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Lactic acid bacteria target NF-κB signaling to alleviate gastric inflammation

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Helicobacter pylori (*H. pylori*) infection and the resulting gastric inflammation are major contributors to gastric cancer development. Probiotics, particularly *Lactobacillus*, are promising for their anti-inflammatory potential, yet their exact mechanisms in inhibiting *H. pylori*-induced inflammation are unclear. In our previous study, *Lactiplantibacillus plantarum* ZJ316 (*L. plantarum* ZJ316) demonstrated strong anti-inflammatory effects against *H. pylori* infection *in vivo*, but its precise mechanisms were not fully understood. Here, we aimed to investigate how *L. plantarum* ZJ316 inhibits the inflammatory response to *H. pylori* infection. Our results demonstrated that *L. plantarum* ZJ316 effectively reduced the expression of pro-inflammatory cytokines in *H. pylori*-infected AGS cells. Mechanistically, *L. plantarum* ZJ316 inhibited the NF-κB signaling pathway by preventing the degradation of IκBα, suppressing p65 phosphorylation, and blocking the nuclear translocation of phosphorylated p65. Treatment with the NF-κB inhibitor BAY 11-7082 further decreased tumor necrosis factor-α (TNF-α), interleukin-8 (IL-8), and interleukin-1β (IL-1β) levels, confirming the inhibitory effect of *L. plantarum* ZJ316 on the NF-κB pathway. In *H. pylori*-infected mice, oral administration of *L. plantarum* ZJ316 significantly alleviated inflammatory cell infiltration, reduced TNF-α and pepsinogen II (PGII) levels, and increased interleukin-10 (IL-10) levels in serum. A comparative metagenomic analysis of the gastric microbiota revealed a decrease in *Prevotella* and *Desulfovibrio*, alongside an increase in *Ligilactobacillus* and *Akkermansia*, supporting the protective effects of *L. plantarum* ZJ316 and correlating with their decreased inflammatory response. In summary, administration of *L. plantarum* ZJ316 demonstrated robust anti-inflammatory effects against *H. pylori* infection by suppressing NF-κB signaling and promoting favorable changes in the gastric microbiota composition. Therefore, *L. plantarum* ZJ316 holds promise as a novel functional food for protecting the body against *H. pylori* infection.

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1. Introduction

Helicobacter pylori (*H. pylori*) is the most significant risk factor for gastric cancer, which ranks as the third leading cause of cancer-related deaths globally.[1] While only 2% of individuals harboring *H. pylori* eventually develop malignancies, the bacterium is also implicated in other gastric conditions such as ulcers and gastritis.[2] The etiology of *H. pylori* involves several factors, including genetic predispositions, the host environment, and specific bacterial virulence factors.[3] Moreover, elevated levels of inflammatory responses among long-standing *H. pylori* infestation are particularly critical, playing a substantial role in the development and advancement of gastric cancer.[2]

H. pylori colonization and disease establishment involve several key steps that enable the bacterium to thrive in the acidic stomach environment and induce gastric damage.[4] Initially, *H. pylori* adapts to the stomach's acidity by producing urease, which neutralizes gastric acid, allowing survival in that harsh environment.[5] The bacterium then uses its flagella to navigate through the mucus layer and adhere to gastric epithelial cells[6] *via* adhesins such as blood group antigen-binding adhesin (BabA) and sialic acid-binding adhesin (SabA).[7,8] Once attached, *H. pylori* secretes virulence factors like cytotoxin-associated gene A (CagA), which disrupts cellular signaling, weakens tight junctions between epithelial cells, and increases mucosal barrier permeability.[9,10] This triggers a chronic

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inflammatory response, with the release of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6,[11,12] promoting cellular stress, oxidative damage, and DNA mutations in gastric epithelial cells.[13,14] Additionally, *H. pylori* alters host signaling pathways, including NF-κB, MAPK, and JAK-STAT, thereby sustaining inflammation.[15–17] Over time, this persistent inflammation leads to epithelial cell damage, metaplasia, and potential progression to gastritis and even gastric cancer.[7,10]

The persistent inflammation induced by *H. pylori* infection causes ongoing tissue damage, genetic mutations, and carcinogenesis, making it a major risk factor for gastric diseases.[18] NF-κB and STAT3 pathways are prominently activated during carcinogenesis.[19] NF-κB, a ubiquitous inflammatory pathway in animal cells, exhibits constitutive activation in gastric cancer,[20,21] particularly mediated by CagA following *H. pylori* infestation. NF-κB activation induces the secretion of diverse inflammatory cytokines, which are implicated in processes including angiogenesis and the inhibition of apoptosis.[22] Notably, NF-κB activation has been recognized as crucial in linking *H. pylori*-induced inflammation with cancer development. Yang *et al.* demonstrated that *H. pylori* triggers miR-223-3p upregulation *via* CagA-dependent mechanisms, with NF-κB directly stimulating gastric cancer cell proliferation by binding to the miR-223-3p promoter.[23] Moreover, the lipopolysaccharide of this bacterium binds to the toll-like receptor on the surface of gastric epithelial cells,[24] triggering subsequent inflammatory signaling pathways, including NF-κB.[25,26] Inhibiting NF-κB activation could mitigate inflammation and potentially reduce cancer risk. Emerging evidence highlights the crucial importance of the gastric microbiota in regulating the development of *H. pylori*-related chronic diseases.[27] Previous research has shown that in comparison with individuals without *H. pylori*, individuals who have been diagnosed with an *H. pylori* infection exhibit an enrichment of specific gastric bacterial genera, including *Streptococcus*, *Neisseria*, *Prevotella*, *Rothia*, *Fusobacterium*, *Veillonella*, and *Haemophilus*, which contribute to inflammatory processes.[28–30]

Early elimination of *H. pylori* infestation can potentially regress associated diseases.[31–33] However, the effectiveness of eradication regimens has been considerably undermined globally by increasing antimicrobial resistance and uncertain impact on the gut microbiota, hindering widespread screening and eradication efforts.[34–36] Moreover, current treatments for *H. pylori* eradication are beset by numerous drawbacks: high costs, complex protocols, frequent side effects, and low patient adherence due to complex drug regimens.[37] Therefore, exploring alternative or antibiotic-free strategies is crucial to mitigate *H. pylori*-induced gastric diseases.

Over the past few years, the European *Helicobacter* and Microbiota Study Group has proposed updated *H. pylori* treatment guidelines that recommend specific probiotics as adjunct therapies, particularly for patients who experience significant antibiotic intolerance.[38,39] Clinical research has supported the efficacy of probiotic administration, primarily using *Lactobacillus* strains, in alleviating disease severity and reducing antibiotic side effects.[40–43] *Lactobacillus*, widely acknowledged for its health benefits, positively impacts human health by modulating the gut microbiota, enhancing beneficial metabolite production, and regulating immune responses.[44] In recent years, a large number of research studies have underscored its protective role against *H. pylori*-induced gastric diseases through inhibiting bacterial growth, enhancing the gastrointestinal microbiome community, and inducing apoptosis in gastric cancer cells.[45,46] Notably, Urrutia-Baca *et al.* reported that reuterin, a bacteriocin derived from *Limosilactobacillus reuteri*, effectively suppresses the transcription of the VacA and FlaA genes of *H. pylori*.[47] Several *Lactobacillus* species, including *Lacticaseibacillus rhamnosus*, *Lactobacillus acidophilus*, and *Levilactobacillus brevis*, demonstrate the ability to attach to gastric epithelial cells *in vitro* and mitigate *H. pylori*-triggered inflammation in animal models.[48–50] Moreover, certain probiotic LAB strains alleviate *H. pylori*-induced inflammation by suppressing IL-8 secretion, potentially through the inactivation of Smad7 and NF-κB pathways.[51,52] While probiotics, particularly *Lactobacillus* species, have shown promise in mitigating inflammation caused by *H. pylori* infection, the precise molecular mechanisms behind their anti-inflammatory effects remain unclear. Current research often emphasizes the anti-inflammatory effects of probiotics, but lacks an in-depth analysis of how these effects are linked to the changes in the gut microbiota. Additionally, while much of the existing research on *H. pylori* focuses on its direct impact on the gastric epithelium, there is a lack of comprehensive studies exploring how probiotics modulate the host's immune response to *H. pylori*.[7]

Our previous investigations have shown that *L. plantarum* ZJ316 successfully mitigates *H. pylori*-induced inflammatory responses in murine models.[53,54] However, the specific mechanisms have remained elusive. In this study, we delve into the anti-inflammatory properties of *L. plantarum* ZJ316 both *in vitro* and *in vivo* during *H. pylori* infection. Compelling evidence showed that *L. plantarum* ZJ316 substantially suppresses *H. pylori*-induced NF-κB signaling activation, confirmed by inhibiting the NF-κB pathway with BAY 11-7082. Moreover, leveraging multi-omics analysis revealed that supplementation with *L. plantarum* ZJ316 enhances the abundance of *Ligilactobacillus* and *Akkermansia*, a widely recognized probiotic group known for its potential to alleviate inflammation. Overall, this study sets the stage for further exploration of novel probiotic functional foods, offering valuable insights for future research on effective strategies for preventing and managing *H. pylori*-associated inflammatory conditions.

2. Materials and methods

2.1. Bacterial strains and cell culture

Helicobacter pylori (CCTCC M20211218), a gift from Yongliang Zhu (Second Affiliated Hospital of Zhejiang University School of Medicine), was isolated from *H. pylori* positive gastritis

patients. *H. pylori* was cultured on CBA supplemented with 7% v/v sheep blood and antibiotics (Hopebio, Qingdao, China) at 37 °C under microaerobic conditions.[53] *Lactiplantibacillus plantarum* ZJ316 (CCTCC M208077), originally selected from the laboratory, was cultivated in MRS medium at 37 °C for 36–48 hours.

The AGS cells (human gastric adenocarcinoma epithelial cells) were acquired from the National Collection of Authenticated Cell Cultures (cat. no. TCHu232). AGS cells were propagated in Ham's F-12K medium fortified containing 10% fetal bovine serum (FBS, Procell, China) at 37 °C under 5% CO_2.[53] Then, the bacteria mentioned above were centrifuged, diluted in a fresh F-12K medium, and poured into AGS cells at a multiplicity of infection (MOI) of 100 : 1 for 2, 6 or 12 hours for the subsequent experimental assays. Cells were exposed to the NF-κB inhibitor BAY 11-7082 (MCE) at a concentration of 10 μM.

2.2. RNA extraction, reverse transcription and RT-qPCR

Total RNA extraction from cells and tissue specimens was carried out using the TRIzol reagent (Sigma-Aldrich). Subsequently, the isolated RNA was converted into cDNA using the Severn Biotech synthesis kit. RT-qPCR analysis was conducted by using the TB Green promoter RTQ-960 PCR system using the SYBR Green kit (Severn Biotech), following the manufacturer's protocols. The expression patterns and C_t values were quantitatively evaluated *via* real-time PCR, and the gene expression changes were assessed using the $2^{-\Delta\Delta Ct}$ method, normalized to the internal controls, with the control group set as 1 to facilitate relative comparisons between the groups. This standard approach was applied consistently across all the groups to account for inter-sample variations. All experiments were conducted in triplicate for robust statistical validation. The primer sequences used are detailed in Table 1.

Table 1 Sequences of the primers employed for RT-qPCR

Genes	Primers (5′–3′)
h GAPDH-F	GGCGACCTGCTGGAACATTACTG
h GAPDH-R	CATCGTTTAGGGCGTGGACTACC
h IL-6-F	AGACAGCCACTCACCTCTTCAG
h IL-6-R	TTCTGCCAGTGCCTCTTTGCTG
h IL-1β-F	CGGTTGCGTGGAAGACACTATGG
h IL-1β-R	GTGGAAGCGGGTAGGATGTTGTG
h TNF-α-F	TGAAAACCTCCAAGCAGCCCAAC
h TNF-α-R	GGCGTGTTTGATGTTGAGTCTGTTG
h IFN-γ-F	GAGTGTGGAGACCATCAAGGAAG
h IFN-γ-R	TGCTTTGCGTTGGACATTCAAGTC
h MAP3K14-F	CCACCTTTTCAGAACGCATTTTC
h MAP3K14-R	GTAGCATGGGCCACATTGTTG
h IκBα-F	TCCACTCCATCCTGAAGGCTAC
h IκBα-R	CAAGGACACCAAAAGCTCCACG
h IL-8-F	CGGATGATGTGATGGATGGTGTGG
h IL-8-R	GGGTATGCACGGTTACGAGTTTGG
m GAPDH-F	GGAGCGAGATCCCTCCAAAA
m GAPDH-R	GGCTGTTGTCATACTTCTCATGG
m IκBα-F	CACTTGGTGACTTTGGGTGCT
m IκBα-R	GCTGTATCCGGGTACTTGGG

2.3. RNA-seq analysis of AGS cells

AGS cells underwent total RNA isolation with the TRIzol reagent (Sigma-Aldrich) as per the manufacturer's guidelines, followed by genomic DNA removal achieved using DNase I (Takara).[55] Subsequently, the cDNA library was prepared following the protocol of the TruSeq™ RNA sample preparation kit,[55] and library preparations were carried out by Nuohe Zhiyuan Biotechnology Co., Ltd. Differential expression analysis between the samples was conducted with the transcripts per million reads method. Genes were classified as differentially expressed if they exhibited a p-value <0.05 and a log2 fold-change ≥1, determined using DESeq2. Enrichment analyses for functional annotation, encompassing GO and KEGG pathway analyses, were conducted with the results reported at a false discovery rate (FDR) <0.05.

2.4. Dual luciferase reporting system and western blot analysis

AGS cells, cultured in 24-well plates, were co-transfected with NF-κB luciferase plasmids and pRL-TK for 24 h. After a 4-hour exposure to *H. pylori*, the cells were harvested, and Renilla and firefly luciferase activities were measured using a dual luciferase reporter assay system. The Renilla luciferase activity was then normalized to the firefly luciferase activity. Cellular proteins were isolated using a RIPA lysis buffer, and their concentrations were assessed by using a BCA reagent kit (Solarbio). Proteins were then resolved by SDS-PAGE and transferred to PVDF membranes, blocked, and subsequently exposed overnight at 4 °C to primary antibodies targeting NF-κB p65 (8242S, 1 : 1000), phosphorylated NF-κB p65 (3033S, 1 : 1000), IκBα (4814S, 1 : 1200), and α-tubulin (2144S, 1 : 1000) (Cell Signaling Technology). After TBST washes, the membranes were incubated with HRP-conjugated secondary antibodies and visualized by the enhanced chemiluminescence method (Omni-ECL).

2.5. Animal experiments

Six to eight-week-old SPF C57BL/6J mice were kept in a 12-hour light/dark cycle and provided *ad libitum* access to food and water. Room conditions were regulated at temperatures ranging from 18 to 26 °C and relative humidity between 40% and 70%. All animal procedures were conducted in compliance with the guidelines approved by the Animal Ethics Committee of Shanghai Public Health Clinical Center (approval number: 2022-A037-01). Following a one-week acclimation period, mice were assigned to four experimental groups in a randomized manner (n = 8 per group).

Control group. Mice were given oral gavage of sterile normal saline every second day for a duration of 5 weeks.

Hp group. Mice were orally dosed with sterile normal saline for a duration of 3 weeks, followed by the oral administration of *H. pylori* (400 μL, 1.0×10^9 CFU per mL) every second day for a duration of 2 weeks.

Hp + ZJ316 group. Mice were orally dosed with *L. plantarum* ZJ316 (400 μL, 1.0×10^9 CFU per mL) every second day for a

duration of 3 weeks, followed by the oral administration of *H. pylori* (400 μL, 1.0×10^9 CFU per mL) every second day for a duration of 2 weeks.

Hp + ZJ316 + BAY group. Based on the Hp + ZJ316 group, from the fourth week, the mice received intraperitoneal injections of NF-κB inhibitor solution (200 μL) every second day, with a standardized dose of 5 μg g^{-1} (Fig. 5A).

2.6. ELISA, hematoxylin–eosin staining and histological immunofluorescence

Serum samples were obtained and subjected to IL-10, TNF-α, and PGII levels using ELISA kits (EK-Bioscience), following the manufacturer's guidelines.

The gastric tissues were preserved in 4% paraformaldehyde, followed by embedding in paraffin, slicing into 4 mm slices,[56] and staining with hematoxylin and eosin (H&E). The immunostaining of gastric tissue was conducted following the established protocols as previously described.[57] After deparaffinization and rehydration, antigen retrieval was executed, and subsequently, blocking was carried out. Slides underwent overnight incubation at 4 °C with primary antibodies, followed by exposure to secondary antibodies and a subsequent 1-hour incubation in darkness. Finally, the samples were examined and photographed using a Zeiss LSM710 confocal microscope. Histological scoring was performed based on the following criteria: scoring was performed for the mucosal layer structure, adenoids, columnar epithelial cells, and inflammatory cell infiltration, divided into four levels, each with a score of 25, to reflect the severity of the injury. A score of 0–25 indicates no tissue damage, 26–50 indicates mild injury, 51–75 indicates moderate injury, and 76–100 indicates severe injury. The evaluation includes mucosal clarity, adenoid atrophy and necrosis, epithelial cell integrity, and inflammatory cell infiltration. The protein levels were quantified by IHC using ImageJ software (National Institutes of Health, Bethesda, USA) and the integrated optical density was analyzed using the color deconvolution method.[58]

2.7. Metagenomic sequencing

Gastric tissue samples from various groups were collected after experiments. DNA isolation was performed using the fast DNA spin kit following the manufacturer's protocol. Beijing Biomarker Biotechnology Co. Ltd (Beijing, China) conducted the library preparation and paired-end sequencing. Gastric microbiota analysis was carried out using BMK Cloud (**https://www.biocloud.net**).

2.8. Statistical analysis

Values are presented as the mean ± standard deviation (M ± SD) from three independent experiments. One-way analysis of variance (ANOVA) was used to compare the differences among the groups, followed by a *post hoc* analysis with the least significant difference (LSD) test. A *p*-value of less than 0.05 was considered statistically significant. Pearson correlation coefficients were employed to assess the relationships between the variables. All statistical analyses were performed using GraphPad Prism software (version 9.0; GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. *L. plantarum* ZJ316 reduces the inflammatory response in AGS following *H. pylori* infection

Persistent *H. pylori* infestation triggers an inflammatory response, contributing to mucosal damage. We established an infection model to investigate how *L. plantarum* ZJ316 modulates the *H. pylori*-triggered inflammatory response in AGS cells. In the context of *H. pylori* infection, we noted a marked elevation in the expression levels of inflammatory factors, including TNF-α, IL-1β, IFN-γ, and IL-6 at 2, 6, and 12 h. Conversely, treatment with *L. plantarum* ZJ316 resulted in notable reductions in the pro-inflammatory cytokine levels compared to the untreated cells. Specifically, TNF-α levels decreased by 49.12%, 75.54%, and 52.48%, IL-1β by 70.04%, 38.35%, and 85.43%, IFN-γ by 78.99%, 21.57%, and 51.55%, and IL-6 by 77.95%, 60.94%, and 36.00%, respectively (Fig. 1A–D). These findings highlight the promising prospects of *L. plantarum* ZJ316 to mitigate cytokine production in AGS cells in the presence of *H. pylori* infection.

3.2. Treatment with *L. plantarum* ZJ316 effectively reversed the alterations in AGS cell transcriptional profiles caused by *H. pylori* infection

To examine the effects of *L. plantarum* ZJ316 on the primary inflammatory signaling pathway following *H. pylori* infection at the transcriptome level, we conducted a high-throughput RNA-seq analysis on AGS cells from three experimental groups, including control, Hp, and Hp + ZJ316. To ensure robustness in our cluster analysis, genes were selected based on the highest coefficient of variation across the entire dataset. Using this criterion, we identified a set of 397 identified differentially expressed genes (DEGs) that exhibited changes consistently across all experimental conditions, as visualized in the Venn plot (Fig. 2A). The volcano plot analysis highlighted the significant differences in the gene expression patterns among the experimental groups. Between the Hp group and the control group, a total of 4328 genes showed differential expressions, with 2404 genes being upregulated and 1924 genes downregulated (Fig. 2B). To elucidate the functions of the identified DEGs, we conducted GO analysis, categorizing DEGs into three primary functional groups: molecular function (MF), biological process (BP), and cellular component (CC) (Fig. 2C). In terms of biological processes, the DEGs were notably enriched in processes related to cell death, protein phosphorylation, phosphorylation, and intracellular signal transduction. In molecular functions, the enriched categories included protein kinase activity, transcription regulator activity and kinase activity (Fig. 2C). In our analysis of KEGG pathways, we observed a significant enrichment of DEGs in the top 15 pathways, notably including the IL-17 signaling pathway, NF-kappa B signaling pathway, and epithelial cell signaling in *H. pylori*

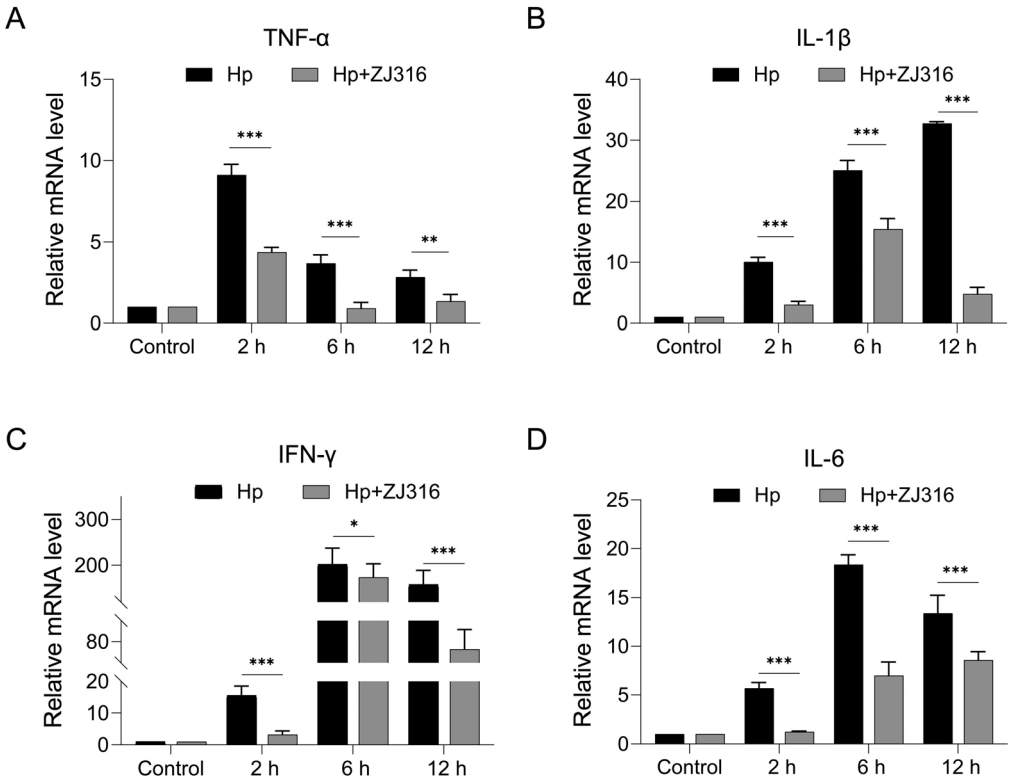

Fig. 1 *L. plantarum* ZJ316 reduces cytokine production following *H. pylori* infestation in AGS cells. (A–D) TNF-α, IL-1β, IFN-γ, and IL-6 mRNA levels. All bar graphs are represented as mean ± SD. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.005$.

infection. These enrichments highlight the alterations in gene regulation and nucleic acid interactions induced by *H. pylori* (Fig. 2D).

Within the *H. pylori*-infected cells, comparison between the Hp group and the Hp + ZJ316 treatment group revealed 3897 differentially expressed genes, including 1546 that were upregulated and 2351 that were downregulated (Fig. 2E). These findings underscored the complex interplay of gene regulation influenced by both *H. pylori* infection and the addition of *L. plantarum* ZJ316, suggesting potential pathways through which *L. plantarum* ZJ316 could potentially alter the host response to *H. pylori* infection at the transcriptional level. The DEGs exhibited notable enrichment in GO function terms, related to protein phosphorylation, phosphorylation, protein kinase activity and kinase activity (Fig. 2F). Overall, these results indicated that the treatments with *L. plantarum* ZJ316 and *H. pylori* lead to significant changes in internal metabolism and transcriptional activities of specific genes in AGS cells. The KEGG pathway analysis revealed top 15 pathways enriched with statistically significant DEGs, which encompassed transcriptional misregulation in cancer, the NF-kappa B signaling pathway, and the IL-17 signaling pathway (Fig. 2G). The analysis revealed that *H. pylori* infection led to increased expressions of genes associated with inflammatory pathways like the NF-kappa B signaling pathway and IL-17 cell differentiation. Further analysis of these genes could provide deeper insights into the specific mechanisms through which *L. plantarum* ZJ316 exerts its effects on the inflammatory signaling pathway in this context.

3.3. *L. plantarum* ZJ316 decreases the *H. pylori*-mediated activation of NF-κB in AGS cells

The NF-κB pathway is known to be constitutively active in various cancer cells.[59–62] *L. plantarum* ZJ316 has shown promise in inhibiting inflammatory factors. KEGG pathway analysis indicated that *L. plantarum* ZJ316 may suppress *H. pylori*-induced inflammation by modulating NF-κB activity. Then, we conducted gene set analysis to delve deeper into NF-κB-related gene changes and visualized the expression patterns of the top 4 significantly altered genes using a heat map. Our findings revealed that *H. pylori* infection dramatically up-regulated MAP3K14 (also known as NF-κB-inducing kinase or NIK), while down-regulating IκBα, a classic NF-κB inhibitory protein. Remarkably, supplementation with *L. plantarum* ZJ316 reversed these gene expression changes, as confirmed by RT-qPCR (Fig. 3A–C).

Activation of the classical NF-κB pathway involves phosphorylation of p65 and degradation of IκBα. As shown in Fig. 3D–F, *H. pylori* infection induced rapid degradation of IκBα within 2 hours, accompanied by increased phosphorylation of p65. In contrast, treatment with *L. plantarum* ZJ316 effectively countered NF-κB activation by attenuating both IκBα degradation and p65 phosphorylation. Phosphorylated p65 nuclear translocation is crucial for initiating the expression of

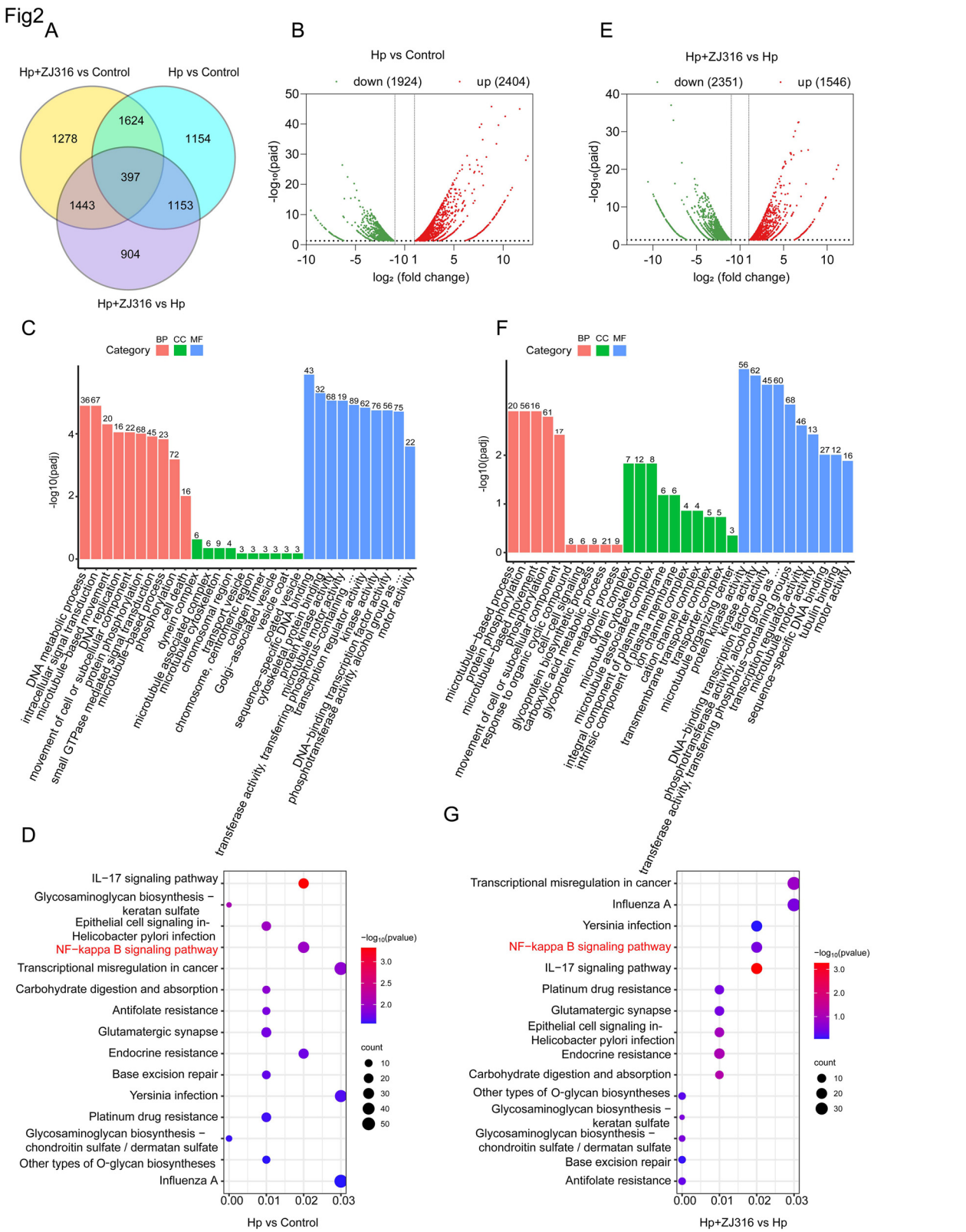

Fig. 2 DEGs in *H. pylori*-infected AGS cells with or without of *L. plantarum* ZJ316 based on RNA-seq. (A) Venn diagram for the control, Hp, and Hp + ZJ316 groups. (B and E) Volcano plot illustrating DEGs comparing the *H. pylori* infection group with the control group (B), and the Hp + ZJ316 group with the Hp group (E), highlighting genes showing up-regulation (red dots) and down-regulation (green dots). (C and F) GO annotation of DEGs between *H. pylori* infection and the control groups (C) and between the Hp + ZJ316 and Hp groups (F), displaying the top 10 GO terms. (D and G) KEGG pathway enrichment analysis comparing the Hp group *vs.* the control and Hp + ZJ316 groups, highlighting the top 15 enriched pathways.

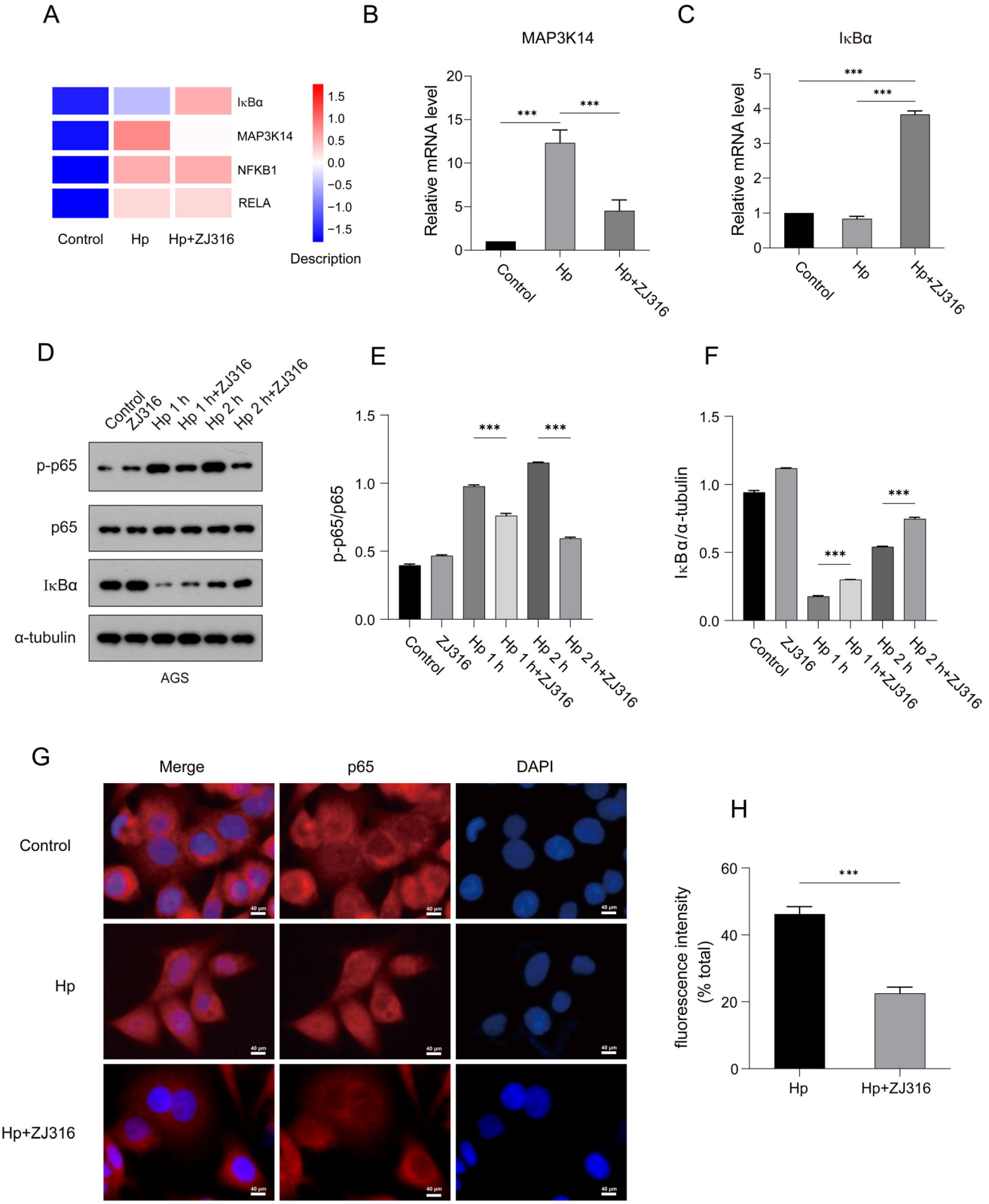

Fig. 3 Effect of *L. plantarum* ZJ316 on the *H. pylori*-induced activation of NF-κB. (A) Heat map of the top 4 DEGs involved in NF-κB signaling. (B and C) mRNA levels of MAP3K14 and IκBα. (D–F) Protein expression levels of p-p65, p65, and IκBα. (G and H) Immunofluorescence assay revealing the nuclear localization of p65 in AGS cells following 1.5 hours of *H. pylori* infection (scale bar: 40 μm). All bar graphs are represented as mean ± SD. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.005$.

inflammatory mediators, including IL-8, IL-6, and TNF-α.[63] To investigate *L. plantarum* ZJ316's role in suppressing NF-κB activation with *H. pylori* infection, we performed an immunofluorescence assay. Our findings showed that in comparison with the control group, *H. pylori* infection robustly elevated the percentage of cells with nuclear p65, whereas treatment with *L. plantarum* ZJ316 markedly reduced this nuclear translocation with *H. pylori* infestation (Fig. 3G and H). The results indicated that *L. plantarum* ZJ316 achieves its anti-inflammatory effects through modulating the NF-κB signaling pathway, specifically by suppressing classical IκBα degradation and p65 phosphorylation as well as its nuclear translocation.

3.4. BAY 11-7082 increases the anti-inflammatory effects of *L. plantarum* ZJ316

The degradation of IκBα and subsequent release of NF-κB hinge on phosphorylation at Ser32 and Ser36 residues.[64,65] BAY 11-7082 selectively and irreversibly impedes IκBα phosphorylation.[66] To explore the involvement of NF-κB signaling in the response of *L. plantarum* ZJ316 to *H. pylori*-induced inflammation, AGS cells were either left untreated or treated with 10 μM BAY 11-7082. The results indicated that *H. pylori* infection led to a significant increase in IL-8, TNF-α, and IL-1β levels at 2, 6, and 12 h compared to the control group, whereas treatment with *L. plantarum* ZJ316 led to a reduction in the levels of these inflammatory markers (Fig. 4A–C). Additionally, treatment with BAY 11-7082 augmented the protective effect of *L. plantarum* ZJ316 by suppressing IL-8, IL-1β, and TNF-α levels. Conversely, IL-10 levels, recognized for their anti-inflammatory cytokine role, displayed a contrasting trend (Fig. 4D). To delve into the impact of *L. plantarum* ZJ316 on the downstream targets of NF-κB, we examined IκBα degradation, p65 phosphorylation and NF-κB transcriptional activity. Western blot analysis additionally validated the heightened phosphorylation of p65 and IκBα degradation upon *H. pylori* stimulation, which was dampened by *L. plantarum* ZJ316 intervention. Importantly, additional administration of BAY-117082 also reduced these markers compared to the incubation with *L. plantarum* ZJ316 alone under *H. pylori* conditions (Fig. 4E–G). To confirm the impact of *L. plantarum* ZJ316 on the *H. pylori*-induced activation of NF-κB, we transfected a luciferase reporter controlled by an NF-κB promoter (NF-κB-Luc). The results showed that *H. pylori* infection triggered NF-κB-dependent transcriptional activation. Notably, NF-κB transcriptional activity was reduced by *L. plantarum* ZJ316 treatment alone and was significantly further attenuated when *L. plantarum* ZJ316 was combined with BAY 11-7082 (Fig. 4H). These findings strongly demonstrate that *L. plantarum* ZJ316 mitigates inflammation following *H. pylori* infection, partly through the modulation of the NF-κB pathway.

3.5. Regulation of NF-κB signaling by *L. plantarum* ZJ316 in attenuating *H. pylori*-induced inflammation *in vivo*

To explore the underlying mechanism by which *L. plantarum* ZJ316 dampens NF-κB activation in the presence of *H. pylori in vivo*, C57BL/6 mice were inoculated with *H. pylori* and received *L. plantarum* ZJ316 supplementation, with or without BAY 11-7082 (Fig. 5A). Notably, exposure to *H. pylori*, *L. plantarum* ZJ316, and BAY 11-7082 did not significantly affect the mouse body weight, as shown in Fig. 5B. Inflammatory molecules are pivotal in the pathogenesis of *H. pylori* infection, contributing significantly to tissue damage. Then, we evaluated the gastric tissue morphology using H&E staining. The control group exhibited a normal gastric mucosal structure, an intact glandular morphology, and a preserved columnar epithelium (Fig. 5C). Conversely, *H. pylori* infection induced notable histopathological changes characterized by increased inflammation and infiltration of inflammatory cells, resulting in tissue damage. Treatment with *L. plantarum* ZJ316 reduced infiltration of inflammatory cells in the infected gastric tissues. Furthermore, inhibition of NF-κB signaling using BAY 11-7082 further mitigated tissue damage compared to the treatment with *L. plantarum* ZJ316 alone (Fig. 5C). The assessment scores for tissue damage showed consistent results across the experiments (n = 5) (Fig. 5D). Furthermore, a detailed analysis of inflammatory cytokine levels in the mouse serum revealed a notable decrease in IL-10 and a pronounced elevation of TNF-α levels in the *H. pylori*-infected group, in comparison with the normal control group (Fig. 5E and F), while treatment with *L. plantarum* ZJ316 reversed these trends by increasing IL-10 levels and reducing TNF-α levels (Fig. 5E and F). Pepsinogens (PGs), enzyme precursors secreted by the gastric mucosa, serve as indicators of atrophic gastritis and are associated with a heightened gastric cancer risk.[67,68] Previous studies have associated elevated PG II levels with chronic inflammation in *H. pylori*-related gastritis and gastric ulcers[69,70] (Fig. 5G). Consistent with these findings, our study demonstrated elevated PG II levels following *H. pylori* infection, which were effectively reversed by treatment with *L. plantarum* ZJ316. Furthermore, administration of BAY 11-7082 augmented the protective effect of *L. plantarum* ZJ316 by further lowering PG II and TNF-α levels, while increasing IL-10 levels in serum (Fig. 5E–G).

Finally, RT-qPCR and IHC were employed to elucidate the mechanisms underlying *L. plantarum* ZJ316's inhibition of NF-κB activity. Our results demonstrated that *H. pylori* infection significantly decreased the IκBα expression at both mRNA and protein levels (Fig. 5H and J), concurrently increasing the expression of phosphorylated p65 (Fig. 5I). Treatment with *L. plantarum* ZJ316 alone or in combination with BAY 11-7082 further reduced the IκBα levels, while enhancing the phosphorylated p65 expression (Fig. 5H–J), with statistical analysis confirming consistent experimental results (Fig. 5K). These comprehensive findings underscore the potential of *L. plantarum* ZJ316 in modulating NF-κB signaling in response to *H. pylori* infection, suggesting its role as a promising therapeutic agent for mitigating inflammation associated with gastrointestinal diseases.

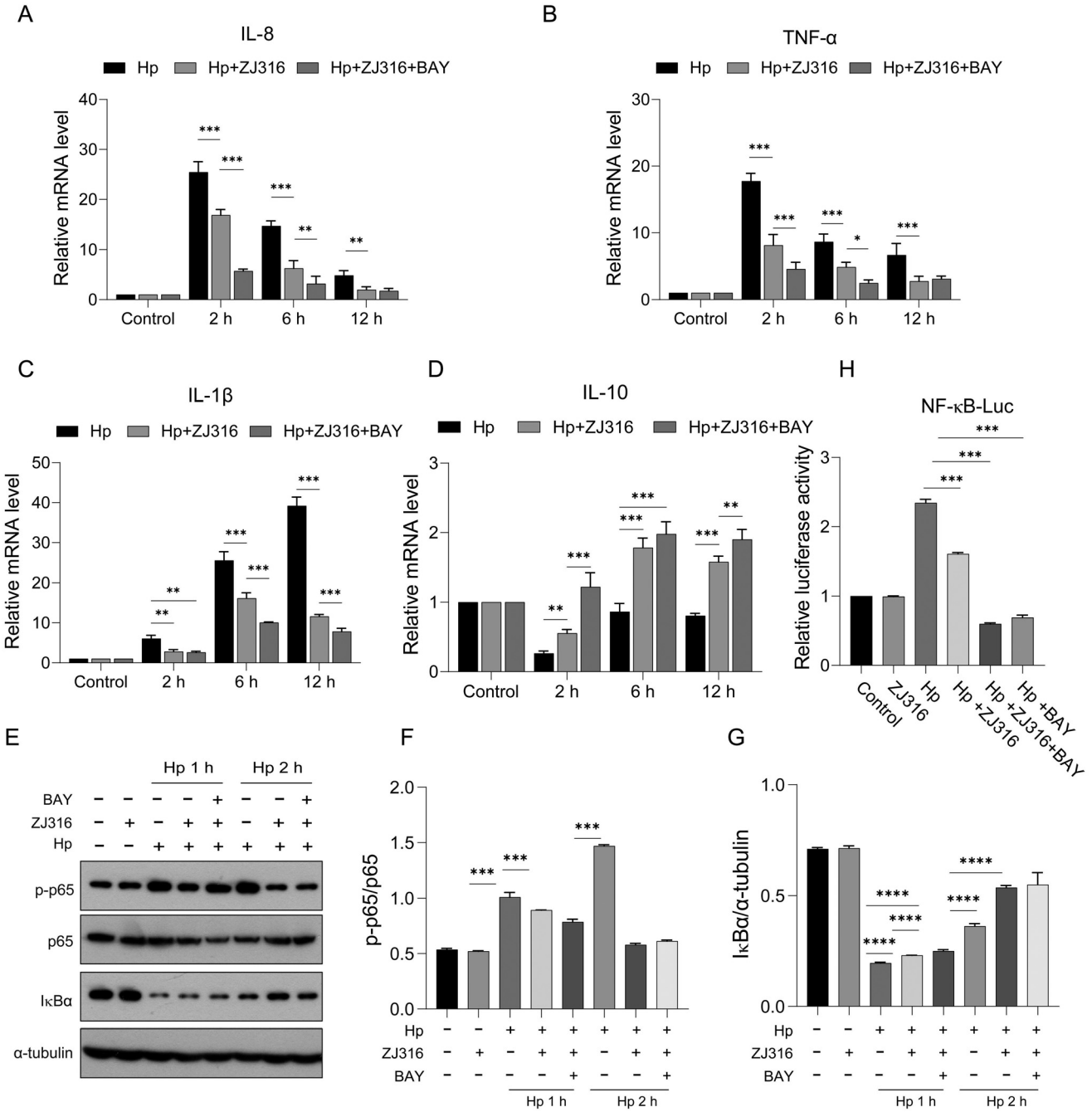

Fig. 4 Effect of BAY 11-7082 on the regulation of *L. plantarum* ZJ316 on the *H. pylori*-induced activation of NF-κB. (A–D) RT-qPCR analysis of the expressions of IL-8, TNF-α, IL-1β and IL-10 in AGS cells treated with *H. pylori* for 2, 6, and 12 h, with *L. plantarum* ZJ316 alone or in combination with the NF-κB inhibitor BAY 11-7082. (E–G) Western blot analysis for determining the NF-κB protein level in AGS cells infected with *H. pylori* for 1 and 2 h, with *L. plantarum* ZJ316 alone or in combination with BAY 11-7082. (H) Luciferase reporter assays showing NF-κB-dependent transcriptional activation in AGS cells across the different treatment groups. All bar graphs are represented as mean ± SD. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.005$.

3.6. Effect of *L. plantarum* ZJ316 on the gastric microbiota in mice with *H. pylori* infection

Recent studies have highlighted the crucial role of gastric microbes beyond *H. pylori* in gastric cancer progression. *H. pylori* infection has been linked to significant alterations in the microbiota composition, particularly in gastric cancer patients.[71] In our investigation, we explored the microbial composition and changes in the gastric microbiome in *H. pylori*-infected mice treated with *L. plantarum* ZJ316, with or without BAY 11-7082 treatment. At the genus level, *Lactobacillus* and *Prevotella* were invariably predominant across all treatment groups. *H. pylori* treatment notably increased the abundance of *Prevotella* and *Desulfovibrio*, while decreasing

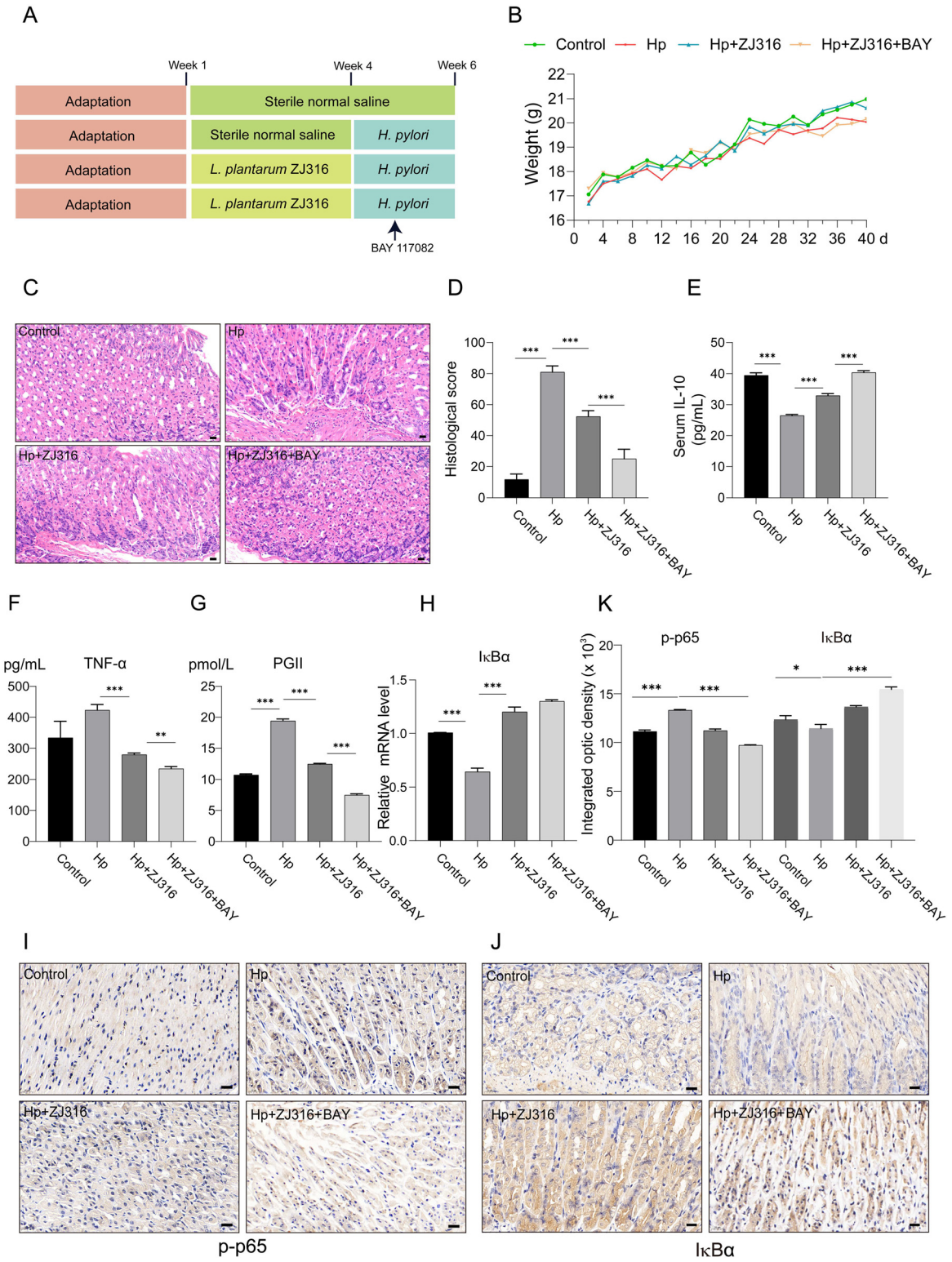

Fig. 5 Effects of *L. plantarum* ZJ316 and NF-κB inhibition on *H. pylori*-induced gastric inflammation in C57BL/6 mice. (A) Refining of the animal experiment flowchart. Data were collected from n = 5 mice per group, with an initial group size of n = 8 mice. (B) Body weights of mice across the four groups. (C) H&E staining of gastric tissues (magnification: ×40, scale bar: 20 μm). (D) Histopathological scores. (E–G) Levels of TNF-α, IL-10 and PG II in serum across the four groups (n = 5). (H) mRNA level of IκBα of the gastric tissue. (I and J) IHC of phosphorylated p65 and IκBα (magnification: ×40, scale bar: 20 μm). (K) Quantification of p-p65 and IκBα levels across the treatment groups. All bar graphs are represented as mean ± SD, n = 5. *p < 0.05; **p < 0.01; and ***p < 0.005.

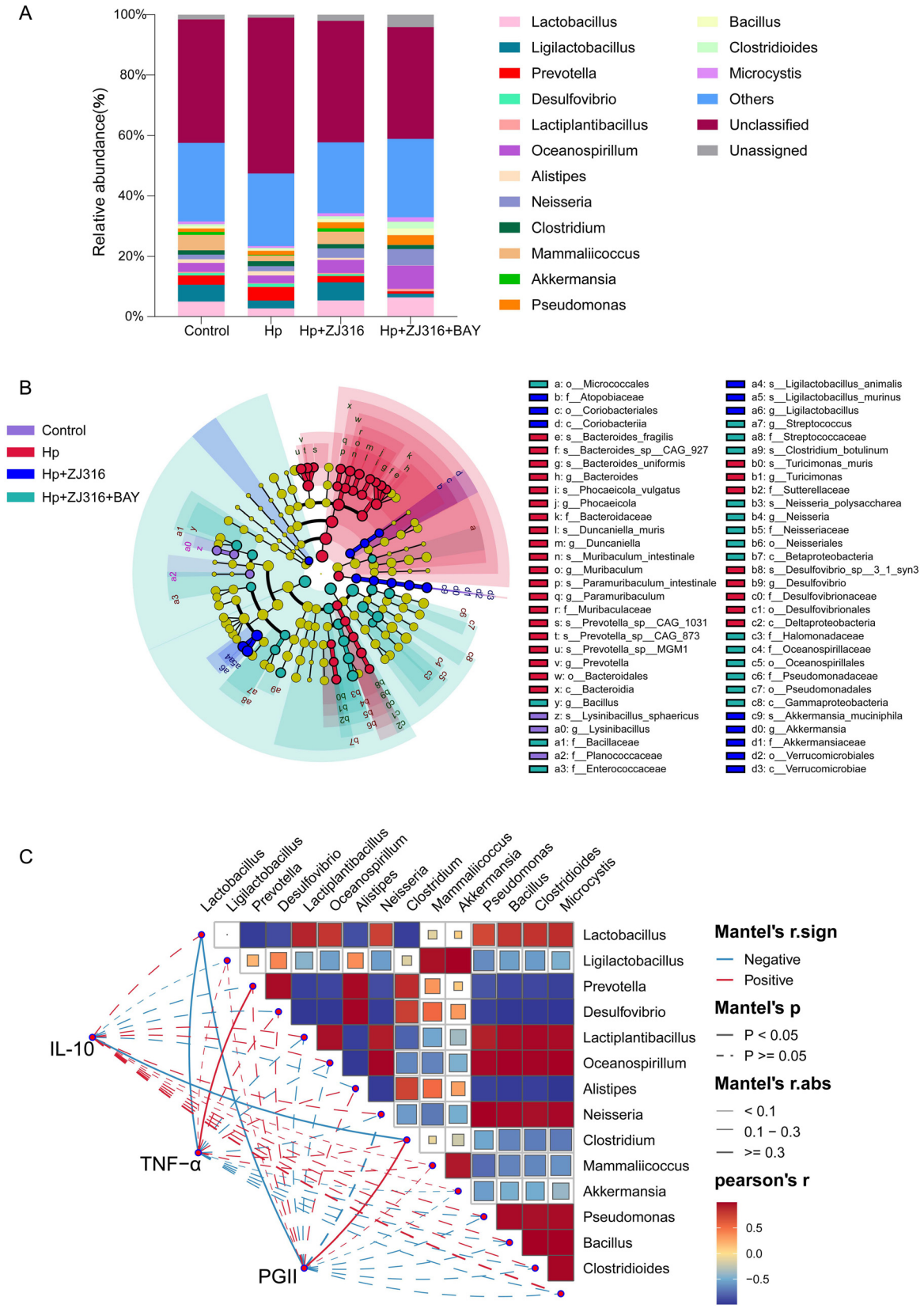

Fig. 6 Effect of *L. plantarum* ZJ316 on the gastric microbiota in mice with *H. pylori* infection. (A) Genus level gastric microbiota. (B) Cladogram analysis across the four groups. (C) Spearman's correlation analysis between the gut microbiota (top 15 genus levels) and cytokines. The red color signifies a positive relationship and the blue color indicates a negative relationship. $n = 5$. Pearson's correlation coefficients were employed to assess the relationships between the variables. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$.

the levels of *Lactobacillus*, *Akkermansia*, *Oceanospirillum*, and *Ligilactobacillus* compared to the control group. Treatment with *L. plantarum* ZJ316 completely mitigated these effects. Furthermore, when comparing *H. pylori*-infected mice treated with *L. plantarum* ZJ316 alone to those additionally treated with BAY 11-7082, BAY 11-7082 treatment significantly increased *Lactobacillus* abundance and reduced *Prevotella*, thus restoring the gastric microbiome towards a profile more akin to the control group (Fig. 6A). To discern the key taxa driving distinctions among the four groups, we employed a cladogram analysis across the four groups (Fig. 6B). We observed higher abundances of *Alistipes*, *Prevotella*, and *Desulfovibrio* in the Hp group. Conversely, in the Hp + ZJ316 group, *Ligilactobacillus* were notably more abundant. To elucidate the relationship between the top 15 genera of the gastric microbiota and inflammatory responses, as well as prostaglandins (PGs), we conducted Spearman's correlation analysis. We found that *Clostridium* strongly correlated with reduced IL-10 levels but increased PG II levels ($p < 0.05$). *Lactobacillus* was linked to lower TNF-α and PG II levels ($p < 0.05$). Additionally, *Prevotella* showed a significant association with TNF-α ($p < 0.05$) (Fig. 6C).

4. Discussion

H. pylori infection is widespread globally, particularly in emerging nations, where infection rates can exceed 80%.[72] Chronic inflammation resulting from *H. pylori* infestation is a primary driver that contributes to the onset of gastric cancer.[12,73] Infected individuals are at a markedly increased risk of developing gastric cancer compared to uninfected individuals. The pathogenicity of *H. pylori* is mainly attributed to several virulence factors, including lipopolysaccharides, and vacuolating toxins such as VacA and CagA. These factors activate inflammatory signaling pathways such as NF-κB and MAPK.[74,75] Extensive research underscores the central role of CagA in *H. pylori*-triggered inflammation and tumorigenesis, primarily through its activation of the NF-κB transcription factor.[74,76–78] This chronic inflammation fosters a microenvironment that promotes the development of cancer-prone cells.[79] Quadruple therapy is the established treatment regimen for eradicating *H. pylori*, yet it confronts significant challenges, including rising drug resistance and potential adverse effects such as gastrointestinal disorders and microbiota imbalance due to antibiotic misuse.[80]

Lactobacillus strains hold promise as a potential solution to these challenges. Earlier research has pointed to a potential relationship between *Lactobacillus* strains and bolstering host immunity.[81–83] Our earlier studies demonstrated that administration of *L. plantarum* ZJ316 improved gastric microbial balance and effectively reduced the inflammatory reaction triggered by *H. pylori* infection in mice.[54] Nevertheless, the exact mechanisms driving these effects remain elusive. This study elucidates that *L. plantarum* ZJ316 markedly alleviates inflammation following *H. pylori* infestation by blocking NF-κB activation, both *in vitro* and *in vivo*. Furthermore, supplementation with *L. plantarum* ZJ316 improves gastric microbial diversity by reducing the abundance of inflammation-associated microbes such as *Prevotella* and *Desulfovibrio*, while increasing *Ligilactobacillus* and *Akkermansia*, which are linked to anti-inflammatory effects (graphical abstract). These findings suggest that *L. plantarum* ZJ316 holds promise as a therapeutic agent for alleviating *H. pylori*-induced inflammation.

Genetic variations in pro-inflammatory cytokine genes, comprising IL-1β and TNF-α, contribute significantly to the risk of gastric cancer.[84–86] Specific combinations of polymorphisms in IL-1β, IL-1RN, TNF-α, and IL-10 can elevate the risk of gastric cancer by 27-fold,[87] underscoring the pivotal role of these inflammatory cytokines in gastric cancer development.[88–90] Research studies have shown that *H. pylori* infection upregulates the aforementioned inflammatory cytokines.[91,92] Consistent with previous findings, our study demonstrated that *H. pylori* induces TNF-α, IL-1β, IFN-γ, and IL-6 production from co-infected AGS cells at 2 h, 6 h, and 12 h. Furthermore, administration of *L. plantarum* ZJ316 effectively reduced the production of these inflammatory cytokines induced by *H. pylori* across all observed time points. Similar findings have been reported for other probiotic *Lactobacilli*, including *Lactobacillus johnsonii*,[93] *Ligilactobacillus salivarius*,[94] and *Lactiplantibacillus plantarum*,[46] which demonstrate the suppression of TNF-α, IL-1β as well as IL-6 secretion or the modulation of gene expressions induced by *H. pylori*. This study underscores the beneficial impact of *L. plantarum* ZJ316 on AGS cells, highlighting its potential to mitigate the inflammatory response triggered by *H. pylori*.

RNA-Seq, an advanced method for profiling the transcriptome using deep-sequencing technologies, has found widespread application in genomic and molecular biology research.[95] To delve deeper into how *L. plantarum* ZJ316 inhibits *H. pylori*-induced inflammation, we administered *L. plantarum* ZJ316 and *H. pylori* to AGS cells for 2 h and employed the RAN-Seq technology to explore the underlying signaling pathways and mechanisms. Our study identified 3897 DEGs between the Hp and Hp + ZJ316 treatment groups, with 1546 genes upregulated and 2351 genes downregulated, highlighting notable changes in the metabolic and transcriptional activities within AGS cells due to the treatments. Subsequent GO and KEGG analyses provided deeper insights into the specific mechanisms through which *L. plantarum* ZJ316 affects the inflammatory signaling pathways. Specifically, we discovered that *L. plantarum* ZJ316 treatment attenuated the up-regulation of genes triggered by *H. pylori* infection, particularly those enriched in the NF-κB signaling pathway and IL-17 signaling pathway according to the KEGG analysis. Notably, NF-κB, a transcription factor involved in inflammation, cell proliferation, and carcinogenesis, remained consistently activated during *H. pylori* infection[96,97] and emerged as a potential target of *L. plantarum* ZJ316 in mitigating the inflammatory response.

NF-κB, initially bound to IκB proteins in the cytoplasm, undergoes activation upon phosphorylation and degradation

of IκB proteins. The phosphorylation of p65 allows the p50–p65 complex to move into the nucleus, enabling NF-κB to bind to specific DNA sequences.[98–101] This process facilitates interactions with transcriptional coactivators like p300/CBP, thereby promoting the activation of genes crucial for immune responses, apoptosis, inflammation, and cell survival.[98–101] Therefore, inhibiting NF-κB signaling has become a promising approach for diverse medical conditions, given its pivotal role demonstrated in numerous disease studies.[102] Yong *et al.* showed that *Lactobacillus acidophilus* improved *H. pylori*-induced gastric inflammation by blocking the Smad7 and NF-κB pathways. Additionally, conditioned media from *Ligilactobacillus salivarius* B101, *Lacticaseibacillus rhamnosus* B103, and *Lactiplantibacillus plantarum* XB7 were identified as protective agents against *H. pylori*-induced gastric inflammation in AGS cells, achieved through suppression of the NF-κB pathway.[52] Our study revealed that probiotic therapy restored the transcriptional levels of certain NF-κB up-regulated genes following *H. pylori* infection, involving MAP3K14 and IκBα. The western blot analysis definitively showed that *L. plantarum* ZJ316 reversed NF-κB activation triggered by *H. pylori*, evidenced by decreased p65 phosphorylation and IκBα degradation in AGS cells. Upon phosphorylation, NF-κB translocates to the nucleus to activate various inflammatory genes. Immunofluorescence analysis confirmed that *L. plantarum* ZJ316 effectively blocks the *H. pylori*-induced nuclear translocation of p65, substantiating its inhibition of IκBα degradation and p65 phosphorylation. This mechanism reduces the NF-κB entry into the nucleus and suppresses inflammatory cytokine production. Similar effects were observed with human gastrogenic *Weizmannia coagulans* BCF-01, which inhibited NF-κB signaling in *H. pylori*-induced inflammation by blocking IκBα degradation and p65 phosphorylation in macrophages.[103] In our study, suppressing the NF-κB activity with BAY 11-7082 demonstrated that treatment with BAY 11-7082 enhances the beneficial effects of *L. plantarum* ZJ316 on AGS cells against inflammatory damage induced by *H. pylori* infection. These findings strongly suggest that *L. plantarum* ZJ316 alleviates inflammation triggered by *H. pylori*, partly through modulation of the NF-κB pathway. However, the specific regulatory mechanism of *L. plantarum* ZJ316 on the NF-κB signaling pathway remains unclear. Generally, upon activation of Toll-like or TNF receptors in response to bacterial infection, the IKK complexes (IKKα, IKKβ and IKKγ) are activated,[65,101] phosphorylating IκB proteins, which leads to their degradation and the subsequent release of NF-κB dimers.[63,65,101] In our study, whether *L. plantarum* ZJ316 regulates the activation of the IKK complex, thereby regulating IκB phosphorylation and alleviating *H. pylori*-induced gastric inflammation, requires further investigation. *H. pylori* infection has been shown to activate STAT3, MAPKs, and AP-1, which interact with NF-κB signaling, forming a complex regulatory network in response to pathogens and inflammatory stimuli.[76,104] The *Lacticaseibacillus paracasei* strain 06TCa19 and BIFICO capsules, which contain a blend of *Enterococcus faecalis*, *Bifidobacterium animalis*, and *Lactobacillus acidophilus*, have been reported to reduce the activation of NF-κB and p38 MAPK signaling pathways.[105,106] Additionally, a combination of probiotics such as *Lactiplantibacillus plantarum*, *Lacticaseibacillus rhamnosus*, and *Lactobacillus acidophilus* has the potential to alleviate *H. pylori*-induced inflammation by upregulating SOCS-2 and SOCS-3 expressions or inhibiting JAK2 activation *via* the JAK-STAT signaling pathway.[107] It is possible that *L. plantarum* ZJ316 is involved in regulating the MAPK and JAK/STAT pathways to suppress *H. pylori*-induced inflammation, but further investigation is needed.

Treating mice with *H. pylor*i-induced gastritis using *L. plantarum* ZJ316 resulted in reduced infiltration of inflammatory cells and decreased secretion of TNF-α, alongside with an elevated IL-10 level compared to mice infected with *H. pylori* alone. These findings align with prior studies demonstrating the efficacy of probiotics, including *Lacticaseibacillus rhamnosus*,[55,108] *Ligilactobacillus salivarius*[55] and *Lacticaseibacillus rhamnosus* JB3,[109] in suppressing *H. pylori*-induced inflammation in mouse models. PG II, a precursor enzyme secreted by the gastric mucosa, along with serological markers including PG I, PG I/PG II ratio, and gastrin-17, are utilized in epidemiological investigations to assess the risk of gastric cancer.[110–113] Gastric inflammation increases the release of both pepsinogens into the bloodstream, with PG II levels showing a more pronounced elevation than PG I.[114] Studies consistently showed that infection with CagA-positive *H. pylori* significantly increases PG II levels and reduces the PG I/PG II ratios.[115,116] Furthermore, our findings indicated that *H. pylori* colonization enhances serum PG II levels, while treatment with *L. plantarum* ZJ316 decreases PG II levels. Importantly, we observed an additive effect between the *L. plantarum* ZJ316 treatment group and the *L. plantarum* ZJ316 + inhibitor group.

Dysbiosis of the gastric microbiota has been linked to the development and advancement of several diseases. Research studies have indicated that *H. pylori* invasion can alter the composition of microbiota, especially in individuals with gastric cancer.[117] Our research indicated that *H. pylori* colonization predominantly reduces the abundance of beneficial flora, including *Lactobacillus*, *Akkermansia* and *Ligilactobacillus*, while increasing *Prevotella* and *Desulfovibrio*, and treatment with *L. plantarum* ZJ316 reversed these changes by decreasing *Prevotella* and *Desulfovibrio* and increasing *Ligilactobacillus* and *Akkermansia*. Spearman's correlation analysis revealed that *Prevotella* showed a notable association with TNF-α. These findings align with previous research highlighting *Prevotella* as a Gram-negative anaerobic bacterium found in various human body sites including the mouth, respiratory tract, urogenital tract, and gastrointestinal tract, implicated in infections such as oral cavity issues and heart failure.[118,119] *Desulfovibrio* has been identified as a causative pathogen of bacteraemia and abdominal infections,[120] with its outer membrane vesicles damaging the intestinal epithelial barrier and triggering intrinsic inflammation.[121] Additionally, *Clostridium* was strongly correlated with decreased IL-10 levels but increased PG II levels. In contrast, *Lactobacillus* was associated with decreased levels of both TNF-α and PG II. Cáceres

et al. found that the nutritional form of *Clostridium chauvoei* triggers an inflammatory response in macrophages.[122] Due to the imbalance of the gut microbiota caused by *H. pylori*, *Clostridium difficile* can release toxins during intestinal colonization, leading to local tissue damage and severe inflammatory reactions.[123] Moreover, *Akkermansia* has been shown to strengthen the intestinal barrier function and immune response and is inversely associated with inflammatory bowel disease.[119,124] These findings imply that supplementation with *L. plantarum* ZJ316 reshaped the gastric microbiota by decreasing *Prevotella* and increasing beneficial bacteria such as *Lactobacillus*[104,125] and *Ligilactobacillus*,[126] supporting their protective effects and correlating with a decreased inflammatory response. In this study, we investigated the anti-inflammatory effects of *L. plantarum* ZJ316 in *H. pylori*-induced gastric inflammation, focusing primarily on the NF-κB signaling pathway. However, further investigation is needed to fully understand the specific molecular mechanisms by which *L. plantarum* ZJ316 inhibits NF-κB signaling, particularly its potential effects on the phosphorylation and activation of the IKK complex, a crucial step in the NF-κB pathway.[63,65,101] In general, *H. pylori* infection is known to induce inflammation through various pathways, including STAT3, MAPKs, and AP-1,[104,127] and it remains unclear whether *L. plantarum* ZJ316 also modulates these pathways. Additionally, while *L. plantarum* ZJ316 has been shown to improve the gastric microbiota disrupted by *H. pylori* infection, further research is needed to elucidate how these microbiota changes contribute to the observed anti-inflammatory effects. Finally, while the results from our mouse model are promising, clinical studies are essential to validate the effectiveness and safety of the application of *L. plantarum* ZJ316 in humans. Nonetheless, substantial work remains to be done before these findings can be translated into clinical applications.

5. Conclusions

This study demonstrates that *L. plantarum* ZJ316 effectively inhibits *H. pylori*-induced inflammatory responses by targeting the NF-κB signaling pathway. It achieves this by blocking IκBα degradation, preventing p65 phosphorylation, and inhibiting the nuclear translocation of p65. Additionally, *L. plantarum* ZJ316 reshapes the gastric microbiota by increasing the abundance of beneficial bacteria and decreasing the levels of harmful bacteria, particularly *Prevotella*, which is positively correlated with the secretion of inflammatory cytokines. These findings highlight the potential of *L. plantarum* ZJ316 as a probiotic therapy for mitigating *H. pylori*-induced inflammation and supporting gastrointestinal health. Further research is necessary to optimize dosage, delivery methods, and clinical applications for broader use.

Author contributions

Shiying Wu: supervision, writing – review and editing, conceptualization and funding acquisition; Yuenuo Luo: writing – original draft, investigation and formal analysis; Fangtong Wei: investigation and formal analysis; Yanan Li: methodology; Jiayi Fan: data curation; Yang Xu: investigation; Yongqiang Chen: data curation; Wenjie Zhang: methodology; Xuelong Li: methodology; Ziqi Chen: validation; Chenlan Xia: validation; Mingyang Hu: formal analysis; Ping Li: supervision; and Qing Gu: resources, funding acquisition, and project administration.

Data availability

The original data presented in the study are included in the article. For additional information, please contact the corresponding author.

Conflicts of interest

The authors declare that there are no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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