1 for the variables that differed; the tables show raw day-30 means $\pm$ SD for descriptive purposes together with the ANCOVA P values. Pairwise comparisons after a significant ANCOVA used Bonferroni-adjusted contrasts of the adjusted means. Within-group day-0 versus day-30 comparisons are not reported, because they do not address the treatment question. End-of-trial variables were compared by one-way ANOVA followed by the Tukey HSD test when parametric assumptions were met (digestibility coefficients, faecal pH, acetate, propionate, acetate and propionate proportions, A:P ratio and ammonia-N), or by the Kruskal-Wallis test with Dunn's post-hoc test and Bonferroni correction otherwise (faecal consistency score, butyrate, total SCFA and butyrate proportion). In addition, because the three treatments represented equally spaced, ordered MSM levels (0, 4 and $8\text{ g}/\text{head}/\text{day}$), prespecified orthogonal polynomial contrasts were used to test linear and quadratic dose-response trends. These were estimated from the same linear model (on the baseline-adjusted estimates for ANCOVA outcomes, with standard errors from the model residual variance) and are reported for all variables, including those whose omnibus test was non-parametric, as a descriptive trend statistic. No adjustment was made for the number of outcomes tested; results with P values close to 0.05 are therefore regarded as exploratory and hypothesis-generating rather than confirmatory. No value was missing and no outlier was removed. Exact P values are reported throughout. Significance was set at $P < 0.05$, and results are presented as mean $\pm$ standard deviation.

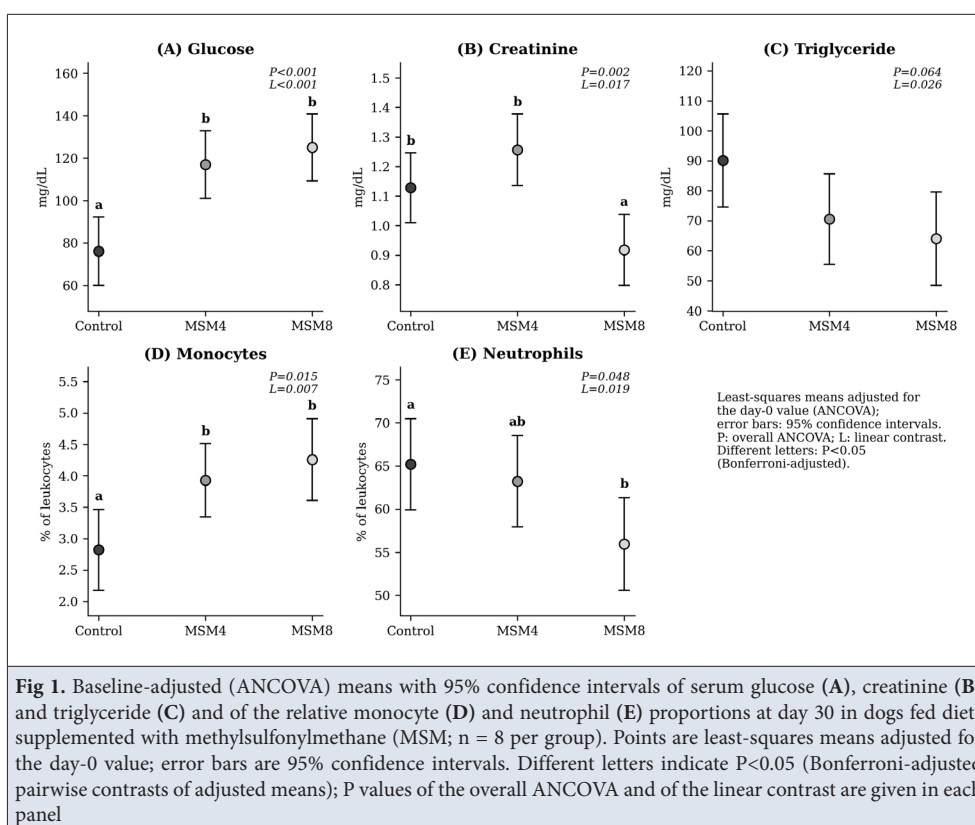


Fig 1. Baseline-adjusted (ANCOVA) means with 95% confidence intervals of serum glucose (A), creatinine (B) and triglyceride (C) and of the relative monocyte (D) and neutrophil (E) proportions at day 30 in dogs fed diets supplemented with methylsulfonylmethane (MSM; $n = 8$ per group). Points are least-squares means adjusted for the day-0 value; error bars are 95% confidence intervals. Different letters indicate $P<0.05$ (Bonferroni-adjusted pairwise contrasts of adjusted means); P values of the overall ANCOVA and of the linear contrast are given in each panel

RESULTS

Nutrient Digestibility

Apparent total-tract digestibility coefficients are shown in Table 2. MSM did not significantly affect the digestibility of dry matter, organic matter, crude protein or crude fat ($P>0.05$), although mean values were numerically higher in both MSM groups for all four fractions, the highest being recorded in MSM4. Body weight, re-measured on day 30, did not change in any group, and the ration was consumed completely throughout.

Serum Biochemistry

Day-30 serum biochemical values are presented in Table 3 (raw means; baseline-adjusted means with 95% CI for glucose, creatinine and triglyceride in Fig. 1); groups did not differ at baseline for any analyte (day-0 ANOVA, $P>0.05$).

Serum glucose and creatinine differed among groups at the end of the trial (ANCOVA $P<0.001$ and $P=0.002$). Glucose increased with MSM level in a significant linear dose-response (adjusted means 76, 117 and 125 mg/dL; linear $P<0.001$), whereas creatinine was lowest in MSM8 (adjusted 1.13, 1.26 and 0.92 mg/dL) and described a significant quadratic pattern ($P=0.004$). Triglyceride decreased with dose and, although the overall group effect was not significant ($P=0.064$), the decline was significant as a linear contrast ($P=0.026$). Liver enzymes, amylase, total protein, albumin, cholesterol, calcium, phosphorus and magnesium did not differ among groups ($P>0.05$); GGT showed a borderline linear trend (overall $P=0.094$; linear $P=0.050$).

Haematology

Haematological findings are summarised in Table 4. The leukocyte differential is reported both as relative

Table 2. Apparent total-tract nutrient digestibility (%) of dogs fed diets supplemented with methylsulfonylmethane (MSM)

Item	Control	MSM4	MSM8	Combined P-values	Contrast Analyses	
					L	Q
Dry matter	81.60±11.17	85.20±3.79	82.88±6.29	0.833	0.743	0.385
Organic matter	88.13±7.09	90.40±2.44	88.95±4.18	0.785	0.743	0.397
Crude protein	81.71±10.48	86.37±3.29	83.78±5.29	0.508	0.564	0.247
Crude fat	94.55±3.12	95.60±2.28	95.52±1.58	0.645	0.454	0.603

Values are mean±standard deviation ($n = 8$ per group). Control: no MSM; MSM4: 4 g MSM/head/day; MSM8: 8 g MSM/head/day. Combined P values: overall group effect (one-way ANOVA or Kruskal-Wallis); Contrast analyses: P values for the linear (L) and quadratic (Q) orthogonal polynomial contrasts across MSM dose

Table 3. Serum biochemical variables (day 30) of dogs fed diets supplemented with methylsulfonylmethane (MSM)

Parameter (unit)	Control	MSM4	MSM8	Combined P-values	Contrast Analyses	
					L	Q
BUN (mg/dL)	10.64±3.63	13.60±12.69	12.75±2.80	0.930	0.708	0.960
Creatinine (mg/dL)	1.12±0.11 ^b	1.29±0.25 ^b	0.89±0.14 ^a	0.002	0.017	0.004
Glucose (mg/dL)	77.75±35.84 ^a	115.62±7.31 ^b	124.62±8.26 ^b	<0.001	<0.001	0.096
Cholesterol (mg/dL)	161.0±99.7	122.8±71.1	158.1±69.7	0.537	0.965	0.271
Triglyceride (mg/dL)	91.88±32.11	70.62±14.86	62.12±4.12	0.064	0.026	0.473
Total protein (g/dL)	3.93±1.74	4.07±2.57	5.01±1.08	0.662	0.490	0.566
Albumin (g/dL)	2.44±0.92	2.16±0.79	1.91±0.37	0.370	0.174	0.769
AST (U/L)	10.00±4.99	11.50±6.65	12.88±4.22	0.599	0.649	0.387
ALT (U/L)	49.75±20.00	46.38±11.35	47.00±8.82	0.930	0.899	0.724
GGT (U/L)	5.75±3.11	5.62±1.77	7.38±2.07	0.094	0.050	0.339
Amylase (U/L)	926.8±217.5	879.0±191.5	794.3±166.7	0.442	0.210	0.847
Calcium (mg/dL)	6.14±2.61	6.09±2.28	7.80±1.14	0.251	0.248	0.241
Phosphorus (mg/dL)	4.97±0.88	4.58±0.87	5.33±0.82	0.259	0.832	0.107
Magnesium (mg/dL)	2.00±0.49	1.79±0.72	1.55±0.30	0.160	0.060	0.737

Values are raw day-30 mean±standard deviation (n = 8), shown for descriptive purposes; P values derive from the baseline-adjusted (ANCOVA) models. Combined p values: overall group effect from analysis of covariance adjusted for the day-0 baseline; Contrast analyses: P values for the linear (L) and quadratic (Q) polynomial contrasts across MSM dose, computed on the baseline-adjusted estimates. Means within a row with different superscript letters differ (P<0.05, Bonferroni-adjusted pairwise contrasts of adjusted means). The homogeneity-of-slopes assumption did not hold for BUN; its ANCOVA result is descriptive. Total protein, albumin and calcium were below the reference interval in all groups at both sampling days and are not interpreted in absolute terms (see Discussion)

Table 4. Haematological variables (day 30) of dogs fed diets supplemented with methylsulfonylmethane (MSM)

Parameter (unit)	Control	MSM4	MSM8	Combined P-values	Contrast Analyses	
					L	Q
WBC (10 ⁹ /L)	6.87±1.47	6.58±1.42	6.43±1.44	0.739	0.442	0.955
Neutrophils (10 ⁹ /L)	4.48±0.95	4.22±1.22	3.61±1.17	0.325	0.147	0.733
Lymphocytes (10 ⁹ /L)	2.11±0.68	1.94±0.49	2.22±0.57	0.675	0.442	0.632
Monocytes (10 ⁹ /L)	0.17±0.05	0.26±0.09	0.29±0.09	0.054	0.026	0.425
Eosinophils (10 ⁹ /L)	0.09±0.04	0.16±0.08	0.30±0.24	0.225	0.234	0.278
Neutrophils (%)	65.54±4.99 ^a	63.64±7.45 ^{ab}	55.24±9.05 ^b	0.048	0.019	0.405
Lymphocytes (%)	30.46±4.89	29.98±7.12	34.66±5.71	0.249	0.166	0.352
Monocytes (%)	2.58±0.74 ^a	3.91±1.00 ^b	4.53±0.76 ^b	0.015	0.007	0.270
Eosinophils (%)	1.38±0.44	2.41±1.02	5.49±4.94	0.220	0.101	0.701
RBC (10 ¹² /L)	7.26±1.37	6.32±0.94	6.97±0.76	0.144	0.948	0.053
Haemoglobin (g/dL)	17.39±3.22	15.40±2.40	16.99±2.04	0.234	0.939	0.093
Haematocrit (%)	47.79±8.06	42.64±6.37	46.81±5.46	0.196	0.979	0.075
MCV (fL)	66.03±3.30	67.49±3.61	67.11±2.92	0.135	0.615	0.052
MCHC (g/dL)	36.33±0.72	36.16±0.54	36.26±0.40	0.873	0.755	0.690
Platelets (10 ⁹ /L)	247.8±66.5	221.4±45.5	234.1±97.7	0.902	0.689	0.857
MPV (fL)	9.80±1.12 ^{ab}	10.21±0.71 ^a	9.46±0.97 ^b	0.032	0.031	0.122

Values are raw day-30 mean±standard deviation (n = 8), shown for descriptive purposes; P values derive from the baseline-adjusted (ANCOVA) models. Leukocyte differentials are given as relative proportions (compositional; sum ≈ 100%) and as absolute counts. Combined p values: overall group effect from analysis of covariance adjusted for the day-0 baseline; Contrast analyses: P values for the linear (L) and quadratic (Q) polynomial contrasts across MSM dose, computed on the baseline-adjusted estimates. Means within a row with different superscript letters differ (P<0.05, Bonferroni-adjusted pairwise contrasts of adjusted means); for MPV the omnibus test was significant but only MSM8 versus MSM4 remained significant after adjustment. The homogeneity-of-slopes assumption did not hold for eosinophil %, platelet count and plateletcrit; their ANCOVA results are descriptive

proportions, which are compositional and sum to approximately 100%, and as absolute counts. The relative monocyte proportion increased with MSM and was higher in both MSM groups than in the control ($P=0.015$), rising linearly with dose, while the relative neutrophil proportion decreased and was lower in MSM8 ($P=0.048$). Absolute monocyte counts showed a linear trend only (overall $P=0.054$; linear $P=0.026$) and absolute neutrophil counts did not differ ($P=0.33$). Mean platelet volume differed overall (ANCOVA $P=0.032$; linear $P=0.031$); after Bonferroni adjustment MSM8 differed from MSM4 ($P=0.048$) but not from the control ($P=0.092$). Eosinophils followed similar numerical trends that were not significant after adjustment for baseline, reflecting a higher eosinophil proportion already present in MSM8 at day 0. The overwhelming majority of erythrocyte, leukocyte and platelet indices remained within the reference intervals for dogs.

Faecal Consistency and pH

Faecal consistency score and faecal pH are shown in Table 5. Neither variable differed among groups ($P>0.05$). Mean faecal consistency scores were similar across groups (2.46-2.73 on the five-point scale) and faecal pH was close to 6.5 in all groups. Thus, MSM supplementation did not alter stool quality or the faecal acidity of the dogs.

Faecal Short-Chain Fatty Acids and Ammonia

Faecal SCFA concentrations, the derived fermentation profile and faecal ammonia are presented in Table 6 and Fig. 2. Acetate was the predominant acid in all groups. Faecal acetate was lower in MSM8 than in the control (29.1 vs 62.1 $\mu\text{mol/g DM}$; overall $P=0.021$, Tukey) and declined linearly with dose ($P=0.011$). Propionate differed among groups overall ($P=0.045$) and showed a linear decline ($P=0.024$), although no single pairwise comparison reached significance after Tukey adjustment. Butyrate and total SCFA did not differ in the overall Kruskal-Wallis test ($P=0.073$ and $P=0.052$) but both showed a significant linear contrast ($P=0.017$ and $P=0.011$).

The relative composition of the SCFA pool changed in parallel: the share of propionate rose from 28.3% to 37.2% of total SCFA (overall $P=0.082$; linear $P=0.046$) while the shares of acetate and butyrate fell, and the acetate-to-propionate ratio decreased from 2.48 to 1.69 (overall $P=0.059$; linear $P=0.027$). Because the absolute propionate concentration also decreased, this represents propionate forming a larger proportion of a smaller measured SCFA pool rather than an increase in propionate. Valeric, isocaproic and hexanoic acids were below the detection limit in the great majority of samples (detected in 1, 1 and 4 of 24 dogs, respectively) and were not analysed statistically.

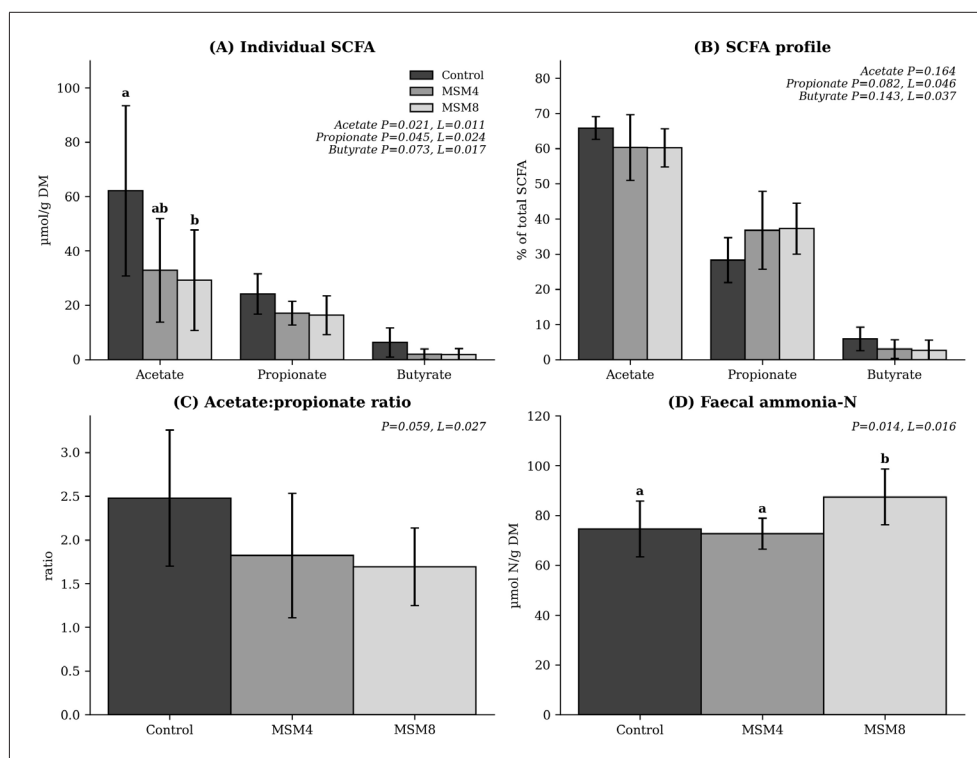


Fig 2. Faecal short-chain fatty acid (SCFA) concentrations (A), SCFA profile (B), acetate-to-propionate ratio (C) and faecal ammonia nitrogen (D) at the end of the trial in dogs fed diets supplemented with methylsulfonylmethane (MSM; $n = 8$ per group). Bars are raw means and error bars are standard deviations, expressed per g faecal dry matter. Different letters above bars indicate $P<0.05$ (Tukey HSD); P values of the overall test and of the linear contrast are given in each panel

Table 5. Faecal consistency score and faecal pH of dogs fed diets supplemented with methylsulfonylmethane (MSM)

Item	Control	MSM4	MSM8	Combined P- values	Contrast Analyses	
					L	Q
Faecal consistency score (1–5)	2.73±0.30	2.46±0.34	2.65±0.44	0.350	0.653	0.172
Faecal pH	6.59±0.21	6.53±0.15	6.60±0.18	0.698	0.946	0.403

Values are mean±standard deviation (n = 8). Faecal consistency was scored on a five-point scale (1 = liquid/formless; 2 = soft, poorly formed; 3 = soft, formed and moist; 4 = well-formed, firm; 5 = hard and dry). Combined P values: overall group effect (one-way ANOVA); Contrast analyses: linear (L) and quadratic (Q) polynomial contrasts across MSM dose. Scores of 3–4 are generally regarded as desirable on this scale; the observed means (about 2.5) lie at the soft end of the range. Faecal score was compared by Kruskal-Wallis and faecal pH by one-way ANOVA

Table 6. Faecal short-chain fatty acid concentrations, fermentation profile and ammonia nitrogen of dogs fed diets supplemented with methylsulfonylmethane (MSM)

Item	Control	MSM4	MSM8	Combined P- values	Contrast Analyses	
					L	Q
Acetate (μmol/g DM)	62.1±31.3 ^a	32.8±19.1 ^{ab}	29.1±18.5 ^b	0.021	0.011	0.227
Propionate (μmol/g DM)	24.1±7.4	17.0±4.4	16.3±7.1	0.045	0.024	0.264
Butyrate (μmol/g DM)	6.35±5.38	1.98±1.95	1.78±2.20	0.073	0.017	0.188
Total SCFA (μmol/g DM)	92.6±42.5	51.9±24.9	47.3±27.5	0.052	0.011	0.214
Acetate (% of total)	65.85±3.24	60.30±9.36	60.18±5.45	0.164	0.097	0.347
Propionate (% of total)	28.27±6.36	36.72±11.07	37.23±7.24	0.082	0.046	0.292
Butyrate (% of total)	5.88±3.36	2.98±2.62	2.58±2.86	0.143	0.037	0.340
Acetate: propionate	2.48±0.78	1.82±0.71	1.69±0.44	0.059	0.027	0.367
Ammonia-N (μmol/g DM)	74.6±11.2 ^a	72.7±6.2 ^a	87.4±11.2 ^b	0.014	0.016	0.065

Values are mean±standard deviation (n = 8), expressed per gram of faecal dry matter after conversion from the acidified extract (see Methods); ammonia-N is given as μmol N per g DM (× 14.007 = μg N/g DM). Total SCFA is the sum of acetate, propionate and butyrate; proportions are calculated on this total. Combined P values: overall group effect by one-way ANOVA (acetate, propionate, acetate %, propionate %, A:P ratio, ammonia-N) or Kruskal-Wallis (butyrate, total SCFA, butyrate %); Contrast analyses: P values for the linear (L) and quadratic (Q) polynomial contrasts across MSM dose. Means within a row with different superscript letters differ (P<0.05; Tukey HSD). Propionate differed overall (P=0.045) but no pairwise comparison reached significance after Tukey adjustment

Faecal dry-matter content did not differ among groups (P=0.500), indicating that the concentration differences were not a consequence of differences in faecal moisture.

Faecal ammonia-N moved in the opposite direction to the SCFA: it was higher in MSM8 (87.4 μmol/g DM, equivalent to 1224 μg N/g DM) than in either the control (74.6) or MSM4 (72.7 μmol/g DM) (overall P=0.014, Tukey) and increased linearly with dose (P=0.016).

DISCUSSION

To the authors' knowledge, this is among the first studies to characterise the effects of dietary MSM on apparent nutrient digestibility together with serum biochemistry, haematology, faecal quality and hindgut fermentation in dogs. In this small 30-day study, MSM at 4 or 8 g/head/day was not associated with detectable differences in nutrient digestibility, faecal consistency or faecal pH, within the limits of a design that could detect only large effects. Several clinical-pathology variables and faecal metabolites differed among groups or showed dose-related trends: faecal acetate and total SCFA were lower and faecal ammonia-N higher at the highest dose, and

serum glucose, creatinine and the leukocyte differential also changed. Whether any of these shifts is biologically important cannot be decided from the present data, and they are discussed below as observations requiring confirmation rather than as established effects.

The absence of significant digestibility differences agrees with recent canine and feline work, in which MSM did not change feed intake or faecal output in poodles^[1] and did not alter growth in Ragdoll kittens^[12], whereas it improved nutrient digestibility in broilers^[10]. The poultry result should not be extrapolated to dogs, whose digestive physiology and diet differ substantially. The numerical values obtained here were slightly higher in the supplemented groups for all four fractions, but the extruded diet was already highly digestible, leaving limited scope for improvement, and the between-animal variation typical of client-quality dogs reduced statistical power. Absence of a significant difference must not be read as evidence of equivalence: with eight dogs per group only differences of roughly 12 percentage points in dry-matter digestibility, or about 4 points in crude-fat digestibility, would have been detected reliably, and no equivalence margin was predefined. Any digestibility benefit of MSM

would plausibly act through support of mucosal integrity rather than a direct enzymatic effect ^[9], a mechanism proposed from rodent work that remains untested in dogs and is more likely to become apparent under digestive challenge than in healthy dogs. The coefficients obtained here fall in the same range as those reported previously for Golden Retrievers fed extruded diets ^[23,32], indicating that the acid-insoluble ash method ^[21,22] behaved consistently in this setting, although the present design does not itself validate the marker.

Faecal quality is a key acceptability criterion for a functional pet-food ingredient, and neither consistency score nor pH was affected, although the mean scores of about 2.5 lay at the soft end of the scale in all groups, including the control. This should be kept in mind when the absence of a treatment effect is interpreted. Faecal SCFA are increasingly used as a readout of hindgut fermentation in dogs, where they respond to the type and amount of fermentable substrate reaching the colon ^[33-36]. Faecal concentrations are, however, the net result of microbial production, mucosal absorption, transit time and dilution, so a lower faecal concentration may reflect reduced production, increased absorption or both. The present data therefore describe a change in measured faecal concentrations, not a demonstrated change in colonic production. Acetate and total SCFA fell as dose rose, every acid showed a linear contrast, and faecal dry matter did not differ among groups, so dilution by a wetter stool is excluded. This contrasts with Ragdoll kittens fed 0.2-0.4% MSM for 65 days, in which faecal SCFA were unaffected ^[12], and with laying hens, in which dietary MSM did not alter the proportions of ileal SCFA ^[6]. The inclusion rates used here were higher (about 0.8 and 1.5% of the daily ration as fed), and whether the faecal SCFA response is dose-dependent or specific to dogs cannot be determined from the present data. The higher proportion of propionate should be interpreted accordingly: absolute propionate also declined (24.1 to 16.3 $\mu\text{mol/g DM}$), so propionate simply formed a larger share of a smaller pool, and the lower acetate-to-propionate ratio was driven mainly by the larger fall in acetate. One hypothesis for such a pattern is that MSM-derived sulfur reaching the colon supports sulfate-reducing bacteria, which compete with acetogens for hydrogen; MSM has also been reported to modify the canine and feline faecal microbiota ^[1,12]. Neither sulfur species, sulfide, microbiota composition nor hydrogen metabolism was measured here, however, and this explanation remains untested speculation.

Faecal ammonia-N was higher at 8 g/head/day while the SCFA were lower. Ammonia is a product of microbial protein breakdown and, in dogs, rises when more protein reaches the colon and falls when fermentable carbohydrate increases ^[37]; in canine faecal microbiota fermented

in vitro, SCFA are used as markers of saccharolytic and ammonium and branched-chain fatty acids as markers of proteolytic fermentation ^[38]. The present pattern is therefore consistent with, but does not demonstrate, a relative shift from saccharolytic towards proteolytic activity: faecal ammonia also depends on mucosal absorption and on the nitrogen reaching the colon, and apparent crude-protein digestibility did not change. Faecal pH did not differ among groups, which may reflect the modest size of these shifts relative to the buffering capacity of canine faeces. The ammonia difference was about 17% above the control, all groups remained within a narrow range, and no clinical sign of large-bowel disturbance was observed over 30 days. The absolute concentrations measured here (acetate 29-62, total SCFA 47-93 $\mu\text{mol/g DM}$; ammonia-N 73-87 $\mu\text{mol/g DM}$) are of the same order as those obtained with the same extraction and chromatographic procedure in a previous canine trial ^[23], but lower than values reported by other laboratories using different extraction, dilution and calibration schemes, for example 167 $\mu\text{mol/g DM}$ for acetate, 288 for total SCFA and 187 for ammonia in healthy adult dogs fed a low-fibre control diet ^[31]. Butyrate also formed a smaller share of the SCFA pool here (3-6%) than in that study (about 14%) ^[31], although similarly low faecal butyrate (about 3 $\mu\text{mol/g DM}$) has been reported in dogs fed extruded diets and analysed with the same chromatographic procedure ^[39]. Between-laboratory comparison of absolute faecal SCFA concentrations is therefore not straightforward, and the fermentation findings reported here should be confirmed with fully documented and validated analytical methods; the within-study comparison among groups, analysed under identical conditions, is not affected by this limitation. Whether a lower faecal SCFA concentration is beneficial or unfavourable is not settled by these data: butyrate is the principal energy source for colonocytes, but faecal concentrations reflect what escapes absorption rather than what is produced.

Among the serum variables, glucose rose with MSM dose and remained higher after adjustment for baseline (adjusted means 76, 117 and 125 mg/dL), the two MSM groups lying at the upper limit of the laboratory reference interval (76-119 mg/dL). A comparable rise was reported in feed-restricted laying hens ^[13]; whether the present finding reflects altered glucose handling, a sampling or fasting artefact, or chance in a small study cannot be decided here, and it warrants targeted follow-up with fasting insulin and fructosamine. Creatinine was lowest in MSM8 (adjusted 0.92 versus 1.13 and 1.26 mg/dL) with a quadratic pattern; all group means lay within the reference interval (0.5-1.7 mg/dL) and no other renal analyte changed, so a renal effect is not indicated, but the difference is statistically robust and should not be

dismissed ^[40]. Triglyceride fell linearly, consistent with reports of improved hepatic lipid handling in rodents ^[41,42], although it rose in hens ^[13], so the lipid response is species-dependent and the canine effect is unconfirmed. Liver enzymes did not differ; in a 30-day study this is compatible with, but does not establish, hepatic safety ^[15], and the antioxidant and cytoprotective actions attributed to MSM ^[3,4,6,43] have been shown in rodents and *in vitro* rather than in dogs.

Serum total protein, albumin and calcium lay below the laboratory reference interval in every group and did so already at day 0, before supplementation; none differed among groups or with dose, so the pattern is independent of MSM and indicates a uniform analytical offset affecting the whole batch rather than a biological change. Sampling order and tube type, anticoagulant carry-over, dilution and analyser calibration are recognised pre-analytical and analytical sources of such systematic shifts in canine clinical chemistry, and chelation by EDTA in particular lowers measured calcium ^[44]. Serum here was drawn into plain tubes before the EDTA tube and separated before freezing, and the dogs showed no oedema, weight loss or other sign of hypoproteinaemia or hypocalcaemia. These three analytes are therefore interpreted only comparatively among groups, which were sampled and analysed under identical conditions, and not against the reference interval.

The clearest haematological change was a dose-dependent rise in the relative monocyte proportion with a lower relative neutrophil proportion in MSM8. Because the differential percentages are compositional, a lower neutrophil share arises arithmetically when the monocyte share rises; the absolute monocyte count showed only a linear trend and the absolute neutrophil count did not differ, so the change is small in absolute terms. These shifts are compatible with the immunomodulatory activity of MSM, which regulates monocyte and macrophage inflammatory signalling, including inhibition of nuclear factor- κ B and inflammasome pathways ^[8,9,11], and a recent 30-day MSM trial in horses likewise modified exercise-responsive inflammatory gene expression in skeletal muscle ^[45]. These are, however, rodent, *in vitro* and equine observations. Immune modulation has not been demonstrated in dogs, and a differential count alone cannot establish it. Values remained largely within reference intervals and no leukocytosis, left shift or anaemia developed. Mean platelet volume was lower in MSM8 than in MSM4 after Bonferroni adjustment but did not differ from the control, and the difference (about 1 fL) is within analytical variation. The apparent rise in eosinophils did not survive adjustment for baseline, since eosinophil proportions were already higher in MSM8 at day 0, which is why the day-0 value was used as a covariate.

This study has limitations. The trial lasted 30 days in a single breed, a restricted age range and at two fixed daily doses, in healthy adult dogs housed under kennel conditions. It therefore cannot establish safety for other breeds, for longer use, for clinical populations or for routine commercial use, and it was powered to detect only large effects. Adverse events were monitored by daily clinical observation of appetite, demeanour, vomiting and faeces, and none was recorded; however, no structured adverse-event instrument was used and the feeding staff were not blinded; body weight, re-measured on day 30, was unchanged in all groups. Faecal SCFA and ammonia were measured on a single four-day composite sample per dog, and branched-chain fatty acids, microbiota composition and systemic antioxidant status were not determined. The absolute faecal metabolite concentrations also depend on the extraction, dilution and calibration scheme, and formal recovery and limit-of-quantification validation of the SCFA and ammonia assays was not performed; these values therefore require confirmation.

In conclusion, in this small 30-day study of healthy adult Golden Retrievers, supplementation with 4 or 8 g MSM per dog per day was not associated with detectable differences in apparent total-tract digestibility or faecal consistency. Several clinical-pathology and faecal metabolite measures differed or showed dose trends, but their biological significance is uncertain. The faecal SCFA and ammonia findings require confirmation with fully documented and validated analytical methods; these data do not establish changes in microbial composition or fermentation flux.

DECLARATIONS

Availability of Data and Materials: The datasets generated and analysed during the current study are available from the corresponding author (ZSI) on reasonable request.

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

Declaration of Generative Artificial Intelligence (AI): The authors declare that generative AI and AI-assisted technologies were not used in the design or conduct of the study, in the collection or analysis of the data, or in the creation of the tables and figures. AI-assisted tools were used only to improve the readability and language of the manuscript; the authors reviewed and edited the text and take full responsibility for the content of the publication.

Ethical Compliance Declaration: The authors declare that the study was conducted in accordance with all applicable animal welfare and research ethics principles, institutional and national/international

regulations, and the requirements of the relevant ethics committees and funding/supporting institutions, where applicable. The authors accept full responsibility for the ethical integrity of the study.

Authors' Contributions: ZŞİ: Conceptualisation and hypothesis of the study, diet formulation, conduct of the feeding trial, statistical analysis, writing of the original draft and final decision. HDA and OK: Study design, diet formulation, supervision, assessment of the results and critical revision of the manuscript. EY: Laboratory analyses of faecal short-chain fatty acids and ammonia, interpretation of the fermentation data. BY and DŞ: Follow-up of the experimental procedure, sample collection, digestibility and blood analyses. All authors read and approved the final manuscript.

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RESEARCH ARTICLE

Antibacterial Effect of CpAtt Against *Streptococcus agalactiae* by Increasing the Cell Membrane Permeability

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Abstract

This study aimed to explore the in vitro antibacterial effect of CpAtt, a novel attacin from *Conogethes punctiferalis*, against *Streptococcus agalactiae*. The minimum inhibitory concentration (MIC) was measured via broth microdilution method. Scanning electron microscopy (SEM) was applied to assess the alterations in the overall cell envelope of *S. agalactiae* upon CpAtt treatment. Changes in membrane permeability were characterized by detecting the leakage of intracellular nucleic acids, proteins, alkaline phosphatase (ALP), and β -galactosidase, combined with propidium iodide (PI) staining. Additionally, intracellular reactive oxygen species (ROS) levels were quantified in CpAtt-treated *S. agalactiae*. Additionally, intracellular reactive oxygen species (ROS) levels were detected in the CpAtt-treated *S. agalactiae*. The results showed that the MIC of CpAtt against *S. agalactiae* was 18.8 μ g/mL. After 1 h of treatment with CpAtt at the concentration of 2 MIC, SEM revealed bacterial aggregation, ruptured and collapsed cell walls. CpAtt induced the massive leakage of intracellular nucleic acids and proteins in *S. agalactiae*. Even at low concentrations, CpAtt significantly increased extracellular activities of ALP and β -galactosidase activities, alongside elevated intracellular PI fluorescence intensity, which indicates enhanced membrane permeability of *S. agalactiae*. Furthermore, CpAtt significantly downregulated intracellular ROS levels. Therefore, CpAtt exerts antibacterial activity against *S. agalactiae* by increasing bacterial membrane permeability. These findings suggest that CpAtt is a promising candidate for developing novel antibacterial agents targeting *S. agalactiae*.

Keywords: *Streptococcus agalactiae*, Attacin, Antibacterial activity, Minimum inhibitory concentration, Cell membrane permeability

INTRODUCTION

Streptococcus agalactiae, also known as Group B *Streptococcus* (GBS), a Gram-positive facultative anaerobic zoonotic coccus, was first identified as the causative agent of bovine agalactia mastitis. It remains an important pathogen associated with human perinatal disorders, bovine mastitis and aquatic streptococcosis [1,2]. In fish, *S. agalactiae* induces septicemia and meningoencephalitis, posing a severe threat to tilapia aquaculture [3]. It impairs milk production and milk quality in dairy cows, increasing farming costs and restricting dairy development [4]. Furthermore, *S. agalactiae* infection in young and pregnant livestock may lead to septicemia, polyarthritis, and endometritis, resulting in growth retardation, high culling rates, abortion and stillbirth, thereby imposing substantial economic losses on animal husbandry [5].

Currently, β -lactams are the first choice for treating *S. agalactiae* infections, while macrolides are used for allergic individuals [6]. Long-term and extensive application of penicillins and quinolones in veterinary and aquaculture practices has caused a statistically notable rise in *S. agalactiae* isolation and elevated rates of penicillin non-susceptibility [7]. Zhao et al. [8] analyzed 497 mastitis milk samples collected in China and reported that streptococcal isolates exhibited high levels of antimicrobial resistance and harbored multiple virulence genes of concern. The growing prevalence of multidrug-resistant *S. agalactiae*, together with high resistance to macrolides and tetracyclines, narrows therapeutic choices for human perinatal infections, increases maternal and infant health risks, and compromises the efficacy of conventional antibiotics for bovine mastitis and aquatic streptococcosis. Drug-resistant strains from animals can also spread to



other hosts and threaten public health [9-12]. To address this challenge, alongside standardized antibiotic stewardship, priorities include enhanced resistance surveillance, commercial vaccine development, improvement of rearing environments, and discovery of novel natural antibacterial agents with a low propensity to drive bacterial resistance.

CpAtt, a glycine-rich antimicrobial peptide, was identified from *Conogethes punctiferalis* in our laboratory [13]. Insect antimicrobial peptides are central effector molecules of insect humoral immunity [14]. Most of them are small cationic amphipathic polypeptides, featuring prominent advantages with excellent thermal stability, broad antibacterial spectrum, and low tendency to induce bacterial resistance [15,16]. Therefore, insect antimicrobial peptides are well known as ideal substitutes for antibiotics [17]. Our preliminary assays confirmed that CpAtt exerted remarkable antibacterial activity against Gram-positive bacteria [13]. Building on these findings, the present study further characterizes the antibacterial mode of action of CpAtt against *S. agalactiae*, aiming to provide experimental evidence for the future development of antibacterial agents targeting this pathogen.

MATERIAL AND METHODS

Ethical Statement

This study did not require ethical approval.

Test Strains

S. agalactiae ATCC 13813 were preserved in the Henan Joint International Research Laboratory of Veterinary Biologics Research and Application.

Main Reagents

O-Nitrophenyl β -D-galactopyranoside (ONPG) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. Alkaline phosphatase (ALP) assay kit and enhanced ATP assay kit were obtained from Beyotime Biotechnology Co., Ltd. Triton X-100 was supplied by Thermo Fisher Scientific (China) Co., Ltd. Propidium iodide (PI) was purchased from Beijing Dingguo Biotechnology Co., Ltd. The antimicrobial peptide CpAtt was previously prepared and stored in our laboratory [13]. Briefly, CpAtt was expressed in an *E. coli* expression system and purified by affinity chromatography. The purity of CpAtt was confirmed by SDS-PAGE, and the purity was >95%. The CpAtt stock solution was prepared in sterile PBS, with a stock concentration of 150 μ g/mL. The protein solution was aliquoted and stored at -80°C to avoid repeated freeze-thaw cycles before use.

MIC Determination of CpAtt against *S. agalactiae*

The MIC of CpAtt against *S. agalactiae* was determined by the broth microdilution method according to the CLSI

M07 standard. An overnight culture of *S. agalactiae* was diluted with sterile THB broth to a final concentration of 2×10^5 CFU/mL and used as the inoculum. Aliquots of 100 μ L of the bacterial suspension and 100 μ L of the two-fold serially diluted attacin solution were added to each well of a 96-well microtiter plate, resulting in a total volume of 200 μ L per well and an inoculum of approximately 2×10^4 CFU/mL. CpAtt was prepared in sterile PBS at 150 μ g/mL and then serially two-fold diluted in the wells to final concentrations of 75, 37.5, 18.75, 9.37, 4.68, 2.34, and 1.17 μ g/mL, with three replicates per concentration. The MIC value of Kanamycin sulfate against *S. agalactiae* was 125 μ g/mL, so Kanamycin sulfate (500 μ g/mL) and PBS served as the positive and negative controls, respectively. After static incubation at 37°C for 16 h, the optical density at 600 nm (OD₆₀₀) of each well was measured using a microplate reader. The MIC was defined as the lowest attacin concentration at which no significant increase in OD₆₀₀ was observed, i.e., consistent with that of the negative control. Kanamycin sulfate (final concentration 500 μ g/mL) served as the assay validity positive control. OD₆₀₀ measurements from the microplate confirmed complete growth inhibition of *S. agalactiae* under these experimental conditions, verifying the reliability of the MIC system. This concentration of kanamycin has been widely used as a positive inhibition control in related published work [13]. Independent kanamycin susceptibility testing for this strain was not performed in the current study.

SEM Observation

Bacterial cells in the logarithmic growth phase were collected by centrifugation, washed and resuspended with PBS. A total of 600 μ L bacterial suspension was mixed with an equal volume of CpAtt solution to reach a final concentration of 2 MIC. Sterile PBS was used as the negative control. The mixture was incubated in a water bath at 37°C for 1 h. After PBS washing, the bacterial samples were fixed with 2.5% (v/v) glutaraldehyde solution and transported under low temperature to Wuhan Servicebio Technology Co., Ltd. for sample preparation and observation via SEM.

Detection of Nucleic Acid and Protein Leakage

Bacterial cells in the logarithmic growth phase were collected by centrifugation, and washed twice with PBS. The supernatant was discarded, and the pellet was resuspended in sterile PBS to adjust the bacterial concentration to 2×10^8 CFU/mL. Four groups were treated with CpAtt at the concentrations of 0 MIC, 0.5 MIC, 1 MIC, and 2 MIC, respectively. After incubation in a 37°C water bath for 1 h, samples were centrifuged at 12,000 rpm for 1 min. The absorbance values of the supernatant at 260 nm (A₂₆₀) and 280 nm (A₂₈₀) were determined using

an ultra-micro spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, USA)

Determination of ALP Activity

Bacterial suspensions were treated with 0 MIC, 0.5 MIC, 1 MIC, and 2 MIC CpAtt, respectively, while 0.3% (v/v) Triton X-100 was used as the positive control. All mixtures were incubated in a 37°C water bath for 1 h, followed by centrifugation at 12,000 rpm for 1 min. The supernatant was collected and assayed in accordance with the instructions of the ALP assay kit. The OD value of each well was detected at 405 nm using a microplate reader, and the ALP activity of samples were calculated based on the standard curve.

Determination of β -Galactosidase Activity

S. agalactiae was cultured overnight, harvested by centrifugation, washed, and resuspended in sterile PBS buffer. The bacterial suspension of *S. agalactiae* was treated with CpAtt at final concentrations of 0 MIC, 0.5 MIC, 1 MIC, and 2 MIC, respectively. Under dark conditions, 100 μ L supernatant was added to each well of a 96-well plate, followed by 4.5 μ L ONPG solution with a final concentration of 3 mmol/L. The mixture was gently pipetted and blended, then incubated at 37°C for 30 min. The optical density at 420 nm was measured using a microplate reader. All measured values were normalized to the corresponding bacterial density at OD₆₀₀, and each assay was performed in triplicate with independent biological replicates.

PI Staining Assay

Bacterial suspensions were treated with CpAtt at final concentrations of 0 MIC, 0.5 MIC, 1 MIC, and 2 MIC, respectively. Meanwhile, 0.3% (v/v) Triton X-100 was used as the positive control. All mixtures were incubated in a 37°C water bath for 1 h. After incubation, the bacterial cells were centrifuged, washed and resuspended with sterile PBS to a fixed final volume. Subsequently, 4 μ L of PI stock solution (1 mg/mL) was added to each bacterial suspension in the dark. After thorough mixing, 100 μ L sample was transferred to 96-well plate and incubated at 37°C for 30 min. The fluorescence intensity was measured with a microplate reader at an excitation wavelength of 536 nm and an emission wavelength of 617 nm, respectively. All fluorescence values were normalized to the OD₆₀₀ of the corresponding bacterial suspension to eliminate the interference of bacterial density differences among groups.

Detection of Intracellular ROS Level

Bacterial suspensions were prepared according to Section of "Detection of Nucleic Acid and Protein Leakage". DCFH-DA probe was added to the samples to a final concentration of 10 μ M. After incubation at 37°C for 20 min, the bacteria were washed three times with PBS and

resuspended. The bacteria were then treated with CpAtt at the concentrations of 0 MIC, 0.5 MIC, 1 MIC and 2 MIC. All samples were incubated at 37°C for 30 min. The fluorescence intensity was detected by a microplate reader at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Statistical Analysis

All data were analyzed and plotted using GraphPad Prism 9.3. Each experiment was performed in triplicate with technical replicates of the same biological sample, and results were presented as mean $\pm$ standard deviation (SD). For experiments with a single independent variable (e.g., concentration gradient only), inter-group comparisons were performed using one-way analysis of variance (one-way ANOVA). $P < 0.05$ indicated a significant difference; $P < 0.01$ indicated a statistically significant difference. For experiments containing two independent variables (concentration and incubation time), statistical analysis was conducted using two-way ANOVA followed by Tukey's post-hoc multiple comparison test; $P < 0.05$ compared with the 0MIC group at the same incubation time; ns indicates no significant difference between 30 min and 60 min groups under the same concentration ($P > 0.05$).

RESULTS

MIC of CpAtt Against *S. agalactiae*

As shown in Fig. 1, robust growth of *S. agalactiae* was observed in the PBS-treated growth control group, whereas bacterial growth was fully suppressed in the kanamycin-treated positive control group. Weak bacterial

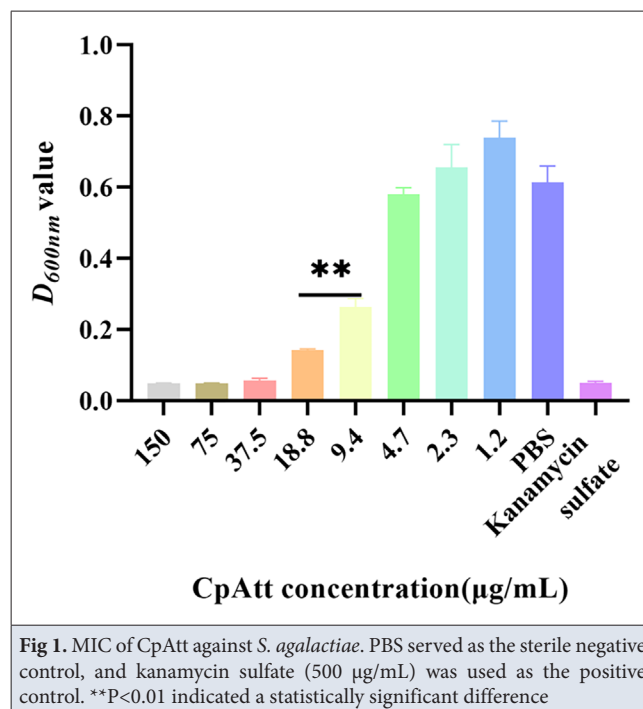
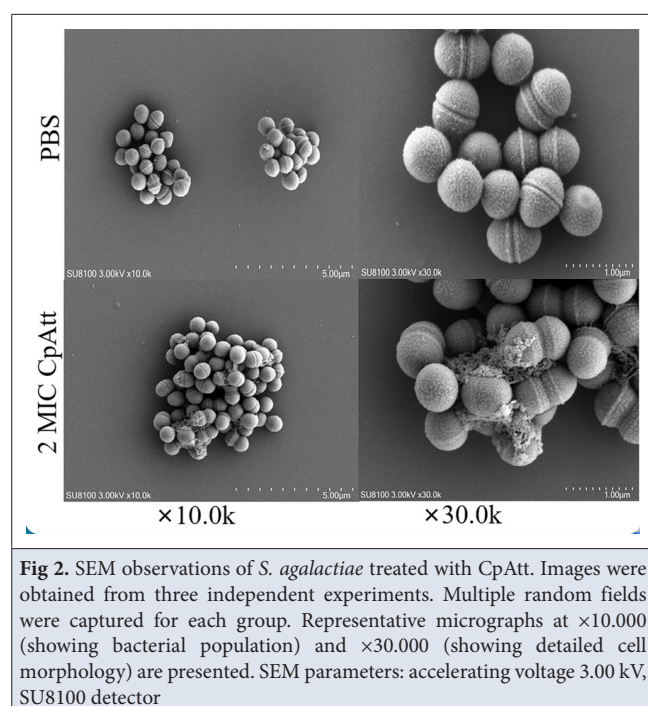


Fig 1. MIC of CpAtt against *S. agalactiae*. PBS served as the sterile negative control, and kanamycin sulfate (500 μ g/mL) was used as the positive control. ** $P < 0.01$ indicated a statistically significant difference



growth was detected at a CpAtt concentration of 9.4 µg/mL. Accordingly, the MIC of CpAtt against *S. agalactiae* was determined to be 18.8 µg/mL.

SEM Observation

After treatment with CpAtt at a concentration of 2 MIC, a large number of *S. agalactiae* cells aggregated and adhered to form dense bacterial clumps under low magnification. The typical short-chain arrangement of normal bacteria disappeared, and severe adhesion among bacterial cells was observed. Under high magnification, the bacterial surface appeared wrinkled and uneven. Cell-envelope damaged and collapsed with ruptured cell margins, and the fragmented debris from lysed bacterial cells could be seen nearby (Fig. 2).

Detection of Nucleic Acid and Protein Leakage

After 1 h of treatment with CpAtt, compared with the 0 MIC group, the 2 MIC group showed statistically significant increase ($P < 0.01$) of nucleic acid leakage in *S. agalactiae* (Fig. 3-A). Similarly, the protein leakage increased markedly ($P < 0.05$) in the 2 MIC group (Fig. 3-B), suggesting that CpAtt could increase the cell membrane permeability of *S. agalactiae*, leading to the leakage of intracellular contents.

Determination of ALP Activity

As shown in Fig. 4, compared with the 0 MIC group, the ALP level gradually increased with the CpAtt concentration increasing in the culture supernatant of CpAtt-treated *S. agalactiae*, indicating that CpAtt destroyed the cell wall of *S. agalactiae* to cause the ALP leakage.

Determination of β -galactosidase Activity

As shown in Fig. 5, the β -galactosidase leakage gradually increased with the rise of CpAtt concentration in all CpAtt-treated groups. The OD₄₂₀ value of the 2 MIC CpAtt-treated group was roughly equivalent to that of the positive control group treated with 0.3% Triton X-100, indicating severe permeabilization of the bacterial membrane.

Results of PI Staining Assay

Compared with the 0 MIC group, the 2 MIC CpAtt treatment significantly increased the PI fluorescence intensity of *S. agalactiae* (Fig. 6), indicating that CpAtt elevates the cell envelope permeability of *S. agalactiae*.

Effects of CpAtt on Intracellular ROS Levels in *S. agalactiae*

As shown in Fig. 7, compared with the 0 MIC group, the fluorescence intensity of all CpAtt-treated groups decreased significantly. Moreover, for all CpAtt-treated groups, the fluorescence intensity after treatment for 60 min did not significantly increase than the one of after treatment for 30 min, suggesting that CpAtt did not induce the ROS accumulation.

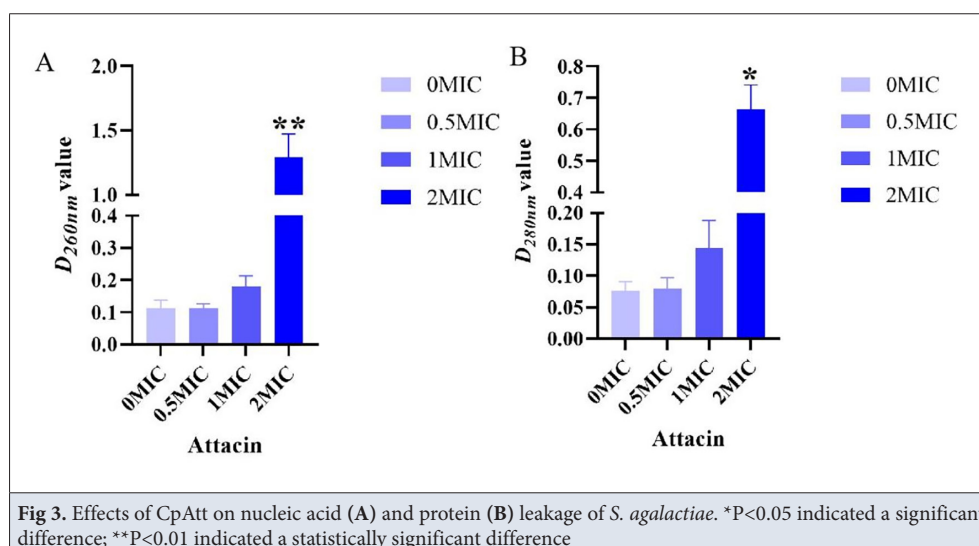


Fig 3. Effects of CpAtt on nucleic acid (A) and protein (B) leakage of *S. agalactiae*. * $P < 0.05$ indicated a significant difference; ** $P < 0.01$ indicated a statistically significant difference

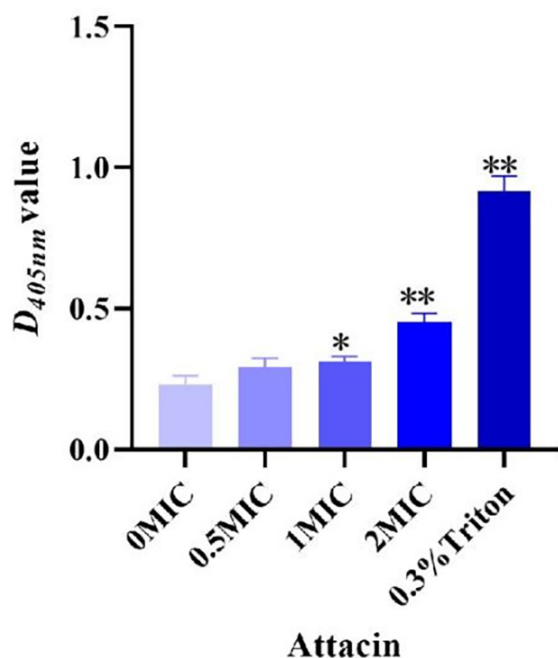


Fig 4. Effects of CpAtt on the ALP leakage in *S. agalactiae*. * $P < 0.05$ indicated a significant difference; ** $P < 0.01$ indicated a statistically significant difference

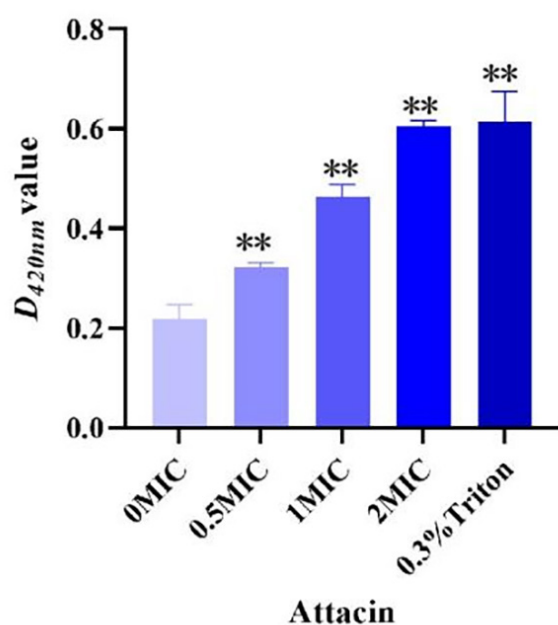


Fig 5. Effects of CpAtt on the β -galactosidase leakage in *S. agalactiae*. ** $P < 0.01$ indicated a statistically significant difference

DISCUSSION

S. agalactiae represents a serious hazard to the health of aquaculture species and livestock, with a continuous

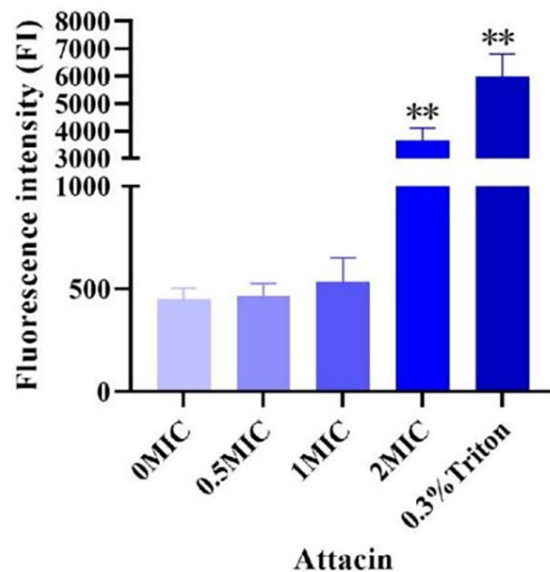


Fig 6. PI fluorescence intensity of *S. agalactiae* after CpAtt treatment. *S. agalactiae* was incubated with CpAtt at 0, 0.5, 1, and 2 MIC. PI fluorescence intensity was determined to evaluate bacterial cell envelope permeability. A broken y-axis was used to clearly visualize subtle variations among low-concentration groups without compressing the signal of the 2 MIC group. Data are presented as mean $\pm$ SD from three independent biological replicates. Significant difference was observed only at 2 MIC compared with the 0 MIC group. ** $P < 0.05$

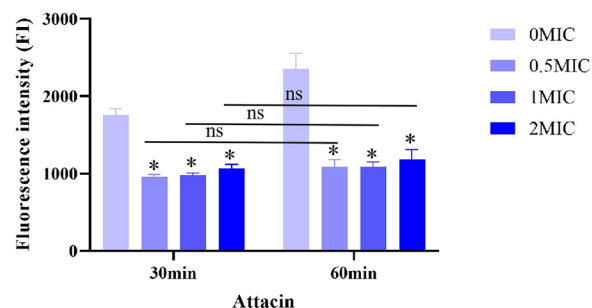


Fig 7. Effects of CpAtt on intracellular ROS Levels in *S. agalactiae*. Intracellular ROS was detected using DCFHDA staining. Fluorescence intensity was normalized to OD_{600} to correct for differences in bacterial density. *S. agalactiae* was treated with CpAtt at 0, 0.5, 1, and 2 MIC for 30 min and 60 min, respectively. Data are presented as mean $\pm$ SD from three independent biological replicates. * $P < 0.05$ compared with the 0 MIC group at the same incubation time by two-way ANOVA followed by Tukey's posthoc test; ns indicates no significant difference between 30 min and 60 min groups under the same CpAtt concentration ($P > 0.05$)

increase in antimicrobial resistance. This pathogen has become an urgent challenge for public health and aquaculture sectors [18]. Therefore, discovering natural antibacterial agents with novel scaffolds, high potency, low toxicity and low risk of resistance induction, together with systematic characterization of their antibacterial mode of action, is a key research priority for the green prevention and control of pathogenic bacteria. Attacins constitute a

unique family of insect-derived antibacterial proteins, with favorable attributes including broad-spectrum activity, high biosafety and low potential to select for bacterial resistance. Accordingly, attacins hold great promise for developing novel antibacterial biopreparations as alternatives to conventional antibiotics ^[19]. Attacins originating from different insect species display substantial differences in antibacterial activity and molecular targets ^[14,15,20]. Attacin from *Hermetia illucens* specifically binds to LPS, disrupts the outer membrane and forms membrane pores, thereby exerting dual antibacterial effects against methicillin-resistant *Staphylococcus aureus* (MRSA) and Gram-negative bacteria ^[21]. Recombinant attacin A from *Drosophila melanogaster*, expressed in *E. coli*, targets bacterial cell membranes and inhibits the growth of the Gram-negative strain *E. coli* DH5 α ^[22]. Given these interspecies differences, it is essential to characterize the antibacterial activity and associated mode of action of CpAtt, an attacin derived from *Conogethes punctiferalis*, against *S. agalactiae*.

The bacterial cell membrane acts as an essential physiological barrier that maintains morphological integrity, osmotic homeostasis, and stable substance metabolism in bacteria ^[23,24]. Structural destruction and permeability dysfunction of the cell envelope represent the primary and canonical mode of action by which natural antimicrobial peptides and proteins exert antibacterial activity. In this study, multiple detection approaches, including morphological observation, fluorescent staining, intracellular macromolecule leakage analysis, and characteristic enzyme activity determination, were integrated to systematically demonstrate that CpAtt effectively impairs cell envelope integrity and elevates the membrane permeability of *S. agalactiae*. SEM provides intuitive visual evidence for evaluating bacterial cell envelope damage. Microscopic results revealed that treatment with 2 MIC CpAtt induced obvious bacterial agglutination in *S. agalactiae*, accompanied by shrunken and irregular cell surfaces, collapsed and damaged cell envelopes, blurred cell margins, and even complete bacterial lysis. These morphological alterations confirm that CpAtt induces irreversible structural damage to the *S. agalactiae* cell envelope.

Nucleic acids and proteins are indispensable intracellular macromolecules that cannot freely diffuse out of bacterial cells under normal physiological conditions with intact membrane barriers. In the present study, treatment with 2 MIC CpAtt significantly increased extracellular nucleic acid and protein contents in the bacterial supernatant, indicating that CpAtt disrupts bacterial membrane integrity and triggers the release of intracellular components, thereby interfering with bacterial metabolism and proliferation. ALP is a typical marker enzyme, while β -galactosidase is a

cytoplasmic enzyme; both are rarely released extracellularly when the bacterial cell envelope remains intact. Our results showed that the extracellular ALP level was significantly elevated after treatment with 1 MIC CpAtt, and β -galactosidase activity increased markedly even under 0.5 MIC CpAtt intervention. These biochemical results further verify that CpAtt alters cell envelope permeability and impairs membrane barrier function in *S. agalactiae*. PI is a nucleic acid-specific fluorescent dye that cannot penetrate intact membranes of viable bacteria. Therefore, increased PI fluorescence exclusively indicates membrane damage and enhanced membrane permeability. In this study, the PI fluorescence intensity of *S. agalactiae* was significantly enhanced after 2 MIC CpAtt treatment, further confirming the loss of membrane barrier properties. Collectively, consistent evidence obtained from morphological observation, fluorescent staining, and quantitative biochemical assays demonstrates that CpAtt inhibits *S. agalactiae* growth primarily by disrupting bacterial cell envelope integrity and increasing membrane permeability, which represents the core antibacterial mode of action of CpAtt against *S. agalactiae*.

Furthermore, intracellular ROS detection further elucidated the unique antibacterial characteristics of CpAtt, which differs from the action pattern of conventional antibacterial agents. Most antibacterial substances exert bactericidal activity by inducing excessive intracellular ROS accumulation, triggering oxidative stress damage and degrading intracellular biomacromolecules ^[25]. In contrast, the present study showed that intracellular ROS levels in *S. agalactiae* were markedly decreased after 30 min of 1 MIC CpAtt treatment, and this regulatory effect was not obviously dependent on treatment duration. Notably, the reduction in fluorescent intensity cannot be exclusively attributed to decreased ROS levels, as it may also be affected by changes in bacterial viability and metabolic activity. Taken together, these results suggest that the antibacterial activity of CpAtt against *S. agalactiae* is not mediated by ROS accumulation and oxidative stress activation.

In summary, this study suggested that CpAtt exerted the antibacterial effects on *S. agalactiae* by increasing the cell membrane permeability to induce the leakage of intracellular substances. These results offer a preliminary mechanistic basis, although further studies with additional clinical isolates, toxicity testing and in vivo experiments are needed to explore its potential antibacterial application.

DECLARATIONS

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

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Competing Interests: The authors declare no competing interests.

Declaration of Generative Artificial Intelligence (AI): The authors confirm that no generative AI created the manuscript text, tables or figures.

Ethical Compliance Declaration: The study was conducted in accordance with all applicable animal welfare and research ethics principles. The authors accept full responsibility for the ethical integrity of the study.

Author Contributions: KQ L, and RH P conceptualized and designed the study. LL Z, KQ L, YH W, SM M, and YK L performed experiments and analyses. LL Z, and KQ L wrote the manuscript. RH P edited the manuscript. All authors approved the manuscript.

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RESEARCH ARTICLE

Clinical, Diagnostic, and Therapeutic Features of Nasopharyngeal Stenosis in Cats: A Retrospective Evaluation of Modified Balloon Dilation Procedures

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Abstract

Nasopharyngeal stenosis is a rare cause of upper respiratory tract obstruction in cats and may be difficult to treat. This retrospective study evaluated the clinical and diagnostic features of affected cats and the outcomes of modified balloon dilation. Records of 52 cats diagnosed between May 2021 and May 2026 were reviewed. Diagnosis was established under general anesthesia using pharyngeal examination, computed tomography, and/or retroflex nasopharyngoscopy. Dilation was performed by progressively inflating cuffed red rubber endotracheal tubes with internal diameters of 2.5, 3.0, and 3.5 mm at the stenotic site. Median age was 24 months, and sex distribution was equal. Stertor occurred in all cats, while purulent rhinorrhea occurred in 24 (46.2%). Computed tomography was used in 38 (73.1%) cases, pharyngeal examination in 36 (69.2%), and nasopharyngoscopy in 18 (34.6%). Dilation was performed in 30 cats (57.7%); treatment was declined for 22 cats. Thirteen treated cats underwent one session, 17 underwent multiple sessions, and eight required additional surgery. Early complications occurred in 10.0%. After a mean follow-up of 2.9±2.7 months, sustained clinical improvement was observed in 29 cats (96.7%), whereas one cat developed restenosis. Age and initial stertor severity were not associated with the number of sessions. Modified balloon dilation appears feasible and clinically effective; however, longer follow-up is required to determine its long-term efficacy.

Keywords: Modified balloon dilation, Nasopharyngeal stenosis, Nasopharyngoscopy, Restenosis, Throat examination

INTRODUCTION

The nasopharynx is the portion of the upper respiratory tract located dorsal to the soft palate and caudal to the choanae ^[1]. Nasopharyngeal stenosis (NPS) is a general term describing congenital or acquired pathological conditions characterized by partial or complete narrowing or obliteration of the nasopharyngeal lumen by fibrous, membranous, or osseous tissues ^[1-6]. While congenital NPS may be associated with developmental anomalies such as choanal stenosis or atresia, acquired stenosis can develop secondary to infectious, inflammatory, traumatic, or iatrogenic processes ^[1,2,4].

Nasopharyngeal stenosis is a rare upper respiratory tract disease in cats that can markedly compromise quality of life ^[2,4,7]. In a substantial proportion of cases, the condition is reported to develop secondary to scar tissue formation during the resolution of chronic upper respiratory tract infections and nasopharyngeal inflammation ^[4,6]. Feline herpesvirus and feline calicivirus infections, chronic rhinitis,

gastroesophageal reflux-induced aspiration, nasopharyngeal trauma, and previous surgical interventions represent the primary etiologies that may contribute to stenosis development ^[1,4,6].

The most frequently reported clinical sign in cats with nasopharyngeal stenosis is stertor, which becomes particularly pronounced during closed-mouth breathing. Depending on the severity of the disease, stertorous breathing, increased inspiratory effort, open-mouth breathing, dyspnea, sneezing, reverse sneezing, and chronic serous or purulent rhinorrhea may be observed ^[1,2,4,7]. In cases with advanced obstruction, dysphagia, regurgitation, exercise intolerance, and intermittent respiratory crises can also develop ^[1].

Although skull radiographs can be utilized for the preliminary assessment of the nasopharyngeal region in the diagnostic approach, they provide limited information due to the superimposition of anatomical structures ^[3,6].



Computed tomography (CT) enables the determination of the localization, length, and grade of the stenosis, as well as the evaluation of choanal atresia, sinonasal disease, nasal meatus obliteration, middle ear disease, and concurrent craniofacial anomalies [3,5]. Retroflex nasopharyngoscopy (RN), on the other hand, allows for direct visualization of the stenotic aperture, assessment of mucosal and membranous changes, and simultaneous intervention when necessary [1,2]. However, because thick CT slices or the presence of dense secretions within the nasopharynx can hinder visualization of the stenotic area, the combined use of CT and endoscopic evaluation may enhance diagnostic accuracy [4].

Mechanical dilation, surgical resection of the stenotic membrane, laser ablation, transpalatal or longitudinal palatal approaches, choanoplasty, and metallic or silicone stenting have been described for the treatment of NPS [2,4]. Balloon dilation is a less invasive therapeutic option compared with open surgical approaches, providing controlled expansion of the stenotic tissue. Nevertheless, due to the risk of restenosis, repeated sessions, additional surgical interventions, or antifibrotic therapies may be required [2,4,7]. Treatment selection should be individualized according to the location and extent of the stenosis, whether the stenotic membrane is perforate or imperforate, the presence of concurrent anatomical abnormalities, and the response to previous interventions [1,2,4]. Minimally invasive dilation may be preferred for perforate stenoses, whereas surgical reconstruction may be required for caudally located or imperforate lesions and for cases in which previous minimally invasive treatment has been unsuccessful [1,2,4].

Published evidence on feline NPS remains limited and consists largely of individual case reports and retrospective case series focusing on selected diagnostic or therapeutic aspects [2-7]. Consequently, data integrating demographic and clinical characteristics, diagnostic findings, anatomical features of the stenosis, treatment modalities, complications, and clinical outcomes within the same cohort remain limited. The primary objective of this study was to retrospectively evaluate the demographic and clinical features, computed tomography and endoscopic examination findings, applied therapeutic modalities, complications, and clinical outcomes of cats diagnosed with NPS. The secondary objective was to analyze the number of sessions, concurrent surgical interventions, developing complications, and treatment outcomes in cases undergoing balloon dilation, as well as to determine potential associations between initial clinical characteristics and treatment requirements.

MATERIALS AND METHODS

Ethical Approval

This retrospective study was reviewed by the Istanbul University-Cerrahpaşa Local Ethics Committee for

Animal Experiments, which determined that additional ethical approval was not required (decision date: 29 November 2024; decision no.: 2024/94). Signed informed consent forms were obtained from the owners of all cats included in the study.

Study Design and Case Selection

In this retrospective study, clinical records of 52 cats diagnosed with NPS that presented to the Ear, Nose, and Throat Clinic, Department of Surgery, Faculty of Veterinary Medicine, Istanbul University-Cerrahpaşa between May 2021 and May 2026 were evaluated. Recurrent examination, follow-up, imaging, and treatment records belonging to the same patient were merged under a single case.

Cats that were diagnosed with NPS via pharyngeal examination under general anesthesia, CT, RN, or a combination of these modalities, and whose records included demographic characteristics, clinical findings, diagnostic evaluations, therapeutic procedures, and follow-up outcomes, were included in the study. Cases with only suspected NPS whose stenosis presence could not be confirmed in available physical examination, imaging, or endoscopic records, as well as patients with insufficient clinical data for evaluation, were excluded from the study.

For each case, age, breed, sex, initial stertor score, presence of purulent rhinorrhea, diagnostic modalities used, presence of choanal atresia, treatment modality performed, number of modified balloon dilation sessions, additional operative interventions, complications, restenosis status, and clinical outcomes were recorded. Follow-up outcomes were obtained from follow-up examination records and/or telephone interviews with the owners. The duration between the initial presentation date and the last recorded clinical examination or telephone interview was defined as the total clinical follow-up period.

Clinical Evaluation

The medical history and clinical examination records of the cases at initial presentation were retrospectively reviewed. Initial stertor severity and the presence of purulent rhinorrhea were evaluated.

The presence of purulent rhinorrhea was assessed based on anamnesis and clinical examination records. Cases documented to have purulent nasal discharge were classified as “purulent rhinorrhea present,” whereas those explicitly noted to lack purulent nasal discharge were classified as “purulent rhinorrhea absent.”

Stertore severity was graded from 0 to 4 using a semi-quantitative scoring system developed for this retrospective study, based on findings documented in the medical

history and clinical examination records. Cases with an absence of stertor and normal respiratory sounds at rest and during sleep were scored as 0; cases where stertor occurred only during sleep, excitement, stress, or physical activity and was inconspicuous at rest were scored as 1; cases where stertor was present at rest and audible near the patient without marked respiratory effort or open-mouth breathing were scored as 2; cases with continuous and prominent stertor at rest audible from a distance accompanied by increased inspiratory effort were scored as 3; and cases where severe stertor was accompanied by open-mouth breathing, marked dyspnea, cyanosis, or intermittent respiratory crises were scored as 4.

Diagnostic Evaluation

Pharyngeal examination under general anesthesia, CT, RN, or different combinations of these modalities were used in the diagnostic evaluation of the cases.

Pharyngeal Examination Under Anesthesia

Pharyngeal examination was performed under general anesthesia. During the examination, the oral cavity, soft palate, oropharynx, tonsils, epiglottis, larynx, and nasopharyngeal opening were evaluated. To visualize the nasopharyngeal region, the soft palate was carefully retracted rostradorsally using tissue forceps, and the nasopharyngeal aperture was directly inspected. The nasopharyngeal opening and the stenotic segment were directly evaluated during the examination.

Computed Tomography

CT images were retrospectively reviewed to confirm NPS and evaluate the presence of choanal atresia.

During CT examinations, patients were positioned in sternal recumbency to ensure symmetrical alignment of the cranial region. Scans were performed using a 128-slice Siemens SOMATOM AS+ scanner (Siemens Healthineers, Erlangen, Germany) with a slice thickness of 0.6 mm. Axial images were evaluated in conjunction with dorsal and sagittal multiplanar reconstructions.

Retroflex Nasopharyngoscopy

During RN, a single-use flexible videoendoscope (FIVE S, 3.5 × 65; outer diameter 3.5 mm; working channel 1.2 mm; REF 091361-01; KARL STORZ SE & Co. KG, Tuttlingen, Germany) was advanced through the oral cavity and directed caudodorsally over the soft palate into the nasopharyngeal region. The nasopharyngeal opening and the stenotic segment were directly evaluated.

Because of the retrospective design, the three diagnostic modalities were not applied systematically to every cat. The diagnostic approach was influenced by clinical requirements, owner financial constraints, availability

of the CT and endoscopy units, and the feasibility of performing repeated procedures under general anesthesia. When more than one modality was used, the findings were reviewed descriptively; formal concordance analysis was not performed because the records did not contain a standardized grading system applicable across modalities.

The degree of stenosis was not quantified using aperture diameter, cross-sectional area, or a standardized grading system. Because body size and craniofacial anatomy varied among cats and no validated grading system for feline NPS was available, severity was assessed subjectively from clinical signs and the visual findings obtained during pharyngeal examination and/or RN. In some cats, patency was also assessed qualitatively by instilling sterile saline through the nostril and observing its passage into the nasopharynx. This was used only as an adjunctive clinical assessment and was not considered an objective or standardized measurement of stenosis severity.

Classification of Treatment Modalities

Cases were classified according to the treatment modality performed as follows:

1. Cases treated with modified balloon dilation alone,
2. Cases treated with modified balloon dilation and case-specific operative procedures performed during the subsequent treatment course,
3. Cases in which modified balloon dilation was recommended but the owner declined treatment. Additional operative procedures were recorded as case-specific interventions and were not assumed to have been performed during the same anesthetic session as the initial dilation.
4. Modified balloon dilation was preferred as the initial intervention when a residual nasopharyngeal opening permitted safe passage of the dilation tube. In Case 13, complete membranous occlusion prevented initial tube placement; therefore, the stenotic membrane was surgically removed to create an opening, and modified balloon dilation was performed during subsequent treatment sessions. In Case 26, choanal atresia required initial transpalatal choanoplasty, followed by modified balloon dilation of the nasopharyngeal opening during later sessions. Other operative procedures were selected individually according to concurrent disorders, including otitis media, inflammatory aural polyps, and chronic rhinosinusitis, and according to case-specific anatomical findings.

Preanesthetic Evaluation and Anesthesia Protocol

In cases undergoing pharyngeal examination under general anesthesia, RN, or surgical treatment, the general health status and anesthetic risk were assessed prior to the procedure. Complete blood count, serum biochemistry

analysis, thoracic radiography, and other deemed necessary preanesthetic evaluations were performed according to available clinical records.

In cases undergoing modified balloon dilation, intravenous access was established. For premedication, medetomidine HCl (40 µg/kg, IV; Delax®, AlbaFarma İlaç San. ve Tic. A.Ş., İstanbul, Türkiye) was administered, and ketamine HCl (2 mg/kg, IV; Keta-Control®, Doğa İlaç, İstanbul, Türkiye) was used for anesthetic induction. Following induction, endotracheal intubation was performed, and anesthesia was maintained with isoflurane (Isoflurane USP®, Piramal Critical Care, Pennsylvania, USA).

Heart rate, respiratory rate, peripheral oxygen saturation, end-tidal carbon dioxide, noninvasive arterial blood pressure, and body temperature were monitored throughout anesthesia using a uMEC12 Vet® patient monitor (Shenzhen Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China).

Modified Balloon Dilation Technique

In this study, the term “modified balloon dilation” was used to define the pneumatic dilation technique performed by progressively inflating the cuffs of cuffed red rubber endotracheal tubes instead of a conventional balloon catheter. Cases scheduled for modified balloon dilation were placed in sternal recumbency. The mouth was maintained open using gauze strips positioned around the canine teeth. The soft palate was retracted rostr dorsally with an appropriate instrument to ensure visibility of the nasopharyngeal aperture.

The five-min inflation duration was selected with reference to the protocol described by Champetier et al.^[4], in which each stepwise balloon pressure was maintained for five min. Initially, a cuffed red rubber endotracheal tube with an internal diameter of 2.5 mm (Endotracheal Red Rubber Tubes®, KRUUSE A/S, Langeskov, Denmark) was placed into the stenotic opening. Because cuffed red rubber endotracheal tubes were used instead of a pressure-calibrated balloon catheter, inflation pressure could not be objectively standardized or recorded. Each cuff was manually inflated with air using a syringe until its maximum achievable expansion was reached, as judged by resistance to further injection, and remained continuously inflated throughout the five-minute application.

Each selected tube size was used for a single inflation only; repeated inflation cycles were not performed with the same tube. The procedure was continued sequentially, when appropriate, using cuffed endotracheal tubes with internal diameters of 3.0 and 3.5 mm. Thus, each five-minute inflation was followed by one five-minute resting interval before the next selected tube size was used (Fig. 1). At the end of each inflation, the cuff was completely deflated, the dilation tube

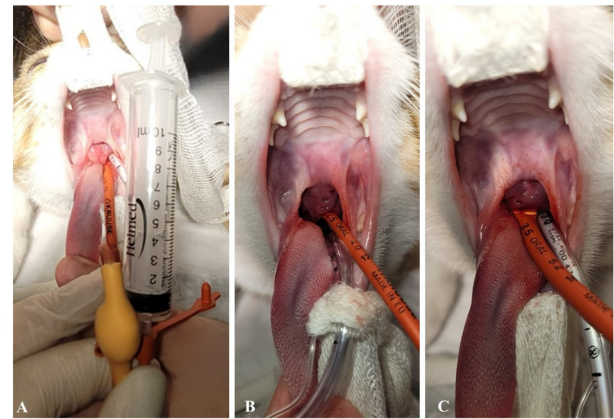


Fig 1. Placement of the endotracheal tubes in modified balloon dilation and sequential performance of the procedure using tubes with internal diameters of 2.5 (A), 3 (B), and 3.5 (C) mm

was removed, the gauze strips maintaining mouth opening were released, and the jaw was allowed to return to a near-closed position around the orotracheal tube. This position was maintained for five minutes to permit reperfusion of compressed tissues, interrupt prolonged mouth opening, and allow assessment for hemorrhage, edema, or mucosal injury. The same stepwise inflation-resting protocol was used in all treated cats; however, the initial and maximum tube sizes were individualized according to the width of the stenotic aperture, patient anatomy, tissue resistance, and tissue response. Progression to a larger tube was discontinued if marked resistance, hemorrhage, edema, or suspected mucosal injury was observed.

At the end of the procedure, the nasopharyngeal aperture was inspected via pharyngeal examination and/or RN. Aperture diameter and cross-sectional area were not measured objectively. Immediate procedural response was assessed qualitatively from the visually observed increase in luminal patency and the ability to introduce progressively larger dilation tubes.

The number of modified balloon dilation sessions and the intervals between sessions were not standardized across all cases. The requirement for repeated sessions was determined by the persistence of stertor and other clinical signs, insufficient nasopharyngeal aperture on follow-up pharyngeal examination or RN, or the development of restenosis. The total number of modified balloon dilation sessions performed for each case was recorded.

Following modified balloon dilation, all cats routinely received ceftriaxone (30 mg/kg, IM, q24h), methylprednisolone (1 mg/kg, PO, q24h), and sucralfate (250 mg/cat, PO, q24h) for seven days because of the potential for mucosal injury or tearing at the dilation site. In cats undergoing additional operative procedures, further medical treatment was selected individually according

to the concurrent disease, procedure performed, and postoperative clinical findings; therefore, these additional treatments were not standardized across cases.

Evaluation of Complications and Clinical Outcomes

Soft palate laceration, hemorrhage, nasopharyngeal and lingual edema, subcutaneous or generalized emphysema developing during the procedure or in the early postoperative period, and the requirement for tracheostomy due to these complications were recorded as complications. Restenosis was evaluated as a late adverse treatment outcome rather than a perioperative complication.

A marked reduction or complete resolution of clinical signs such as stertor, respiratory distress, open-mouth breathing, and purulent rhinorrhea following the procedure was considered clinical improvement.

Treatment outcomes in cases undergoing modified balloon dilation were classified as clinical improvement and restenosis. Restenosis was defined as recurrent narrowing documented during follow-up pharyngeal examination and/or RN, together with recurrent clinical signs when recorded. Restenosis was not quantified using standardized aperture measurements. Due to variability in follow-up duration, clinical outcomes were assessed from the status documented at the last follow-up examination or final telephone interview with the owner. In cases followed solely via telephone interview, the outcome was recorded as owner-reported clinical improvement. Cases in which modified balloon dilation was recommended but declined by the owner were evaluated separately.

Statistical Analyses

Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 31.0 (IBM Corp., Armonk, NY, USA). Continuous variables were presented as mean $\pm$ standard deviation or median, interquartile range, and minimum-maximum values according to their distribution characteristics, whereas categorical variables were presented as frequencies and percentages.

The relationship between age and initial stertor score was evaluated using Spearman's rank correlation analysis. Ages of cases with and without purulent rhinorrhea were compared using the Mann-Whitney U test. Breeds were categorized as purebred and crossbred/domestic; the associations between breed group and severe stertor (scores 3-4) and purulent rhinorrhea were examined using Fisher's exact test. Odds ratios (OR) were reported with 95% confidence intervals.

In cases undergoing modified balloon dilation, the relationships between initial stertor score and number of sessions, as well as between age and number of sessions, were evaluated using Spearman's rank correlation analysis.

Analyses regarding the number of sessions were performed exclusively on cases undergoing modified balloon dilation (n=30).

All tests were two-tailed. Analyses were considered exploratory, and the Benjamini-Hochberg correction was applied for multiple comparisons. An adjusted $P < 0.05$ was considered statistically significant.

RESULTS

A total of 52 cats diagnosed with NPS were evaluated in the study. Of the cases, 26 (50.0%) were female and 26 (50.0%) were male. The ages of the cases ranged from 4 to 144 months. The median age was 24 months, and the mean age was 37.4 ± 37.2 months. Ten cases (19.2%) were under one year of age, 21 (40.4%) were 1 to <3 years old, 15 (28.8%) were 3 to <7 years old, and 6 (11.5%) were seven years of age and older.

During the standardization of breed records, cats documented by coat color or pattern rather than breed -such as Tabby, Ginger, Tuxedo, Calico, or Dark Calico- along with cats specified as crossbred, were combined into the crossbred/domestic group. Accordingly, 46 cases (88.5%) were included in the crossbred/domestic group, and 6 cases (11.5%) were in the purebred group. The purebred group comprised four British Shorthairs, one Scottish Fold, and one Turkish Angora.

Initial stertor score was evaluated as 2 in 41 cases (78.8%), 3 in 10 cases (19.2%), and 4 in 1 case (1.9%). Purulent rhinorrhea was present in 24 cases (46.2%) and absent in 28 cases (53.8%) (*Table 1*).

In the diagnostic evaluation of NPS, pharyngeal examination under general anesthesia was utilized in 36

Table 1. Demographic and Initial Clinical Characteristics of the Cases

Variables	Categories	n (%)
Age group	<1 year	10 (19.2)
	1 to <3 years	21 (40.4)
	3 to <7 years	15 (28.8)
	≥ 7 years	6 (11.5)
Breed group	Crossbred/Domestic	46 (88.5)
	Purebred	6 (11.5)
Sex	Female	26 (50.0)
	Male	26 (50.0)
Initial stertor score	2 - Moderate	41 (78.8)
	3 - Severe	10 (19.2)
	4 - Very severe	1 (1.9)
Purulent rhinorrhea	Present	24 (46.2)
	Absent	28 (53.8)

cases (69.2%), CT in 38 cases (73.1%), and RN in 18 cases (34.6%) (Fig. 2). Multiple diagnostic modalities could be applied in the same case. When the combinations of diagnostic modalities were analyzed, all three modalities were used in 15 cases (28.8%). Pharyngeal examination alone and CT alone were performed separately in 13 cases (25.0%) each, whereas pharyngeal examination combined with CT was utilized in 8 cases (15.4%), CT combined with RN in 2 cases (3.8%), and RN alone in 1 case (1.9%) (Table 2). Choanal atresia was detected in only one case (1.9%; Case no. 26) (Fig. 3). In these 15 cats, the findings were not clinically contradictory and were considered complementary. Because stenosis severity was not documented using a standardized grading system for all modalities, formal concordance or correlation analysis was not performed.

The available retrospective records did not permit reliable classification of the cases as congenital or secondary NPS. Information regarding the presence of clinical signs since birth and previous infectious, inflammatory, traumatic, or iatrogenic events was not documented consistently. Therefore, etiology-specific treatment outcomes were not calculated. The single case of CT-confirmed choanal atresia was recorded separately as an anatomical developmental abnormality and was not used alone to establish an etiologic subgroup.

Modified balloon dilation was performed in 30 cases (57.7%) (Fig. 4). In 22 cases (42.3%) where modified balloon dilation was not performed, the treatment had been recommended, but the owner declined the procedure (Table 3).

In 8 of the 30 cases (26.7%) undergoing modified balloon dilation, at least one case-specific operative procedure was performed during a subsequent treatment session. Case 13 underwent surgical removal of a completely occluding stenotic membrane before subsequent modified balloon dilation. Case 26 underwent transpalatal choanoplasty for choanal atresia before subsequent modified balloon



Fig 3. Sagittal-plane computed tomography image of a case diagnosed with choanal atresia. The nasopharyngeal lumen is obliterated by a soft-tissue-attenuating structure, and the choana is continuous with a bony framework. Choanal atresia and nasal secretions filling the nasal cavity secondary to the obstruction are indicated by the red arrows

dilation. Other recorded interventions reflected concurrent disease or case-specific anatomical findings and included vomer resection, soft palate reconstruction, ventral bulla

Table 2. Distribution of diagnostic modalities

Variables	Category	n (%)
Diagnostic modality performed	Pharyngeal examination	36 (69.2)
	CT	38 (73.1)
	RN	18 (34.6)
Combination of diagnostic modalities	Pharyngeal examination alone	13 (25.0)
	CT alone	13 (25.0)
	RN alone	1 (1.9)
	Pharyngeal examination + CT	8 (15.4)
	CT + nasopharyngoscopy	2 (3.8)
	Pharyngeal examination + CT + nasopharyngoscopy	15 (28.8)

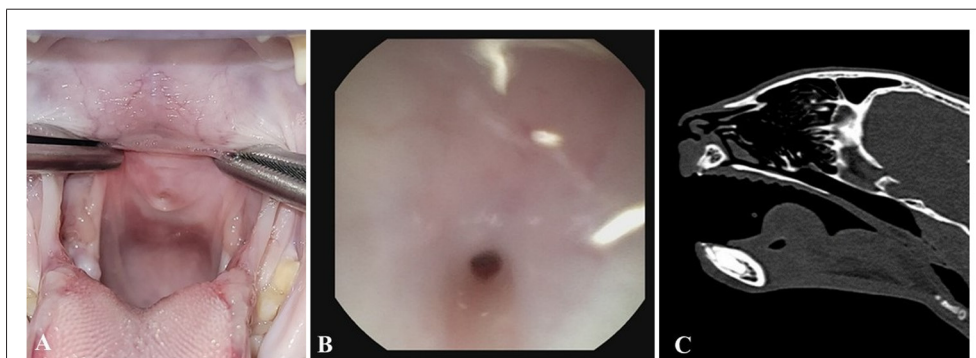


Fig 2. Diagnosis of nasopharyngeal stenosis via pharyngeal examination (A), RN (B), and sagittal CT view in the soft tissue window (C)

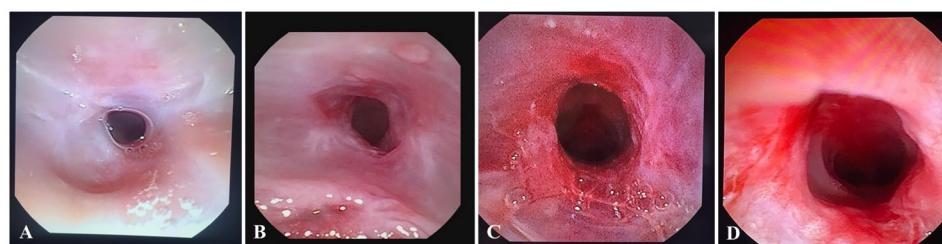


Fig 4. Gradual increase in nasopharyngeal patency following four balloon dilation sessions performed at two-week intervals: first session (A), second session (B), third session (C), and fourth and final session (D), demonstrating progressive widening of the nasopharyngeal meatus

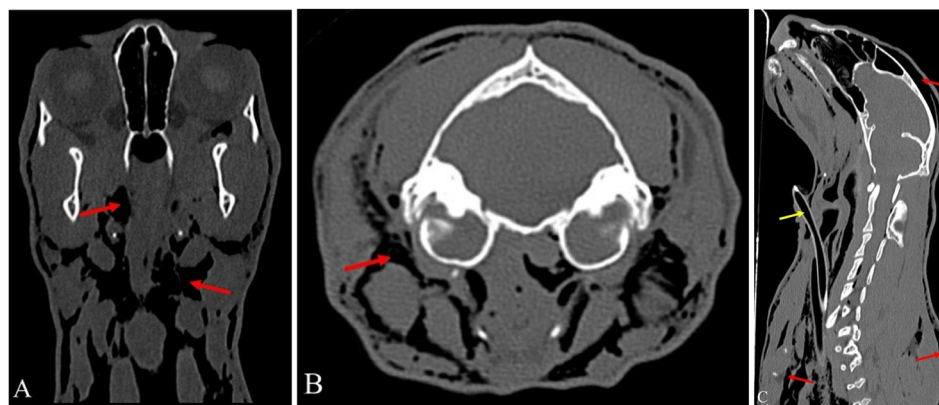


Fig 5. Sagittal (A), axial (B), and coronal (C) computed tomography images showing severe subcutaneous emphysema following balloon dilation in Case No. 26. The air accumulations extending from the nasopharyngeal region into the subcutaneous tissues of the neck and thorax are indicated by red arrows. The yellow arrow in panel C indicates the tracheostomy tube

osteotomy, polypectomy, epiglottectomy, staphylectomy, tonsillectomy, epiglottopexy, and sinonasal aspiration.

Early complications associated with the dilation procedure were recorded in 3 of the 30 cases (10.0%) undergoing modified balloon dilation. Intensive care support was required in Case 25 due to the development of widespread emphysema. Marked edema was observed in the nasopharyngeal region and tongue in Case 28. In Case 26, emphysema developed and tracheostomy tube placement was required (*Fig. 5*).

Table 3. Distribution of the number of sessions in cases undergoing modified balloon dilation

Modified Balloon Dilation Session	n (%)
1 session	13 (43.3)
2 sessions	4 (13.3)
3 sessions	4 (13.3)
5 sessions	4 (13.3)
6 sessions	2 (6.7)
7 sessions	1 (3.3)
9 sessions	2 (6.7)

Furthermore, following nasopharyngeal reconstruction, which was evaluated separately from modified balloon dilation, bilateral mydriasis and transient vision loss developed in Case 13.

In the 30 cats that underwent modified balloon dilation, the mean total clinical follow-up period was calculated as 2.9 ± 2.7 months. The median total clinical follow-up duration was determined to be 2.0 months (interquartile range: 1.5-3.4 months; range: 0.1-11.1 months).

In 29 of the 30 cases (96.7%) undergoing modified balloon dilation, clinical improvement persisted at the final follow-up evaluation. In Case 26, although clinical improvement was initially reported, recurrent narrowing was identified at the second treatment session, approximately one month after the initial intervention, and the case was classified as restenosis in the final assessment (*Table 4*). The restenosis rate was therefore 1/30 (3.3%). Because only one restenosis event occurred, a mean time to recurrence was not calculated.

No significant correlation was found between age and initial stertor score (Spearman's $\rho = -0.083$; raw $P = 0.559$). The median ages of cases with and without purulent rhinorrhea were 18 and 24 months, respectively, with no

Table 4. Treatments, additional procedures, and clinical outcomes

Variable	Category	n (%)
Modified balloon dilation status ¹	Performed	30 (57.7)
	Recommended but declined by owner	22 (42.3)
Additional operative procedure during the treatment course ²	Present	8 (26.7)
	Absent	22 (73.3)
Clinical outcome ²	Clinical improvement	29 (96.7)
	Restenosis	1 (3.3)

¹ Percentages were calculated over all cases (n=52)² Percentages were calculated over cases undergoing modified balloon dilation (n=30)

significant difference between the groups (Mann-Whitney U=302.5; raw P=0.541).

Severe or very severe stertor was detected in 2/6 purebred cats (33.3%) and 9/46 crossbred/domestic cats (19.6%). No significant association was found between breed group and severe or very severe stertor (OR=2.06; 95% CI: 0.32-13.04; raw P=0.595). Purulent rhinorrhea was observed in 4/6 purebred cats (66.7%) and 20/46 crossbred/domestic cats (43.5%). The association between breed group and purulent rhinorrhea was also not statistically significant (OR=2.60; 95% CI: 0.37-18.10; raw P=0.397).

In cats undergoing modified balloon dilation, initial stertor score was not significantly correlated with the number of sessions (Spearman's ρ =0.050; raw P=0.791). Age was also not significantly correlated with the number of sessions (Spearman's ρ =-0.104; raw P=0.586). After Benjamini-Hochberg adjustment for multiple comparisons, none of the evaluated relationships remained statistically significant (Table 5).

DISCUSSION

Mestrinho and Fonseca [3], Kang and Brash [5], and Masuyama et al. [6] documented NPS in cats from different age groups. Cats in the present study were generally younger than those in these previous populations. Earlier referral for advanced imaging or endoscopic evaluation

may partly explain this difference. In addition, the specialized ENT referral setting of the present study may have increased the likelihood of identifying NPS in younger cats.

Congenital and secondary NPS may differ in anatomical configuration, tissue composition, response to dilation, and risk of restenosis. However, the retrospective records in the present study did not permit reliable etiological classification. Only one cat had CT-confirmed choanal atresia, whereas the presence of clinical signs since birth and previous infectious, inflammatory, traumatic, or iatrogenic events was not documented consistently in the remaining cases. Young age at diagnosis alone was not considered sufficient evidence of congenital NPS, and the absence of a documented preceding disorder was not interpreted as evidence against secondary disease. Consequently, subgroup-specific comparisons of clinical success, number of dilation sessions, recurrence, complications, additional treatment, and follow-up duration could not be performed without a substantial risk of etiological misclassification.

O'Neill et al. [7] found no difference in the overall prevalence of common disorders between purebred and crossbred cats, although they identified upper respiratory tract diseases more frequently in purebred cats. In contrast, crossbred/domestic cats constituted the great majority of the present population. The large regional stray-cat population and the predominance of crossbred cats within this population may explain this distribution. Therefore, the breed distribution in the present study probably reflects the geographic characteristics and referral population rather than a specific biological predisposition.

Stertor is an important clinical finding suggestive of nasopharyngeal airway obstruction; however, it is not specific to NPS, as nasopharyngeal polyps, neoplasia, foreign bodies, chronic rhinitis/rhinosinusitis, and brachycephalic upper airway syndrome can also present with stertor [8-10]. The reported frequency of stertor at approximately 90%-100% in previous NPS studies demonstrates

Table 5. Relationships between demographic/clinical characteristics and treatment variables

Variables Compared	Test	Test Statistic/Effect Size	Raw P	Adjusted P
Age - initial stertor score	Spearman's correlation	$\rho = -0.083$	0.559	0.714
Age - purulent rhinorrhea	Mann-Whitney U	U = 302.5	0.541	0.714
Breed group - severe stertor	Fisher's exact test	OR = 2.06	0.595	0.714
Breed group - purulent rhinorrhea	Fisher's exact test	OR = 2.60	0.397	0.714
Initial stertor score - number of sessions	Spearman's rank correlation	$\rho = 0.050$	0.791	0.791
Age - number of sessions	Spearman's rank correlation	$\rho = -0.104$	0.586	0.714

¹ P-values were adjusted for six comparisons using the Benjamini-Hochberg procedure² The analysis was performed on cases undergoing modified balloon dilation (n = 30)CI, confidence interval; OR, odds ratio; ρ , Spearman's rank correlation coefficient

its importance in raising clinical suspicion [3,5,6]. Similarly, the presence of stertor in all cases in the present investigation supports this finding as a strong clinical indicator for the recognition of NPS and the referral of cases for advanced diagnostic workup. In addition to the presence of stertor, its severity was graded in the current records as mild, moderate, severe, and very severe based on subjective clinical observation. To our knowledge, no other study in the available literature has graded stertor severity in cats with NPS. This approach enabled the comparison of cases in terms of clinical severity and allowed the evaluation of a potential relationship between stertor severity and treatment requirement. Applying this grading system to obstructive airway diseases in the future may contribute to the differential diagnosis and help delineate variations in stertor severity across different conditions. However, because this system was developed within the scope of the present study, its inter-observer agreement, reproducibility, and clinical validity need to be verified in prospective studies.

Clinical signs such as stertor, nasal discharge, open-mouth breathing, and dyspnea are shared across various upper respiratory conditions; therefore, reaching a definitive diagnosis based only on clinical examination is challenging. Therefore, advanced diagnostic methods are required in the differential diagnosis of nasopharyngeal diseases [5,11,12]. CT, one of these methods, enables cross-sectional examination of the nasopharyngeal meatus and surrounding bone and soft tissue structures, providing detailed information regarding the localization, morphology, and anatomical extent of stenosis, choanal atresia, nasopharyngeal polyps, inflammatory disease, and neoplasia [5,11-14]. In cats with NPS, the most frequently reported CT finding is a marked and sudden meatal narrowing associated with a soft tissue density band in the caudal third of the nasopharynx. Focal dorsal deviation of the soft palate at the level of the stenosis has also been reported. The anatomical relationship between the soft palate and the stenotic membrane can be evaluated more clearly, particularly in sagittal reconstructions [5,12]. However, patient and mouth positioning during scanning, potential displacement of the soft palate by the endotracheal tube, secretions accumulating in the nasopharyngeal region, and concurrent neoplastic formations can affect the CT appearance and make accurate evaluation of the stenosis difficult. The use of CT in the majority of cases in the present study supports the importance of this method in the diagnosis and anatomical characterization of NPS. Furthermore, CT is considered to contribute to the differential diagnosis of conditions that can cause similar clinical signs and to the identification of pathologies accompanying NPS. Nevertheless, due to the potential limitations of the method, scans should be performed with the mouth open to allow a more objective examination of

the nasopharyngeal meatus, and CT findings must always be interpreted in conjunction with clinical findings and other diagnostic methods.

Choanal atresia is a congenital anomaly characterized by partial or complete obliteration of the choana with bony, membranous, or mixed tissue [12,15,16]. CT allows the differentiation of choanal atresia from NPS by demonstrating in detail the composition of the obstruction, its anatomical extent, and its relationship with the surrounding bony structures [5,9]. This distinction is critically important because the surgical management of choanal atresia differs from that of classical NPS [17-19]. In the present study, the diagnosis of choanal atresia established in Case 26 via CT examination following pharyngeal inspection further underscores the clinical importance of CT in differential diagnosis and surgical planning.

CT measurements can be utilized for nasopharyngeal stent selection and for determining the target balloon diameter in conventional balloon catheter interventions [20,21]. However, in advanced stenosis, the very small residual lumen may reduce measurement reliability; relying strictly on these measurements to directly use a large-diameter balloon may increase the risk of mucosal trauma, fibrosis, and restenosis. Based on our clinical experience, initiating dilation with a small internal diameter cuffed endotracheal tube and gradually increasing the tube diameter according to tissue resistance and the achieved luminal patency provides a more controlled approach.

Pharyngeal examination under general anesthesia enables direct visualization of the caudal nasopharynx and the stenotic membrane in this region via soft palate retraction [9,22]. Nevertheless, the primary limitations of the method include a field of view predominantly restricted to the caudal nasopharynx and the masking of the stenotic aperture by secretions [23]. In the present study, the use of pharyngeal examination in the great majority of cases demonstrated that the method is useful for rapid, direct assessment, particularly of caudally located stenoses; however, it may remain inadequate on its own in rostral lesions and in the presence of copious secretions. Furthermore, uncontrolled retraction of the soft palate was observed to cause mucosal trauma, edema, and hemorrhage, underscoring the importance of careful manipulation. Conversely, its straightforward feasibility by an experienced team and lack of requirement for advanced instrumentation or specialized equipment were considered significant advantages of systematic pharyngeal examination.

RN has been reported to provide direct evaluation of the nasopharynx and choana by advancing a flexible endoscope transorally into the caudal oropharynx and

retroflexing it dorsally around the caudal free edge of the soft palate^[23,24]. Through this modality, the morphology of the stenotic membrane, the location and approximate diameter of the residual lumen, as well as the presence of secretions, inflammation, polyps, and other soft tissue lesions can be assessed. Magnified, illuminated visualization combined with a deflectable endoscope tip enables the inspection of regions inaccessible on pharyngeal examination, whereas irrigation and suction facilitate clearer delineation of the stenotic site by clearing secretions^[23]. However, the narrow nasopharyngeal space in cats restricts the usable endoscope diameter, and proper navigation of the scope demands technical proficiency^[24]. In the current investigation, although RN offered more detailed visualization than pharyngeal examination, it was considered an operator- and equipment-dependent procedure. Performing the procedure under general anesthesia was deemed essential to ensure an adequate working field, patient immobility, and airway security, as well as to minimize the risks of gagging, coughing, and aspiration.

Diagnostic modalities complement one another in the evaluation of NPS. CT identifies the anatomical extent of the lesion and accompanying structural changes but does not replace direct assessment of nasopharyngeal patency. Pharyngeal examination enables direct clinical assessment of the nasopharyngeal opening, whereas RN provides magnified illumination and improved visualization of the affected region. In the 15 cats examined using all three modalities, no clinically contradictory findings were identified; however, formal concordance analysis was not possible because stenosis type and severity had not been recorded using a standardized grading system. The non-uniform use of the modalities reflected the retrospective clinical setting, including owner financial constraints, equipment availability, clinical need, and the feasibility of repeated general anesthesia.

In the literature, a case undergoing three balloon dilation sessions due to restenosis has been reported, whereas another study evaluating seven cats noted that acceptable clinical control was achieved following one or two interventions in cases with long-term follow-up^[2,25]. In another study, the procedure was performed in 12 cats, with only two cases requiring a second intervention due to recurrence. In a more comprehensive investigation, dilations were scheduled as one to three sessions, and a planned serial dilation approach was reported to be potentially associated with a longer progression-free interval^[6,26]. In the present study, the number of sessions reached five to nine in some cases, which was higher than the number of interventions reported in the literature. Because nasopharyngeal tissues are delicate and prone to tearing, increasing luminal patency in a controlled and

staged manner was preferred over forcing dilation in a single session. Accordingly, the requirement for multiple dilations should not always be construed as restenosis or treatment failure, but rather as an outcome of a safe and stepwise therapeutic approach. Our clinical experience suggested that approximately five sessions may be necessary to achieve adequate nasopharyngeal patency in some cases. However, rather than adhering to a rigid protocol, the number of sessions should be determined on a case-by-case basis by evaluating the degree of patency, tissue response, clinical improvement, and complication risk prior to each intervention.

The treatment sequence was likewise individualized according to anatomical accessibility and concurrent disease. Modified balloon dilation was preferred when a residual opening permitted tube passage. Complete membranous occlusion in Case 13 required surgical removal of the membrane before later dilations, whereas choanal atresia in Case 26 required transpalatal choanoplasty before later dilation sessions. Additional procedures for otitis media, inflammatory aural polyps, chronic rhinosinusitis, or other anatomical abnormalities were performed during subsequent sessions as clinically indicated rather than as routine components of the primary dilation procedure.

In humans, prolonged pressure on the base of the tongue by mouth gags and retractors during palatal surgery has been reported to cause lingual edema due to venous congestion, ischemia, and impaired lymphatic drainage^[27,28]. Subcutaneous emphysema developing after oropharyngeal interventions is explained by the passage of air into deep neck tissues through breaches in the pharyngeal mucosa and constrictor muscles^[29,30]. In the present study, the observed nasopharyngeal and tongue edema was thought to be associated with temporary disruption of venous and lymphatic drainage caused by the pressure exerted on surrounding tissues during soft palate elevation. Widespread emphysema was considered to have developed as a result of air tracking into cervicofacial tissue planes through a small tear in the pharyngeal mucosa and underlying muscular layers. This complication represents the first similar case recorded following interventions in this region at the relevant clinic, and no directly comparable reports were found in the available veterinary literature. These findings highlight the importance of avoiding excessive tissue compression and uncontrolled deep trauma during the procedure, as well as the necessity of perioperative airway monitoring. Restenosis, on the other hand, is an expected outcome attributable to post-procedural inflammation, fibrosis, and recurrent scar tissue formation following dilation or reconstruction. Therefore, therapeutic efficacy should not be evaluated solely based on early clinical

improvement, but rather by taking into account the long-term preservation of nasopharyngeal patency.

Inflation durations reported for feline NPS vary considerably. Pollack et al. [24] used serial inflation periods of 30 or 60 sec whereas Champetier et al. [4] maintained each progressively increased balloon pressure for five minutes. The five-minute inflation period used in the present study was based on the latter protocol. Prolonged inflation may provide sustained radial pressure and facilitate gradual expansion of fibrotic tissue; however, it may also increase the risks of mucosal ischemia, tissue trauma, hemorrhage, edema, and inflammation. The five-minute resting interval between applications allowed the jaw to be returned to a near-closed position around the orotracheal tube and provided an opportunity to assess tissue response before further dilation. This precaution is relevant because maximal or prolonged mouth opening in cats can compress the maxillary arteries and reduce arterial supply to the brain and retina [31-33].

Previous investigations have reported stertor and rhinorrhea as frequent clinical findings, yet these findings have not been shown to predict the number of required dilations [6,25]. Similarly, although planned serial dilation has been suggested to be potentially associated with a longer progression-free interval, no significant correlation was identified between the total number of dilations and progression-free survival [26]. The findings of the present study suggest that age and initial stertor severity have limited value in predicting the number of modified balloon dilation sessions. The number of sessions is considered to be influenced more substantially by case-specific anatomical characteristics, such as the localization and length of stenosis, the subjectively assessed degree of narrowing, the fibrotic nature of the tissue, and the luminal caliber achieved at each intervention.

This study has several limitations. Its retrospective design resulted in incomplete or non-standardized clinical records and prevented uniform application of diagnostic, therapeutic, and follow-up protocols. The absence of a control or comparator group precludes any conclusion that the modified dilation technique is superior to previously reported balloon dilation protocols. Selection bias may have arisen because the study population was derived from a specialized ENT referral clinic and treatment was declined by a substantial proportion of owners. Treatment procedures, maximum tube diameter, number and timing of dilation sessions, adjunctive surgical interventions, and follow-up duration varied among cases. Clinical improvement was assessed partly from owner reports and was not consistently confirmed through repeat CT or RN. Objective, standardized measurements of stenosis diameter, length, severity, immediate post-treatment widening, and subsequent

luminal patency were unavailable; the qualitative saline-passage assessment used in some cats was not a validated measurement method. Formal concordance analysis among CT, pharyngeal examination, and RN was therefore not possible. Furthermore, the available records did not permit reliable classification of most cases as congenital or secondary, which prevented robust etiological subgroup comparisons. The limited number of treated cats, low numbers of complications and restenosis events, and short and variable follow-up periods further restricted statistical analysis and assessment of long-term recurrence.

This study showed that pharyngeal examination under general anesthesia, CT, and RN can be used individually or in combination for the diagnosis of feline NPS. Modified balloon dilation with cuffed red rubber endotracheal tubes was followed by clinical improvement in most treated cats, although some required multiple dilation sessions or adjunctive surgical procedures and early procedure-related complications occurred. Age and initial stertor severity did not predict the number of dilation sessions. These findings support modified balloon dilation as a feasible treatment option but do not establish superiority over conventional balloon dilation or other treatments. Prospective controlled studies using standardized anatomical measurements, treatment protocols, and follow-up examinations are needed to compare therapeutic approaches and determine long-term efficacy.

DECLARATIONS

Availability of Data and Materials: The anonymized data supporting the findings of this study are available from the corresponding author upon reasonable request. Information that may identify animal owners will not be shared in accordance with the Turkish Personal Data Protection Law No. 6698 (KVKK).

Ethical Compliance Declaration: The authors declare that the study was conducted in accordance with all applicable animal welfare and research ethics principles, institutional and national/international regulations, and the requirements of the relevant ethics committees and funding/supporting institutions, where applicable. The authors accept full responsibility for the ethical integrity of the study.

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Authors' Contributions: AD and EBB: Conceptualization, methodology, investigation, data curation, interpretation of the results, and writing-original draft. EMP and CNG: Investigation, data collection, data curation, visualization, and writing-review and editing. All authors critically reviewed and approved the final version of the manuscript.

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The editor-in-chief pays attention to whether there is a conflict of interest or union of interest between editors, reviewers and author (s) for an objective and unbiased evaluation of the article. In addition, the authors should disclose any financial interests or links or any conditions that may raise the bias issue in research and article under the above heading.

• Copyright

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The authors must fill in the "Copyright Agreement Form" and sign it with a wet signature. Authors who submit articles from abroad should scan the signed form and send it to the editor via the system or by e-mail. Original forms that are wet signed for articles sent domestically should be submitted to the journal via mail or cargo. The works of the authors who do not submit the Copyright Agreement Form on time are not published.

• Withdrawal of a Submission

In case of if the authors detect a significant error or deficiency in their article under review or if this error is reported to them by the editor/subject editor/referees they can contact immediately to the editor-in-chief and ask the request to withdraw the article by stating the reason. The decision on this issue is up to the editorial board.

• Erratum

After an article has been published, the corresponding author may request the editor to publish "erratum" for any errors or inaccuracies noticed by the authors, editors or readers. In collaboration with the authors, the editor prepares and publishes the Erratum article in the first upcoming issue. These articles, like other publications, should contain the publication tag and DOI number.

• Retraction

If any ethical problem is detected about the article that cannot be compensated and cannot be eliminated with erratum after the article is published, the editor-in-chief and associate editors prepare a justification about the article and apply the retraction procedure to the article. The text file on the web page of a retracted article is blocked and the reason for retraction is added to the system as a file, ensuring that it is constantly in the archive.

• Advertising

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. 4th ed., 339-447, Lea and Febiger, Philadelphia, 1988.

DOI number should be added to the end of the reference.

In the references can be reached online only, the web address and connection date should be added at the end of the reference information. The generally accepted scientific writing instructions must comply with the other references. Abbreviations, such as “et al” and “and friends” should not be used in the list of the references.

Follow the link below for EndNote Style of Kafkas Universitesi Veteriner Fakultesi Dergisi;

<https://researchsoftware.com/downloads/journal-faculty-veterinary-medicine-kafkas-university>

9- Latin expression such as species names of bacteria, virus, parasite, and fungus and anatomical terms should be written in italic character, keeping their original forms.

10- The editorial board has the right to perform necessary modifications and a reduction in the manuscript submitted for publication and to express recommendations to the authors. The manuscripts sent to authors for correction should be returned to the editorial office within a month. After pre-evaluation and agreement of the submitted manuscripts by the editorial board, the article can only be published after the approval of the field editor and referee/s specialized in the particular field.

11- All responsibilities from published articles merely belong to the authors. According to the ethical policy of our journal, plagiarism/self-plagiarism will not be tolerated. All manuscripts received are 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

