Synergistic Effect of Bovine Lactoferrin with Standard Triple Therapy on Helicobacter pylori Clearance and Virulence Gene Downregulation in a Murine Model

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***Abstract*—** Background: Helicobacter pylori is a widespread gastric pathogen associated with chronic gastritis, peptic ulcer disease, and gastric adenocarcinoma. The increasing antibiotic resistance and treatment failure with standard triple therapy necessitate the exploration of alternative or adjunctive therapeutic agents with immunomodulatory properties.the objective of this study aimed to evaluate the therapeutic efficacy of bovine lactoferrin (bLf) alone and in combination with standard triple therapy (TT) (Amoxicillin (AMX), Clarithromycin (CLR), and proton pump inhibitors PPIs) against H. pylori infection in a BALB/c mouse model, with assessment of bacterial clearance and suppression of virulence factor gene expression. The methods found sixty BALB/c mice were randomly divided into five groups (n=12/group). Groups 1-4 were infected intragastrically with H. pylori clinical isolate (1×10^9 CFU/mL), while Group 5 served as uninfected negative control. One-week post-infection, two-week daily treatment was administered as follows: Group 1 (positive control, infected untreated), Group 2 (triple therapy alone), Group 3 (bovine lactoferrin alone, 100 mg/kg), Group 4 (triple therapy plus bovine lactoferrin), and Group 5 (negative control, PBS only). Gastric tissue samples were collected for bacterial colony counting and quantitative real-time PCR analysis of cagA and vacA virulence gene expression. The results found a bacterial load was significantly reduced in all treatment groups compared to positive control ($P < 0.0001$). The greatest reduction was observed in Group 4 (triple therapy plus bLf; 5.457 ± 0.024 $\log_{10}$ CFU/mL), representing an 86.9% reduction compared to Group 1 (6.340 ± 0.015 $\log_{10}$ CFU/mL). Gene expression analysis revealed significant suppression of cagA and vacA in treated groups, with maximal suppression in Group 4 (83.5% reduction for cagA; 79.0% reduction for vacA; $P < 0.05$). In conclusion the study found combination therapy comprising standard triple therapy and bovine lactoferrin demonstrated superior antibacterial efficacy and virulence gene suppression compared to either treatment alone. These findings suggest that bovine lactoferrin may serve as an effective adjunctive agent in H. pylori eradication therapy, potentially through both direct antimicrobial and immunomodulatory mechanisms.

***Keywords* —** Lactoferrin (bLf) alone, *H. pylori* triple therapy; cagA; vacA; bacterial eradication; mouse model.

INTRODUCTION

Helicobacter pylori is a Gram-negative, microaerophilic bacterium that colonizes the gastric mucosa of approximately 50% of the global population, with higher prevalence in developing countries reaching up to 80-90% (1). This pathogen is recognized as a Class I carcinogen by the World Health Organization due to its strong association with the development of gastric adenocarcinoma, mucosa-associated lymphoid tissue (MALT) lymphoma, peptic ulcer disease, and chronic gastritis (2,3).

The pathogenesis of H. pylori infection is mediated by an array of virulence factors that facilitate bacterial colonization, immune evasion, and tissue damage. Among these, the cytotoxin-associated gene A (cagA) and vacuolating cytotoxin A (vacA) are the most extensively characterized and clinically significant (4,5). The cagA gene, located within the cag pathogenicity island, encodes a protein that is translocated into host gastric epithelial cells via a type IV secretion system (T4SS), where it disrupts cellular signaling pathways, leading to cytoskeletal rearrangements, pro-inflammatory responses, and increased carcinogenic potential (6,7). Similarly, vacA induces vacuolation in epithelial cells, modulates immune responses, and promotes bacterial persistence by interfering with T-cell proliferation and antigen presentation (8,9).

Standard first-line therapy for H. pylori eradication typically comprises a proton pump inhibitors (PPIs) combined with two antibiotics, most commonly amoxicillin and clarithromycin, administered for 14 days (5). However, global eradication rates with this triple therapy have declined significantly, falling below 80% in many regions, primarily due to the emergence of antibiotic resistance, particularly

clarithromycin resistance (10,11). Additional factors contributing to treatment failure include poor patient compliance, biofilm formation, and the ability of H. pylori to enter a dormant coccoid form that resists antibiotic action (12,13).

These challenges have prompted intensive research into alternative and adjunctive therapeutic agents. Lactoferrin, an 80-kDa iron-binding glycoprotein belonging to the transferrin family, has attracted considerable attention due to its multifunctional properties, including antimicrobial, immunomodulatory, and anti-inflammatory activities (14,15). Bovine lactoferrin (bLf), which shares high structural and functional homology with human lactoferrin, has demonstrated inhibitory effects against a wide range of pathogens, including H. pylori (16,17). The anti-H. pylori mechanisms of lactoferrin include iron sequestration, direct bacterial membrane disruption, inhibition of bacterial adhesion to gastric epithelial cells, and modulation of host immune responses (9,18).

Previous clinical and experimental studies have suggested that lactoferrin, when used as an adjunct to standard antibiotic therapy, may enhance H. pylori eradication rates. A meta-analysis by (19) reported that supplementation with lactoferrin significantly improved eradication rates compared to triple therapy alone. Similarly, experimental studies have demonstrated that lactoferrin can suppress the expression of H. pylori virulence factors and interfere with the function of the cag type IV secretion system (20). Despite these promising findings, the precise molecular mechanisms underlying the synergistic effect of lactoferrin with conventional antibiotics, particularly its impact on virulence gene expression, remain incompletely understood.

The present study was therefore designed to evaluate the therapeutic efficacy of bovine lactoferrin as an adjunct to standard triple therapy in a well-established BALB/c mouse model of H. pylori infection. Specifically, we aimed to: assess bacterial clearance through quantitative colony counting; investigate the expression of key virulence genes (cagA and vacA) using quantitative real-time PCR; and determine whether combination therapy exerts synergistic effects superior to either treatment alone. Elucidation of these mechanisms may provide a scientific basis for incorporating lactoferrin into future H. pylori eradication protocols, potentially improving treatment outcomes in the face of rising antibiotic resistance.

MATERIALS AND MTHODS

Ethical approved:

The animal study protocol was approved by the Ethics Committee for Animal Research at the College of Veterinary Medicine, University of Baghdad with Protocol Approval Number [14-G. 5/1/2026]. All procedures involving animals were conducted in strict accordance with the committee's ethical guidelines for the care and use of laboratory animals.

Bacterial strains and inoculum preparation:

The H. pylori isolate used in this study was a clinical isolate obtained from a human gastric biopsy. For mouse infection, the bacteria were first cultured on Columbia blood agar plates under microaerophilic conditions at 37°C for 72 hours. Bacterial cells were then harvested and sub-cultured in Brucella broth supplemented with 10% fetal bovine serum for 24–48 hours under microaerophilic conditions with shaking. The bacterial cells were collected by centrifugation, washed twice with sterile phosphate-buffered saline (PBS), and finally re-suspended in PBS. The bacterial density was standardized by adjusting the suspension to an optical density equivalent to a McFarland standard No. 4, which corresponded to a concentration of approximately 1×10^9 CFU/mL as confirmed by serial dilution and plating. This prepared inoculum was used for intra-gastric infection within 30 minutes.

Animal preparation:

Sixty healthy BALB/c mice, aged from 5-7 weeks old male, with an average weight 20-30 grams, were employed in the study. They were purchased from Iraqi Center for Cancer Research and Medical Genetics/ University of Al-Mustansirya. The animals were housed in standards cages in the animal house in the College of Veterinary Medicine/ University of Baghdad, dimensions of the cages around 36.5cm (L) x20.5cm (W) x14.5 cm (H), each contained 12 mice to prevent overcrowding. They had been housed under an air conditioned room (25 ± 2°C) and exposed to 12 hour light/12 hours dark light cycle. They were fed ad libitum on commercially standard available murine pellets and water during the period of experiment

Study design:

Sixty mice were divided randomly into five groups (n=12/group). With the exception of group 5, which served as a negative control and received only Phosphate Buffered Saline (PBS) thought the study period, the other four groups were infected intragastrically with a dose of 1x109 CFU/ml. One week post-infection, daily treatments by oral gavage for two weeks was performed as follows: Group 1(G1), served as positive control group (infected, untreated); Group 2 (G2) treated with triple therapy (TT) alone; Group 3 (G3) received bovine lactoferrin (bLf) alone; Group 4 (G4) treated with combined therapy (TT plus bLf). The samples were collected at one endpoint; at two week post treatment, twelve animals per group were anaesthetized, and blood was collected via cardiac puncture for Enzyme Linked Immunosorbant Assay (ELISA) analysis; blood was collected for ELISA test, and gastric biopsies were excised for bacterial counting and gene expression analysis of bacterial virulence factors genes (CagA and VacA).

Preparation of stock solution and doses of bLf.

The solution of the bLF was prepared by weighing (300 mg) and complete to (30) ml with distilled water and the final dose calculated was 0.1 ml /10gm of mice body weight which was equal to the dose 100 mg/kg (Sun et al., 2016)

Preparation of stock solution and doses of (CLR, AMX, and PPI)

The solutions were prepared by weighing (1gm) of AMX, (500 mg) of CLR and (30 mg) of Lansoprazole and complete to (333.3 ml), (166.7 ml) and (10 ml) with distilled water respectively, and the final dose calculated was 0.1 ml /10gm of

mice body weight (for each drug) which was equal to the doses (30 mg/kg) of Amoxicillin, Clarithromycin and Lansoprazole (Watanabe et al., 2004)

Colonies counting and calculation.

After incubation, colony forming units (CFU) were used to count colonies that were growing both on the surface and inside the agar. The formula below was used to calculate the bacterial load.

CFU/g = (Average of colonies counted x dilution factor x volume of aliquot)/ weight of tissue).

Primers designing for qPCR technique.

The specific primers targeting the genes used for PCR technique were designed baesd on Prim3Plus program. These primers were supplied from Favarogen Biotech (Taiwan) in lyophilized form. The sequences of these primers were listed in Table 1.

Primer		Primer sequence 5'- - 3'	Size of Product (bp)
16S rRNA	F	CTG GAG AGA CTA AGC CCT CC	110
	R	ATT ACT GAC GCT GAT TGT GC	
cagA	F	AAT ACA CCA ACG CCT CCA	400
	R	TTG TTG CCG CTT TTG CTC TC	
vacA	F	ATGGAAATACAACAAACACAC	286
	R	CTGCTTGAATGCGCCAAAC	

Determination of gene expression of CagA and VacA

The expression of important virulence factor genes (CagA and VacA) was examined in order to accurately assess the effect of therapeutic effect on H. pylori pathogenicity at the molecular level. Total RNA extraction was performed on gastric tissue samples that had been maintained in TRIzol reagent to guarantee RNA integrity. Quantitative real-time PCR (qPCR) was carried out after cDNA synthesis. As an internal housekeeping control to account for differences in bacterial load and RNA efficiency between samples, the relative expression of each target gene was normalized to the constitutively expressed bacterial 16S rRNA gene. This method made it possible to precisely compare the virulence gene expression profiles of each experimental group.

Statistical Analysis

Statistical analysis was performed using SPSS software (version 25.0; IBM Corp.). Bacterial colonization counts were compared between groups using Anova test , For the analysis of gene expression data derived from quantitative real-time PCR, the relative expression levels were calculated using the 2–ΔΔCt (Livak) method. A p-value of less than 0.05 was considered statistically significant.

RESULT AND DISCUSSION

Macroscopical examination of H. pylori.

An incubation period (3-7 days) of Columbia agar media under microaerophilic conditions (5% O2, 10% CO2, and 85% N2) showed nonheamolytic small (pinpoint) whitish translucent circular or oval with convex surface colonies of H.pylori (HP1-HP6) as shown in Figure 1.

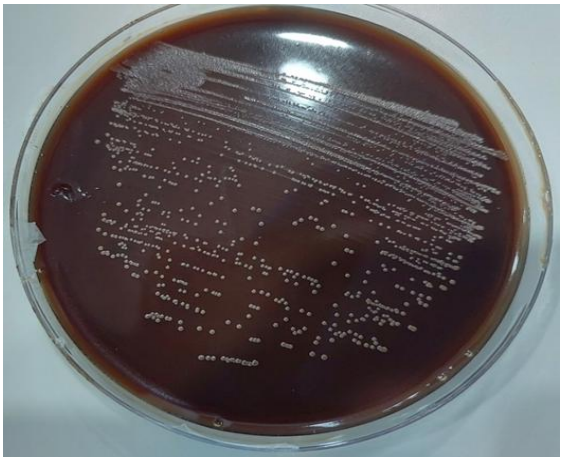

Figure1. Successful isolation of H.pylori on selective media (Columbia agar media).

Analysis of bacterial counting.

The quantitative evaluation of bacterial load through the five experimental groups is showed below. Results are built on the enumeration of colony-forming units (CFU) acquired from gastric samples cultured on Columbia-based selective media shown in figure 2. Statistical comparisons of the bacterial counting detailed in Table 2, shown significant differences between the treatment regimes. These differences are concise in figure 3, which shows the comparative bacterial growth outlines among the groups.

Table 2. Mean (± SEM, n=6) of Log_{10}-Transformed Bacterial Counts (CFU/mL) Across Experimental Groups Following Treatment

Groups	Mean log CFU/mL ± SEM
G1	6.340±0.015 a
G2	6.075±0.012 b
G3	5.788±0.015 c
G4	5.457±0.024 d
G5	0.000±0.000 e
LSD	0.0443
P- value	<0.0001

Different letters indicate significant differences between groups at *P*< 0.05.

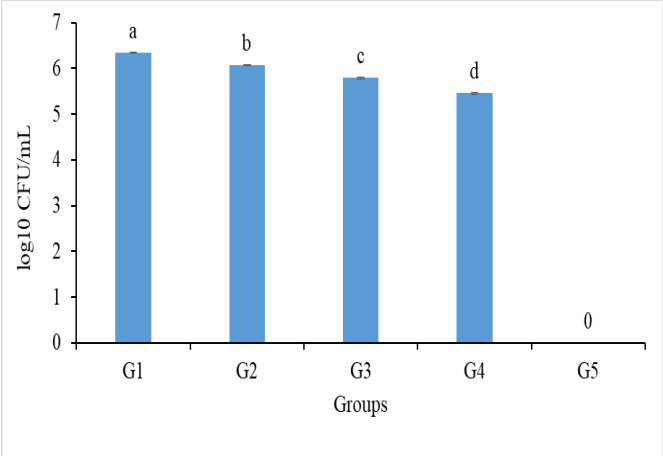

Figure 2. Comparison of bacterial colonization across study groups.

Analysis of the results revealed a statistically significant difference in bacterial counts (log_{10} CFU/mL) among the experimental groups ($P < 0.001$).The highest mean bacterial load was recorded for G1 (6.340±0.015), which indicate the efficacy of infection without treatment. While G2 and G3, showed marked reduction in bacterial load (6.01±0.011 and 5.788±0.015) respectively, G4 showed the highest and greatest reduction in bacterial colonization (5.457±0.024), indicating the immunomodulatory and synergistic effect of bLf.

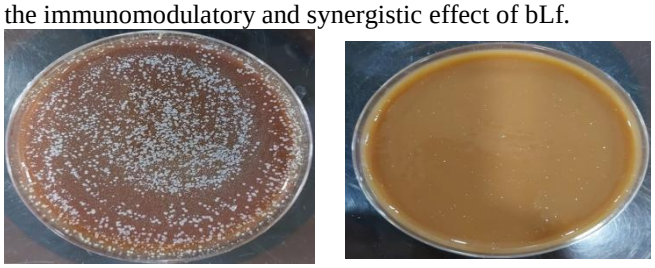

Figure 3. Comparative bacterial growth on Columbia Agar across groups of the study

Concerning the highest bacterial load in g1, which was inoculated with an infective dose and remained untreated with tt regimens, and/or any immunostimulant compounds, this gastric pathogen continued to colonize and replicate freely, establishing infection and biofilm formation. this strategy helps bacterial persistence and evasion of host immune defense, ultimately leading to severe tissue damage (8). the immunological basis for the elevated bacterial load is in accordance with findings reported by (21), who stated that the bacterial cell acts to up-regulate the expression of a protein known as programmed cell death protein 1 (pd-1) on the surface of various immune cells, particularly t lymphocytes. the interaction of pd-1 with its ligand (pd-l1) expressed on surface of h.pylori infected gastric epithelium, inhibitory signals are transmitted to t cells, resulting in immunosuppression of t cell proliferation and reduced production of ifn-γ and il-12.regarding resistance to amx, the outcomes of the study are corresponded with the results reported by (22), who clarified the bacterial ability for overexpression of a protein called an efflux pump. additionally, the resistant to amx is due to mutational changes in penicillin-binding proteins (pbps) (7). furthermore, the outcomes of the study were supported by findings conducted by (10, 23), who was emphasized the significant role of biofilm formation and bacterial switch into dormant (coccoid) form in impairing the penetration of the drug into bacterial cells analysis of gene expression of caga and vaca genes the study showed statistically significant differences among the groups of the study ($p < 0.0001$). the highest level of expression in g1 (1.18 ± 0.181), significantly decreased with other treated groups, the decrease in expression reached 54.7% in g2 (0.534 ± 0.101); 65.5%in g3 (0.407 ± 0.068); and reached 83.5% in g4 (0.195 ± 0.039), indicating the adjunct and synergistic role of blf combined with conventional tt therapy, while g5 (uninfected) showed no caga gene expression. the high expression of caga in (g1) is in accordance with its well-known role as a key virulence factor of h. pylori, associated with establishment and progression of gastric inflammation and cancer (8). also this outcome is in agreement with the findings documented by (11), who revealed that the expression of caga gene in h.pylori isolated from gastric tissue of patients suffered from gastric cancer and peptic ulcer disease are markedly higher than in non-ulcer dyspepsia (nud) individuals (control subjects). additional research by (23) revealed that caga gene plays an important role in h. pylori infection and the interaction between the caga and vaca proteins is a major factor in the development of gastric cancer and infection. in g2, caga expression was significantly reduced after treatment with standard triple therapy, but without complete recovery, the finding which in consistence with the outcomes documented by (24), who stated that the tt therapy was less than the complete eradication threshold, thus the incomplete eradication refers to partial expression to caga protein. treatment of infection with blf alone in g3 also showed reduction in caga expression, the result aligned with (25), who refers to the inhibitory action of blf on cag type iv secretion system (cag t4ss) which has a crucial role in translocation of caga factor into the host cell. on the other hand, analysis of qpcr of vaca gene expression (figure 5) revealed statistically significant differences across the study groups ($p = 0.0168$). the highest expression occurred in g1 (1.49 ± 0.490) compared to g4 (0.313 ± 0.081) and g5 (0.00±0.00), confirming the active production of this cytotoxic virulence factor in g1 and significant suppression in g4. however, the expression was progressively decreased among treated groups; g2: 0.910 ± 0.354 with 38.9% reduction, g3: 0.710 ± 0.261 revealed 52.3% reduction, and g4: 0.313 ± 0.081 with greatest reduction and suppression 79.0% in expression compared with g1. expression of the gene in g5 not detectable table 3. the elevated expression of vaca gene in g1 is agreement with findings reported by (4), who stated that virulence factor vaca acts synergistically with others to establish and develop gastric infections and dyspepsia. these findings also aligned with statements reported by (25), who demonstrated that vaca is an intracellular-acting protein and targets host's mitochondrial inner membrane, thus aiding in colonization and establishment of gastric infection and tissue damage

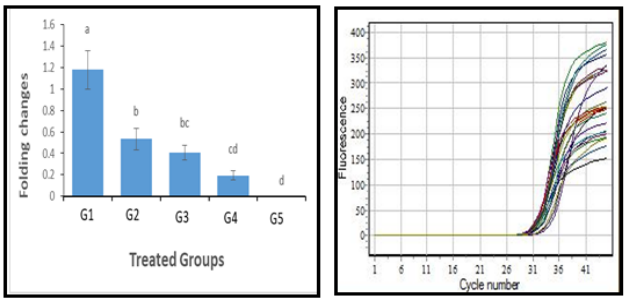

Figure 4. The qPCR reveals the expression of CagA gene in all groups, A-Statistical differences B- Amplification plot of qPCR

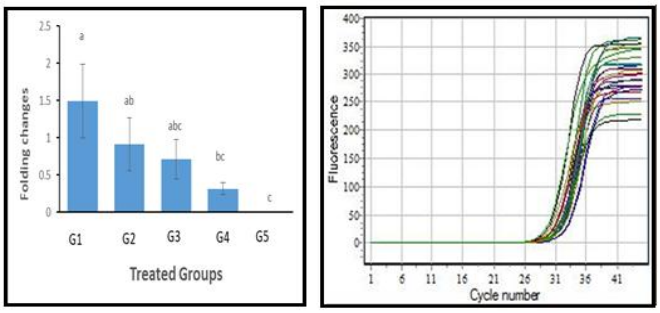

Figure 5. The qPCR reveals the expression of CagA gene in all groups, A-Statistical differences B- Amplification plot of qPCR

Table 3. Relative gene expression with reduction status and Interpretation

Group	Relative Expression (Mean ± SD)	% Reduction vs G1	Interpretation
G1	1.49 ± 0.490	—	Highest expression
G2	0.910 ± 0.354	38.9%	Moderate reduction
G3	0.710 ± 0.261	52.3%	Marked reduction
G4	0.313 ± 0.081	79.0%	Greatest suppression
G5	0.00 ± 0.00	100%	Not detectable

CONCLUSION

Combined therapy (TT plus bLF) demonstrates highly significant bacterial clearance rates comparing to monotherapy of TT and bLF, Molecular detection (RT-qPCR) demonstrates highly significant reduction in bacterial virulence genes in G4 comparing to those related to other treated groups.

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Conflict of Interest

The authors declare no conflict of interest.

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