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Original Article

Therapeutic Potential of Leech Saliva Against Experimental *Salmonella enterica* Serovar Typhimurium Infection in Broiler Chickens

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Abstract

Introduction: *Salmonella enterica* serovar Typhimurium is one of the most common nontyphoidal *Salmonella* serovars affecting poultry production worldwide. Moreover, its increasing resistance to antibiotics has made treatment extremely challenging. Leech saliva contains compounds that confer natural antibacterial and immunomodulatory properties.

Objectives: This study aimed to examine whether treatment with leech saliva alone or in combination with gentamicin reduces the severity of infection in chickens experimentally infected with *Salmonella Typhimurium*.

Methods: A total of 60 Ross 308 chicks were randomly and equally allocated to four groups: a control group, a gentamicin-treated group, a leech saliva-treated group, and a dual therapy group receiving both leech saliva and gentamicin. Bacterial load, gross pathology changes, and histopathological alterations in the spleen, liver, and intestine were examined over five days.

Results: Monotherapy with leech saliva moderately reduced the microbial load and infection-associated tissue damage caused by *S. Typhimurium*, whereas gentamicin monotherapy showed greater efficiency. Compared with either monotherapy, combination therapy yielded the most favorable outcomes, demonstrating significantly enhanced bacterial clearance and a greater reduction in pathological changes ($P<0.05$). Histopathological scores further demonstrated that combination therapy markedly decreased enteritis as well as hepatic and splenic necrosis compared with both monotherapy groups.

Conclusion: The findings indicate that leech saliva exerts a beneficial antimicrobial adjunctive effect. This natural compound may be used as a valuable adjunct in treatments and represents a promising strategy for combating the multidrug resistance of *S. Typhimurium* in poultry.

Keywords: *Hirudo medicinalis*, Leeches, *Salmonella*, *Salmonella* enterica, Poultry, Gentamicin

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Introduction

Salmonella enterica serovar Typhimurium is one of the most common nontyphoidal *Salmonella* (NTS) serovars affecting poultry production worldwide, posing significant threats to both health and food safety (1). This serovar is responsible for causing salmonellosis in multiple species, particularly in chickens and humans (2). *S. enterica* is a Gram-negative, facultatively anaerobic, flagellated, and rod-shaped bacterium (3). *Salmonella* remains a global public health concern due to its zoonotic potential, increasing antimicrobial resistance, and substantial contribution to morbidity and mortality worldwide (4, 5). Nontyphoidal *Salmonella* is estimated to cause approximately 93.8 million cases of gastroenteritis globally and incurs 155,000 deaths annually, with *S. Typhimurium* and *S. Enteritidis* accounting for the majority of infections (1). Furthermore, poultry-associated NTS infections impose a significant public health threat to the population, with an estimated annual burden of $2.79 billion. As the demand for ready-to-eat food continues to rise, these challenges need urgent attention (6). However, traditional treatment strategies have added a layer of complexity.

Salmonella strains, not surprisingly, are exhibiting alarming levels of resistance, significantly reducing the effectiveness of available control measures and posing a serious public health threat.

Although antibiotics are widely used to treat bacterial infections in poultry, resistance to antibiotics is a growing concern and threatens their effectiveness. The misuse and overuse of antibiotics in intensive livestock production, particularly in poultry farming, have always been a concern (7, 8). The inclusion of antibiotics in poultry feed for growth promotion and disease prevention has further contributed to the development of antimicrobial and multidrug resistance, especially in *Salmonella* spp. (e.g., *S. Typhimurium*). Resistance is frequently associated with reduced susceptibility to several antimicrobials, including tetracyclines, sulfonamides, and β-lactams (9-11). Moreover, recent reports on *S. Typhimurium* isolates from poultry revealed resistance to several additional antimicrobials, including tylosin, chloramphenicol, and oxytetracycline (12).

The increasing prevalence of antimicrobial resistance is driven by multiple mechanisms that allow *S. Typhimurium* to survive antimicrobial pressure while maintaining its pathogenicity. These mechanisms have been developed by bacteria, and *S. Typhimurium* acquires resistance to antibiotics through a multitude of mechanisms, including the use of plasmid-mediated resistance genes, efflux pump activity, enzymatic drug inactivation, alterations in antimicrobial target sites, and biofilm formation, which protects a wide range of antimicrobial agents (13).

Extensive research has been conducted on the salivary gland secretions of the medicinal leech (*Hirudo medicinalis*), which contain a diverse range of compounds with potent antimicrobial, anti-inflammatory, anticoagulant, and immunomodulatory properties (14, 15). These effects have been associated with numerous peptides, including hirudin, bdellins, eglins, and destabilases. Additionally, leech saliva exhibits strong antioxidant activity, downregulating proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) (16).

Recent research in Korea has utilized AI-based screening approaches for the discovery of new antimicrobial peptides obtained from leech salivary glands. Importantly, leech antimicrobial peptides such as Hirunipin-2 have demonstrated strong activity against multidrug-resistant and biofilm-producing pathogens, highlighting leech saliva as a promising reservoir of novel antibacterial compounds (17).

Despite the growing interest in the therapeutic potential of leech saliva, a significant knowledge gap remains, particularly regarding its in vivo application against *Salmonella* infections in avian and, more importantly, poultry models. To date, no research has explored the therapeutic efficiency of leech saliva for treating *S. enterica* serovar Typhimurium infection in broiler chickens. Although the antibacterial and immunomodulatory properties of leech saliva have been documented, its in vivo therapeutic application against *S. enterica* serovar Typhimurium in poultry remains unexplored. Addressing this gap is critical for the development of sustainable intervention strategies amid the increasing problem of antimicrobial resistance.

Therefore, the present study investigated the treatment of *S. Typhimurium* infection in broiler chickens using medicinal leech saliva in an in vivo model. The study assessed bacteriological clearance, gross and histopathological changes, and therapeutic interactions, both individually and in combination with gentamicin, to perform an integrated evaluation of the potential of leech saliva as an alternative or complementary antimicrobial treatment in poultry. Furthermore, leech saliva may exert a synergistic effect in the healing process when combined with gentamicin. This study addressed this gap by examining the potential role of leech saliva in promoting sustainable health advances in poultry production.

Materials and Methods

Experimental Animals and Husbandry Conditions

Sixty Ross 308 broiler chickens (mean initial body weight: 180–250 g) were purchased from a commercial hatchery and housed under standardized environmental conditions (temperature: 33 ± 2°C; relative humidity: 60 ± 5%). Broiler chicks were kept in clean, disinfected floor pens and had ad libitum access to water and commercial feed. A prestarter diet was provided for the first three days, followed by a starter diet formulated according to the National Research Council (NRC) recommendations.

Experimental Design and Group Allocation

Following experimental infection with *S. enterica* serovar Typhimurium at five days of age, the chicks were randomly divided into four groups, each comprising 15 birds. The control group received no treatment. The second group was intraperitoneally injected with gentamicin (5% solution; NASR Co., Iran) at a dose of 20 mg/kg body weight per day for five days. The third group received a daily intraperitoneal injection of 0.5 mL of leech saliva containing 400 µg of protein for five days. The fourth group was treated with a combination of gentamicin (20 mg/kg) and leech saliva (0.5 mL) and received coinjections intraperitoneally for five days, as illustrated in Table 1. The administered doses were selected based on pilot studies that established the safety and biological efficacy of the treatments in broiler chicks. The antibiotic susceptibility of *Salmonella* isolates was confirmed using the disk diffusion method, which verified their sensitivity to gentamicin.

Pathogen Culture and Inoculation Procedure

The pathogenic strain of *S. Typhimurium* was cultured in brain heart infusion (BHI) broth. To prepare the media, 3.7 g of BHI powder was dissolved in 100 mL of distilled water and then sterilized using an AV25 autoclave

Table 1. Experimental Groups and Treatment Regimens

Group	Treatment Type	Dose/Concentration	Administration Route	Duration
I	Negative control	None	—	—
II	Gentamicin only	20 mg/kg body weight	Intraperitoneal	5 days
III	Leech saliva only	0.5 mL (400 µg protein)	Intraperitoneal	5 days
IV	Gentamicin + Leech saliva	As above (both agents)	Intraperitoneal	5 days

(Kavosh Co., Iran). Two milliliters of the broth were dispensed into sterile test tubes containing the bacterial inoculum and incubated at 37°C (Pars Azma Co., Iran) for 18–24 hours. Bacterial turbidity was standardized with a 0.5 McFarland standard (optical density at 600 nm, 0.1 OD) using a spectrophotometer (SIGMA, Germany), corresponding to approximately 1.5×10^8 CFU/mL.

All chicks were orally gavaged with 2 mL of the bacterial suspension using a sterile crop tube (5). The challenge dose was determined through pilot titration studies to ensure consistent infection without inducing acute mortality.

Leech Saliva Extraction and Characterization

Leech saliva was collected using a modified protocol described by Abdualkader et al (18). Medicinal leeches were allowed to feed through a parafilm membrane, which was taped onto the lid of a 100 mL Erlenmeyer flask containing 1 mM arginine and 0.15 M sodium chloride. This modified Abdualkader method has been shown to effectively induce salivation while retaining bioactivity (19), and previous studies have identified this solution as a potent feeding stimulus (20). After feeding, the leeches were placed on ice for 10 minutes and subsequently allowed to warm gradually, and a flame was used to stimulate salivation into sterile microtubes, which were stored at −20°C for 72 hours. Protein concentration of the saliva was calculated using a NanoDrop spectrophotometer (Labtron GF-200, Japan), yielding a concentration of 800 µg/mL. For experimental administration, 0.5 mL of saliva containing 400 µg of total protein per chick was injected intraperitoneally once daily. It should be noted that this concentration represents total protein content and was not standardized for specific bioactive components, such as hirudin or bdellins, which may vary depending on the leech's feeding status and extraction methods.

Sample Collection and Microbiological Confirmation

Three chicks from each experimental group were selected at random daily for microbiological and histopathological analyses at 24 hours postinoculation. Experiments were conducted according to institutional ethical guidelines, and euthanasia was performed before sample collection. Target organs were aseptically removed, and samples were collected for bacteriological culture and histological processing. Sampling was performed daily over five consecutive days to monitor disease progression and recovery from infection-induced lesions.

The spleen, intestine, and liver were aseptically excised, and sampling instruments were decontaminated by heating the tips to eradicate surface flora. Tissues were cultured on *Salmonella*–Shigella (SS) agar plates and incubated at 37°C for 24 hours. *Salmonella* colonies are identified based on their diagnostic morphology, including pale to translucent colonies with black centers representing hydrogen sulfide production (5, 21).

Gross Pathological Evaluation

Postmortem changes revealed lesions associated with salmonellosis, such as yolk sac retention, hepatosplenomegaly, pericarditis, perihepatitis, necrotic lesions, and enteritis. Caseous cecal core formation, indicative of typhlitis, indicated the infection establishment (22, 23).

Histopathological Analysis

The tissue samples were fixed in 10% neutral-buffered formalin for 24 hours, after which the fixative was replaced with fresh fixative. Histopathological samples were sent to Imam Khomeini Hospital (Tehran, Iran), where standard histological processing was carried out, including paraffin embedding, microtome cutting at 5 µm thickness, and hematoxylin and eosin staining. The samples were observed microscopically using a compound light microscope at ×4, ×10, and ×40 magnification. Parameters assessed included villous atrophy (scored on a scale of 0-4 based on the degree of shortening), capillary congestion (scored 0-4), and intestinal gland damage (scored 1-4, ranging from partial cellular loss to destruction of the gland). Hepatic changes, including necrosis, inflammatory cell infiltration, and epithelial cell detachment, were evaluated descriptively, as shown in Table 2. All tests were performed in a blinded manner by an experienced pathologist (24-26).

Bacterial Load Enumeration

Bacterial counting was performed following the standard colony-counting method according to the ISO 4833:2003 guidelines. All procedures were conducted aseptically. Briefly, 1 g of tissue sample was placed into a sterile mortar and homogenized with 1 mL of sterile peptone water. The homogenate was then transferred into a test tube containing 9 mL of peptone water to obtain a 10^{-1} dilution. Subsequent serial dilutions (10^{-2} and 10^{-3}) were prepared by transferring 1 mL of the previous dilution into sterile tubes containing 9 mL of sterile peptone water. For each dilution, triplicate samples were obtained. From each dilution, 100 µL was spread onto selective SS agar plates using a sterile glass spreader. All the plated dilutions

Table 2. Histopathological Scoring System for H&E-stained Sections

Parameter	Score	Condition	Description
Villous Atrophy	0	Intact villi	No signs of atrophy
Villous Atrophy	1	Mild involvement	1/4 of the villi affected
Villous Atrophy	2	Moderate involvement	2/4 of the villi affected
Villous Atrophy	3	Severe involvement	3/4 of the villi affected
Villous Atrophy	4	Total atrophy	Complete involvement of all villi
Capillary Congestion	0	No congestion	Capillaries appear normal
Capillary Congestion	1	Mild congestion	1/4 of the capillaries are congested
Capillary Congestion	2	Moderate congestion	2/4 of the capillaries are congested
Capillary Congestion	3	Severe congestion	3/4 of the capillaries are congested
Capillary Congestion	4	Complete congestion	All capillaries congested
Liver Structure	-	Evaluated features	Congestion, necrosis, inflammation, and degeneration
Epithelial Cell Detachment	-	Evaluated	Extent of epithelial cell separation from the epithelial layer
Intestinal Gland Involvement	1	Partial cell detachment	The pericellular space is not visible around some cells
Intestinal Gland Involvement	2	Localized loss	Continuous loss in one part of the gland
Intestinal Gland Involvement	3	Focal damage	A focal lesion and destruction of the gland were observed
Intestinal Gland Involvement	4	Complete destruction	Total glandular destruction

The table presents a semiquantitative grading system used to assess villous atrophy, capillary congestion, epithelial cell detachment, glandular damage, and liver tissue damage. All parameters can be assessed in terms of severity, with the degree of adverse pathological involvement in the tissues increasing as the rate increases.

were incubated aerobically at 37°C for 24 hours. Following incubation, colonies were counted, and bacterial counts were expressed as colony-forming units per gram of tissue (CFU/g) (8).

Statistical Analysis

Quantitative data, including bacterial counts and histopathological scores, are presented as means ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test to compare treatment groups. Statistical significance was set at $P<0.05$, and all analyses were conducted using GraphPad Prism software (8, 27).

Ethical Approval

All animal husbandry was carried out following the ethical guidelines of the Faculty of Veterinary Medicine, Semnan University, Iran. The study protocol received institutional approval and complied with national regulations governing animal welfare in research (Approval No. 990576.09.08.1400).

Results

Bacterial Count in the Spleen

During the five-day experimental period, bacterial colony counts were measured in four groups: control, gentamicin, leech saliva, and combination therapy (leech saliva and gentamicin). The control group exhibited the highest bacterial counts on day 1. At this time point, the gentamicin and leech saliva groups showed similar bacterial loads in the spleen ($P=0.351$). Importantly, the combination group demonstrated significantly lower bacterial counts than all other groups ($P<0.05$). On day 2, the control still had the highest count, while the leech saliva group exhibited a modest reduction compared with day 1 and exhibited bacterial counts similar to those of the gentamicin group in spleen samples ($P=0.112$). However, a statistically significant difference was observed in liver samples ($P=0.0003$). The combination therapy group maintained the lowest bacterial counts ($P<0.05$). On day 3, bacterial counts decreased in the leech saliva, gentamicin, and combination groups. This downward trend continued through days 4 and 5. Comparative analysis among groups revealed significant differences ($P<0.05$). Overall, although leech saliva alone exhibited mild antibacterial activity, it was less effective than gentamicin. In contrast, combination therapy produced the greatest reduction in bacterial colony counts ($P<0.05$), as shown in Figure 1.

Bacterial Count in the Liver

The number of bacteria in the liver was similar to that recorded in the spleen. The highest bacterial loads on day 1 occurred in the control and saliva groups. The combination therapy group exhibited the lowest bacterial load, followed by the gentamicin group, with the overall lowest count ($P<0.05$). On day 2, bacterial counts in the control and leech saliva groups remained significantly higher than those in the combination and gentamicin groups, and the differences were significant. By day 3, the combination group consistently demonstrated the lowest bacterial counts, which remained the lowest until the end of the experiment. On days 4 and 5, a further reduction in bacterial count was also observed in the leech saliva group. *P*-values are also available for each group. Overall, the results of this study, as shown in Figure 2, revealed the

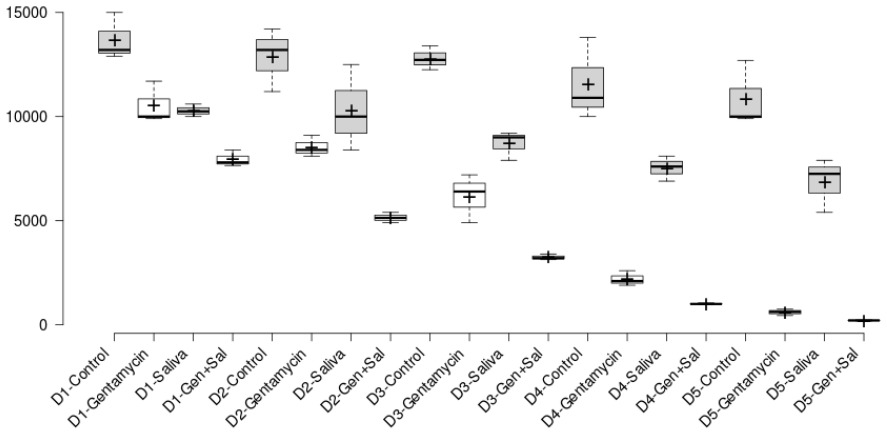

Figure 1. Bacterial Colony Counts in Spleens of Treatment Groups

superior efficacy of combination therapy.

Histopathological Evaluation

Spleen

Histopathological assessment of the spleen revealed that the control group exhibited the most significant damage compared with the other groups ($P<0.05$). The gentamicin, saliva, and combination therapy groups showed similar histological alterations, with no statistically significant differences observed among the groups. These findings are presented in Table 3 and Figure 3.

Intestine

Histological examination of intestinal tissue revealed that the control group sustained the observed injury and developed pronounced enteritis. The degree of injury in the saliva and gentamicin groups was greater than that in the other two groups, with no statistically significant differences between these two groups. In contrast, the combination therapy group exhibited the least degree of intestinal damage and differed significantly from the other three groups ($P<0.05$). The data are presented in Table 3 and Figure 4.

Liver

Histopathological analysis of liver tissues revealed the greatest degree of injury in the control group. The leech saliva group exhibited significantly greater hepatic damage than the gentamicin and combination therapy groups. Both the gentamicin and combination groups demonstrated minimal injuries and were not significantly different from each other ($P<0.05$). The results are summarized in Table 3 and Figure 5.

Discussion

The present study investigated the therapeutic efficacy of medicinal leech (*Hirudo medicinalis*) saliva, administered alone or in combination with gentamicin, in broiler chickens experimentally infected with *S. enterica* serovar Typhimurium. Given the increased prevalence of antimicrobial resistance among poultry pathogens (4, 5, 7-9), these results suggest the potential role of bioactive natural products as adjunct therapeutic agents. The research demonstrates that leech saliva alone provides limited antibacterial efficiency; however, when combined with gentamicin, the applications are more extensive. Synergistically, the combination therapy increased bacterial killing and reduced pathological damage in major organs, including the liver, spleen, and intestine (1, 14-17). In this experimental model, the combined administration of leech saliva and gentamicin resulted in a significantly greater reduction in bacterial load compared with either treatment alone.

Gentamicin, a commonly prescribed aminoglycoside antibiotic, exhibits considerable antimicrobial activity against S. Typhimurium by inhibiting bacterial protein synthesis and penetrating host cells, including macrophages, where *S. Typhimurium* can survive intracellularly (28, 29). However, increasing resistance to gentamicin in poultry pathogens underscores the need for adjunctive measures (30). Leech saliva contains numerous antibacterial compounds against important pathogens. A wide variety of bioactive peptides are present in leech saliva, including hirudin, bdellins, and hirunipins, which exhibit antimicrobial, anti-inflammatory, and immunomodulatory properties (16, 31). Previous studies have demonstrated that the saliva of *Hirudo medicinalis* and Hirudinaria manillensis leeches exhibits inhibitory effects against both Gram-positive and Gram-negative bacteria, including *S.* typhi, *Staphylococcus aureus*, and *Escherichia coli* (15, 18, 32). This suggests that leech saliva may serve as a natural antimicrobial agent. Nevertheless, our findings indicate that despite the presence of antimicrobial peptides in leech saliva, it did not yield significant clinical efficacy in the present poultry model. The combination therapy approach, which involves direct bacterial inhibition via gentamicin and immune modulation and antibacterial effects via leech-derived

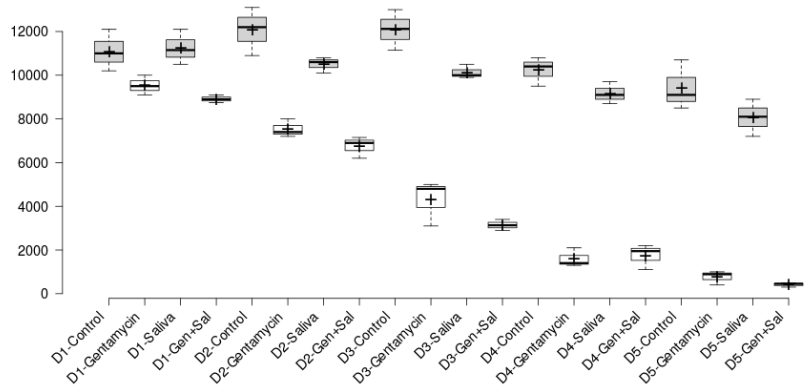

Figure 2. Bacterial Colony Counts in Livers by Colony Size

Table 3. Histopathological Assessments of the Spleen, Liver, and Intestine

Treatment	Histopathology – Spleen (Mean ± SD)	Histopathology – Intestine (Mean ± SD)	Histopathology – Liver (Mean ± SD)
Control	3.4 ± 0.89[a]	3.8 ± 0.44[a]	3.6 ± 0.54[a]
Gentamycin	1.4 ± 0.54[b]	2.4 ± 0.54[b]	1.6 ± 0.54[c]
Saliva	0.2 ± 0.70[b]	2.8 ± 0.44[b]	2.4 ± 0.54[b]
Gentamycin + Saliva	0.1 ± 0.70[b]	1.6 ± 0.54[c]	0.6 ± 0.54[c]

Histopathological assessments of the spleen, liver, and intestine were conducted across four experimental groups: control, gentamycin, saliva, and gentamycin plus leech saliva. Tissue damage was rated based on severity. The combination therapy group presented the most pronounced reduction in tissue damage. Superscript letters ([a], [b], [c]) indicate statistically significant differences between groups ($P < 0.05$).

compounds, likely contributed to the greater clearance of *Salmonella* in the combination treatment group.

Despite these inherent antimicrobial and anti-inflammatory properties, leech saliva alone did not yield significant clinical efficacy in the present poultry model. Bacterial clearance in the leech group was incomplete, and histopathological recovery was modest, suggesting that leech saliva alone may not achieve sufficient pharmacodynamic concentrations when administered alone. This limitation may be due to peptide concentrations, pharmacokinetics of active peptides, or the species composition of saliva. For example, studies suggest that the antimicrobial activity of leech saliva is influenced by the duration of starvation before saliva collection. Ghafoorzadeh et al reported that leeches starved for three months produced saliva with the highest concentration, which was highly effective in killing mutant *Streptococcus* species and inhibiting biofilm formation with overlapping species *S. sobrinus* and *S. mutans* (33). Nonetheless, when combined with gentamicin, leech saliva produced a better therapeutic effect. Therefore, leech saliva should be regarded as an adjunct therapy rather than a replacement for conventional antibiotics in acute systemic infections.

The histopathological findings further supported the benefits of combination therapy, which effectively reduced tissue injury compared to the control and monotherapy groups. Villi atrophy, hepatocellular necrosis, and splenic degeneration were strongly inhibited. A study by Ayhan et al revealed that lyophilized leech saliva extract inhibited the release of TNF-α, IL-6, and GM-CSF from stimulated macrophages, thereby contributing to reduced tissue inflammation (31). Additionally, Bilden et al reported that enzymes and vasodilators in leech saliva facilitate the healing process by stimulating neovascularization, enhancing blood flow, and promoting tissue perfusion after injury (34). Furthermore, Meriaux et al demonstrated the role of lipids and peptides, specifically endocannabinoids and gangliosides, in regulating inflammation within the regenerating leech nervous system (35). Histopathological examination has long been a cornerstone in poultry model systems for evaluating the severity of enteric and hepatic lesions resulting from bacterial infection and therapeutic interventions (36, 37). These findings corroborate the conclusions drawn from the present study and support the potential of adjunctive therapies to mitigate pathogen burden and tissue injury due to host-mediated tissue damage.

Notably, our combination therapy cohort showed a significantly reduced bacterial load in intestinal and liver tissue, as measured by quantitative culturing and histopathological scoring. Specifically, villus atrophy, hepatocellular necrosis, and splenic degeneration were markedly diminished in the combination group compared to both monotherapy groups, indicating enhanced tissue preservation.

The therapeutic potential of the leech saliva extract can be attributed to many compounds. Medicinal leeches have developed a uniquely specialized salivary system containing numerous bioactive molecules that modulate host immune responses and inhibit microbial degradation

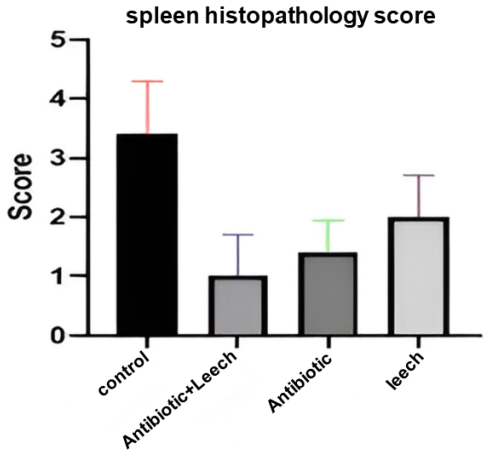

Figure 3. Histopathological Changes in Spleens
This figure depicts histopathological changes in the spleens of the treatment groups. Differences in liver injury levels were detected between the groups. The control group exhibited the most severe liver injury, with statistically significant differences ($P<0.05$).

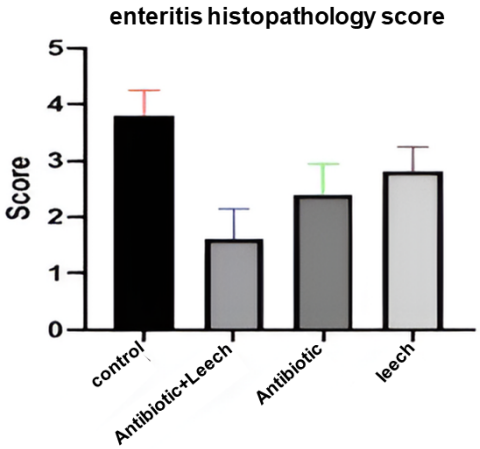

Figure 4. Histopathological Scores of Intestines
Figure 4 displays the histopathological scores of the intestine from each treatment group. The control group showed the greatest degree of tissue damage, whereas the combination treatment group exhibited the least degree of inflammation and structural alteration.

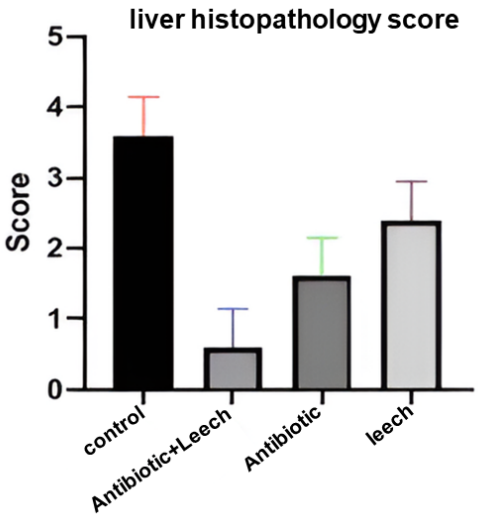

Figure 5. Histopathological Scores of Livers
Figure 5 illustrates histopathological scores of the livers from each group. The greatest degree of tissue damage was observed in the control group, whereas the gentamicin and combination groups showed the lowest degree of inflammation and structural alterations.

in ingested blood. Eglins, for example, are potent inhibitors of neutrophil elastase, cathepsin G, and other proteases that degrade tissue under inflammatory conditions (38). Similarly, bdellins and Eglin C, owing to their protease inhibitory activity, play a crucial role in tissue protection by inhibiting neutral proteinase activity. These proteins effectively block inflammation by limiting proteolytic degradation but can also block structural damage leading to tissue injury, suggesting their therapeutic efficacy in various inflammatory diseases, such as arthritis (14,39). Destabilase, a bioactive component present in leech salivary secretions, is a multifunctional enzyme that has been previously recognized for its antimicrobial activity. It exhibits both enzymatic and nonenzymatic antimicrobial activities, including a defensin-like α1-helical peptide motif. Destabilase hydrolyses the β-1,4 glycosidic bond of bacterial peptidoglycan, thereby exerting lysozyme-like activity and inducing lysis of bacterial cells. Although Destabilase maintains its antimicrobial effectiveness under heat stress, it does not hydrolyze glycosidic bonds. Instead, it retains its ability to inhibit bacterial growth with the defensin-like α1-helical peptide motif, regardless of hydrolytic activity (38). Moreover, Ghawi et al reported that Hirudinaria manillensis salivary gland secretions exhibited dose-dependent free radical scavenging activity, with an IC_{50} value comparable to that of ascorbic acid. The antioxidant activity was attributed to some proteomic components containing phenolic/aromatic residues, thiol groups, and hydrophobic amino acids capable of donating protons to scavenge reactive oxygen species (40).

Cooper and Mologne also reported that the therapeutic efficacy of leech saliva in wound healing is attributed to the combined action of a diverse mixture of over 100 distinct biologically active compounds, including hirudin, calin, and apyrase, which exert anticoagulant, anti-inflammatory, and antioxidant effects. This complex protein mixture regulates thrombin activity and inhibits platelet aggregation, thereby facilitating tissue preservation and enhancing microvascular function (41). Hyaluronidase, another enzyme present in leech saliva, plays an important role in wound healing by cleaving hyaluronan (38, 42).

We speculate that the decreased necrosis observed in the combination therapy group may be partly due to the vasoprotective benefits of leech saliva. Bioactive factors not only inhibit inflammatory mediators but also cause vasodilation and improve circulation, thereby improving the delivery of oxygen and nutrients to damaged tissue (43). The presence of histamine-like substances and acetylcholine in leech saliva, which can act as vasodilators and increase blood flow, further supports this hypothesis (44). Increased perfusion improves tissue recovery,

as evidenced by the histologic improvements. The proposed mechanisms underlying these effects include inhibition of platelet aggregation, changes in vascular permeability, decreased oxidative stress, and inhibition of proinflammatory cytokine release (45,46). The overall activity of leech saliva highlights its potential as a source of novel antimicrobial and immunomodulatory compounds.

There are currently no in vivo studies in chicken broilers to determine the efficacy of leech salivary secretion (alone or in combination with standard antimicrobials) against *S. enterica* serovar Typhimurium. This study addressed this gap for the first time by showing the synergy between medicinal leech (*Hirudo medicinalis*) saliva and gentamicin in a broiler infection model. Although gentamicin is still widely regarded as a first-line aminoglycoside for poultry therapy, its limitations, including nephrotoxicity, tissue residue accumulation, and resistance development, have been well-documented in previous studies. For example, Kamouh et al reported that 21% of chicken meat contained gentamicin residues, raising serious food safety concerns (47). Similarly, Onyeanu et al identified gentamicin residues in 65% of broilers sampled from poultry markets in Nigeria, with hepatic and renal tissues exhibiting the highest concentrations (48). These results underscore the need for the identification of adjunctive therapies that can enhance efficacy while reducing the dose of conventional antibiotics. While the synergy observed in this study is clear, further research is required to elucidate the pharmacokinetic drug interaction between leech saliva and gentamicin.

Previous research has demonstrated promising outcomes. Rassouli et al reported that leech saliva inhibited concentration- and exposure time-dependent inhibitory effects on the growth of *S. aureus* and *E. coli* (32). Another recent study also found that Hirudinaria manillensis leech saliva crude extract displayed strong inhibitory activity against *Klebsiella pneumoniae*, *E. coli*, and *S. enterica*, with antimicrobial activity influenced by the period of starvation of the leeches before extraction (49). These results suggest that the bioactivity of leech compounds is regulated by physiological and environmental conditions. Furthermore, Bilden et al confirmed the antioxidant and anti-inflammatory effects of Hirudo verbana saliva on human endothelial and ovarian epithelial cell lines, supporting its therapeutic potential for non-antimicrobial applications (34). Gerivani et al found that both microbial reduction and inflammation reduction increased when leech salivary extract was administered in conjunction with erythromycin, thereby confirming the adjuvant effects of leech saliva (50). Although these studies show promise, they have not been conducted in an in vivo poultry model, highlighting a translational gap that our study directly addresses. The results of our study illustrate the importance of considering nature-derived bioactive compounds, such as leech salivary extracts, in the control of antimicrobial resistance, especially in food-producing animals where antibiotic overuse remains a pressing concern (51).

Despite the contributions of our study, we acknowledge several limitations. First, our study focused on acute-phase effects, and we did not assess long-term effects such as resistance occurrence. Secondly, while histopathology revealed immune modulation via leech salivary compounds, we did not characterize cytokine signaling (31) or use comprehensive molecular approaches to assess immunosignaling cascades. Thirdly, the bioactive constituents of different leech species vary, especially when considering feeding conditions and extraction conditions, which can reduce reproducibility. Furthermore, we did not profile the toxicity and assess the side effects of leech saliva, precluding definitive safety conclusions. Additionally, we did not conduct dose–response studies, which would enable therapeutic window analysis. We also did not assess the effect of leech saliva on gut microbiota, a significant modulator of host immunity and pathogen defense. Lastly, inter-rater reliability measures, such as Cohen's kappa, were not employed due to single-pathologist scoring. Future studies should address these limitations and include standardized acute dose escalation studies using proteomics approaches to verify leech saliva preparations, dose escalations, microbiome analyses, and toxicity data for clinical translatability. Large cohort, long-term knockout studies are warranted in the future to determine the sustained therapeutic effect, development of resistance patterns following treatment, and safety profile. Both regulatory approval and field usage can inform optimum dosing ranges, and toxicity testing can be used to assess safety if the leech salivary extract is used repeatedly (52).

To further validate the translatable impacts of our findings, future studies should incorporate a controlled field trial approach under real-world farm conditions to estimate the efficacy and safety of leech salivary extract under varying environmental and management conditions. Additionally, single- and repeat-dose optimization studies should be conducted to establish therapeutic thresholds and minimize the risk of potential toxicity. In addition, comparative pharmacokinetic studies of different parenteral delivery routes might help determine bioavailability and inform practical delivery strategies. Long-term monitoring of treated populations is critical to detect any potential resistance patterns, especially in endemic areas where repeated exposure is likely to happen. Such directed research would provide a solid evidence base to seek regulatory approval and effective clinical application.

Conclusion

In conclusion, this study provides strong evidence for the therapeutic antimicrobial effect of leech (*Hirudo medicinalis*) saliva on broiler chickens infected with *S. enterica* serovar Typhimurium. Although leech saliva administered alone resulted in a moderate reduction in

bacterial load and improvement in tissue pathology, the combination of leech saliva and gentamicin yielded the best results. The results of the present study support previous findings that leech bioactive compounds possess anti-inflammatory, antimicrobial, and immunomodulatory properties. Overall, our results support the potential application of leech saliva to combat antimicrobial resistance in food animals while reducing the quantity of high-dose conventional antibiotics such as gentamicin. This study highlights the importance of advancing nature-based therapeutic approaches in addressing the growing concern of antimicrobial resistance.

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Competing Interests

The authors declare no conflict of interests related to this study.

Ethical Approval

All animal procedures were approved by the Institutional Animal Ethics Committee of the Faculty of Veterinary Medicine, Semnan University, and were conducted in full compliance with national and professional guidelines for animal care and welfare in Iran.

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