

# World Journal of *Gastroenterology*

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Editorial Board of *World Journal of Gastroenterology*, Haruhiko Sugimura, MD, PhD, Director, Sasaki Institute, Sasaki Foundation, Tokyo 101-0062, Japan. [hsugimur@po.kyoundo.jp](mailto:hsugimur@po.kyoundo.jp)

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## Basic Study

Linolenic acid-metronidazole inhibits the growth of *Helicobacter pylori* through oxidation

Wen-Ting Zhou, Yuan-Yuan Dai, Li-Juan Liao, Shi-Xian Yang, Hao Chen, Liang Huang, Juan-Li Zhao, Yan-Qiang Huang

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**Wen-Ting Zhou, Li-Juan Liao, Liang Huang, Juan-Li Zhao,** School of Basic Medicine, Youjiang Medical University for Nationalities, Baise 533000, Guangxi Zhuang Autonomous Region, China

**Yuan-Yuan Dai,** School of Basic Medicine, Youjiang Medical University for Nationalities, Affiliated Hospital of Youjiang Medical University for Nationalities, Baise 533000, Guangxi Zhuang Autonomous Region, China

**Shi-Xian Yang,** Department of Laboratory, Affiliated Hospital of Youjiang Medical University for Nationalities, Baise 533000, Guangxi Zhuang Autonomous Region, China

**Hao Chen,** School of Basic Medicine, Department of Pathology, Wannan Medical College, Wuhu 533000, Anhui Province, China

**Liang Huang,** Key Laboratory of the Prevention and Treatment of Drug Resistant Microbial Infecting, Youjiang Medical University for Nationalities, Baise 533000, Guangxi Zhuang Autonomous Region, China

**Yan-Qiang Huang,** School of Basic Sciences, Youjiang Medical University for Nationalities, Baise 533000, Guangxi Zhuang Autonomous Region, China

**Corresponding author:** Liang Huang, Instructor, Key Laboratory of the Prevention and Treatment of Drug Resistant Microbial Infecting, Youjiang Medical University for Nationalities, No. 98 Chengxiang Road, Baise 533000, Guangxi Zhuang Autonomous Region, China. [youyihuangl@163.com](mailto:youyihuangl@163.com)

**Abstract****BACKGROUND**

Resistance to antibiotics is one of the main factors constraining the treatment and control of *Helicobacter pylori* (*H. pylori*) infections. Therefore, there is an urgent need to develop new antimicrobial agents to replace antibiotics. Our previous study found that linolenic acid-metronidazole (Lla-Met) has a good antibacterial effect against *H. pylori*, both antibiotic-resistant and sensitive *H. pylori*. Also, *H. pylori* does not develop resistance to Lla-Met. Therefore, it could be used for preparing broad-spectrum antibacterial agents. However, since the antibacterial mechanism of Lla-Met is not well understood, we explored this phenomenon in the present study.

**AIM**

To understand the antimicrobial effect of Lla-Met and how this could be applied in treating corresponding infections.

**METHODS**

*H. pylori* cells were treated with the Lla-Met compound, and the effect of the compound on the cell morphology, cell membrane permeability, and oxidation of the bacteria cell was assessed. Meanwhile, the differently expressed genes in *H. pylori* in response to Lla-Met treatment were identified.

**RESULTS**

Lla-Met treatment induced several changes in *H. pylori* cells, including roughening and swelling. *In vivo* experiments revealed that Lla-Met induced oxidation, DNA fragmentation, and phosphatidylserine ectropionation in *H. pylori* cells. Inhibiting Lla-Met with L-cysteine abrogated the above phenomena. Transcriptome analysis revealed that Lla-Met treatment up-regulated the expression of superoxide dismutase *SodB* and *MdaB* genes, both anti-oxidation-related genes.

**CONCLUSION**

Lla-Met kills *H. pylori* mainly by inducing oxidative stress, DNA damage, phosphatidylserine ectropionation, and changes on cell morphology.

**Key Words:** *Helicobacter pylori*; Oxidation; Superoxide dismutase; *SodB* genes; *MdaB* genes

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**Core Tip:** The clarithromycin resistant *Helicobacter pylori* (*H. pylori*) is listed by the World Health Organization as the priority bacteria in urgent need of developing new antibiotics. Our previous research found that linolenic acid-metronidazole has a good antibacterial effect on *H. pylori* and is not easy to develop drug resistance. Therefore, we further explored its antibacterial mechanism against *H. pylori*. It was found that it mainly kills *H. pylori* by inducing oxidative stress, DNA damage, phosphatidylserine ectropionation, and changes on cell morphology. This study may provide a theoretical basis for the development and application of new anti *H. pylori* lead compound.

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**INTRODUCTION**

*Helicobacter pylori* (*H. pylori*) is the main pathogen that causes upper digestive diseases, such as chronic gastritis, peptic ulcer, and gastric cancer[1-4]. At present, the treatment options for *H. pylori* infections include standard triple therapy, bismuth-containing quadruple therapy, and sequential therapy[5,6]. Due to the overuse and misuse of antibiotics, the drug resistance rate of *H. pylori*, including multi-drug resistance, is gradually increasing, negatively impacting the control and treatment of *H. pylori* infections[7-10]. Therefore, there is an urgent need to develop new anti-*H. pylori* agents[11].

Due to the long period and significant investment required for developing new antibiotics, the transformation or modification of the existing drugs is more efficient in shortening the drug research and development cycle. Modifying existing drugs could improve their efficacy while reducing the development of antimicrobial resistance. Zinc linolenic acid and liposome linolenic acid are linolenic acid derivatives effective at increasing the sensitivity of drug-resistant *H. pylori*. Resistance against zinc linolenic acid and liposome linolenic acid is minimal[12,13]. Although metronidazole is a widely used and cost-effective drug, its clinical application for *H. pylori* infection treatment is limited by resistance development.

Our previous study found that the minimum inhibitory concentration (MIC) of linolenic acid-metronidazole (Lla-Met) against six strains of the drug-resistant *H. pylori* was 2-4 µg/mL. Additionally, the *H. pylori* strains did not develop resistance against this compound. Therefore, Lla-Met would serve as promising antibiotics. However, its antibacterial mechanism is poorly understood[14], this study explored this mechanism.

## MATERIALS AND METHODS

### Materials

*H. pylori* strain G27 (Courtesy of Prof. Bi Hongkai, Nanjing Medical University), calf serum, a Columbia blood agar base, a brain heart infusion (BHI,OXOID) medium, L-cysteine (L-cys) (AR 99%, MACKLIN), a fluorescence orthomicroscope (OLYMPUS, Tokyo, Japan), reactive oxygen species (ROS) detection kits (Beyotime), cell apoptosis 4',6-diamidino-2-phenylindole (DAPI) detection kits (Beyotime), apoptosis detection kits (Beyotime), reverse transcription kits (Monad), reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) kits (Monad), a Lightcycler96 fluorescence ration PCR instrument (Roche, Germany), and a scanning electron microscope were used in the present study.

### Thawing and culture of *H. pylori* strain

Standard *H. pylori* strain G27 stored at -80 °C were thawed and centrifuged to remove the preservation solution (Glycerin:BHI:serum = 3:6:1). The bacteria were inoculated on a Columbia agar medium, or a brain heart infusion medium supplemented with 10% calf serum and cultured in a microaerophilic environment.

### Cell morphology assay

The effect of the Lla-Met on *H. pylori* morphology was observed by scanning electron microscopy[15-18]. *H. pylori* was treated with 4 and 8 µg/mL of Lla-Met and incubated for 24 h in a three-gas incubator. The bacteria were pelleted by centrifugation and fixed overnight with 2.5% glutaraldehyde. The bacteria suspension was centrifuged to remove glutaraldehyde before dehydration with 30%, 50%, 70%, 90%, and 100% ethanol. The pellet was dried through refrigeration in a vacuum. After that, the *H. pylori* morphology was observed and photographed under a KYKY-EM8100 scanning electron microscope (KYKY, Beijing).

### Membrane damage assay

The *H. pylori* cells were stained as previously described by Hwang *et al*[19]. Briefly, the G27 bacterial suspension ( $1 \times 10^8$  CFU/mL) at the logarithmic phase was treated with Lla-Met for 2 h at the rate of 16 µg/mL. The cell suspension was centrifuged at 12000 r/min for 2 min to pellet the cells. The medium was poured out, and the harvested cells were suspended in phosphate buffered saline (PBS). The cells were stained with a propidium iodide solution (PI, 10 µg/mL, Thermo Fisher) at 37 °C protected from light 30 min and thereafter centrifuged at 12000 r for 5 min. The dye unbound to the harvested cells was washed away with sterile PBS. Thereafter, the cells were suspended in PBS and immediately observed under a fluorescence microscope (Olympus Corporation, Tokyo, Japan).

### Cell membrane pore size assay

FITC-FD was mainly used to evaluate the degree of *H. pylori* cell membrane damage after treatment with Lla-Met. The process was performed as previously described[20]. Briefly, G27 bacterial suspension ( $1 \times 10^8$  CFU/mL) at the logarithmic phase was incubated with Lla-Met (16 µg/mL) for 2 h, centrifuged, and the pellet was suspended in sterile PBS. The cell suspension was protected from light with FITC-labeled glucan FD4, about 4.0 kDa, with a diameter of 1.4 nm (Sigma, United States) and FD10, about 10.1 kDa, with a diameter of 2.3 nm (Sigma, United States), both at a final concentration of 100 µg/mL. After 30 min of incubation at 37 °C, the unbound fluorescent dye was washed off with sterile PBS. The cells were suspended in sterile PBS. Finally, the fluorescence influx of FD4 and FD10 was detected at the excitation and emission wavelengths of 495 nm and 520 nm, respectively, by a multimode reader (BioTek, America).

### Ion channels assay

The G27 bacterial suspension ( $1 \times 10^8$  CFU/mL) at the logarithmic phase was incubated with Lla-Met (16 µg/mL) for 2 h and centrifuged at 12000 r/min for 2 min. The changes in concentrations of extracellular K<sup>+</sup>, Na<sup>+</sup>, Cl<sup>-</sup>, and Ca<sup>2+</sup> were determined by ion-selective electrodes. Three biological repeats were performed for each experiment.

### Intracellular reactive oxygen assay

The level of intracellular ROS in the *H. pylori* cell was detected by the DC-FDH probe, as described by Akhtar *et al*[21-22]. Briefly, G27 bacterial suspension ( $1 \times 10^8$  CFU/mL) was treated with Lla-Met (8 µg/mL and 16 µg/mL) for 2 h and centrifuged to remove the supernatant. The harvested cells were protected from light DCF-DA (10 µM) for 30 min and centrifuged at 12000 r for 2 min. The excess probe was washed off with sterile PBS. Finally, the cells were resuspended in PBS solution, and the fluorescence intensity was analyzed using a multifunctional microplate reader (BioTek, America) at the excitation and emission wavelengths of 485 nm and 520 nm, respectively. The fluorescence intensity was also analyzed using a fluorescent microscope (OLYMPUS, Tokyo, Japan). PBS and polymyxin were the controls.

### Validation of how Lla-Met kills *H. pylori*

The G27 bacterial suspension was incubated with L-cys, a ROS scavenger, to evaluate the effect of ROS on the viability of G27 bacteria. Briefly, G27 bacterial suspension ( $1 \times 10^8$  CFU/mL) at the logarithmic phase was incubated with or without 40 mmol/L L-cys (which did not affect the viability of G27 cells) for 1 h and thereafter with Lla-Met (16 µg/mL) for 8 h. The optical density values were measured at OD<sub>600nm</sub> using a multifunctional microplate reader (BioTek, America). In addition, the fluorescence intensity was analyzed using fluorescence microscopy (OLYMPUS, Tokyo, Japan). G27 bacterial suspension treated with or without 40 mmol/L L-cys was incubated for 1 h. The suspension was treated with

Lla-Met (16 µg/mL) for 2 h and centrifuged to remove the supernatant. The harvested bacteria were incubated with DCF-DA (10 µM) for 30 min and washed with sterile PBS.

### DNA fragmentation assay

When DAPI passes through the intact cell membrane, it binds to bacterial DNA. The bacterial DNA thus stains blue. Damaged DNA appears as dots. Therefore, the fragmentation of *H. pylori* DNA after Lla-Met treatment was detected using the DAPI staining[23]. Briefly, the G27 bacterial suspension ( $1 \times 10^8$  CFU/mL) was incubated with or without 40 mmol/L L-cys for 1 h and thereafter with Lla-Met (16 µg/mL) for 2 h. Thereafter, the suspension was centrifuged to remove the supernatant and treated with DAPI (1 µg/mL) for 30 min. The unbound dye was washed off with PBS, and the cells were resuspended in PBS. Fluorescence intensity was determined at excitation and emission wavelengths of 358 nm and 460 nm, respectively (OLYMPUS, Tokyo, Japan), with PBS as a control.

### Phosphatidylserine ectropion assay

Phosphatidylserine (PS) is usually located on the inner side of the cell membrane. At the early stage of apoptosis, PS is translocated to the cell surface. Annexin-V, a  $Ca^{2+}$ -dependent phospholipid binding protein, bind to PS with high affinity [24]. The G27 bacterial suspension in the logarithmic phase ( $1 \times 10^8$  CFU/mL) was incubated with or without 40 mmol/L L-cys for 1 h and thereafter with Lla-Met (16 µg/mL) for 2 h. Thereafter, the suspension was centrifuged to remove the supernatant, and the pellet was incubated with Annexin-V and incubated for 30 min. The unbound dye was washed off with PBS, and the cells were resuspended in PBS. The fluorescence intensity was analyzed at excitation and emission wavelengths of 490 nm and 520 nm, respectively, using a multifunctional microplate reader (BioTek, America) and a fluorescence microscope (OLYMPUS, Tokyo, Japan), with PBS as a control.

### Transcriptome sequencing

G27 bacterial suspension ( $1 \times 10^8$  CFU/mL) ( $OD_{600} = 0.3$ ) was incubated with 2, 4, 8, and 10 µg/mL Lla-Met for 0 h, 2 h, and 8 h, and the ODs were measured at 600 nm. Three biological repeats were performed for each experiment. When the OD remained constant (0.3), the bacterial RNA was extracted for transcriptome sequencing, which was performed by Nanjing Fengzi Bio-pharm Technology. Three biological repeats were performed for each experiment.

The sequencing was performed using Illumina PE150 technology. The alignment and transcript assembly were performed using Boetie2 and the Rockhopper software. All genes were quantitatively analyzed, and the differentially expressed genes (DEGs) were identified. The biological processes and pathways regulated by the DEGs were then identified. Principal component analysis (PCA) demonstrates principal component analysis, analyzing the composition of different samples can respond to the differences and distances between samples, the more similar the sample composition, the closer the distance in the PCA graph.

### Validation of differential gene expression

Total bacterial RNA was extracted using a Novizan RNA kit, and the expression of mRNA was analyzed by a real-time fluorescent quantitative PCR instrument (Lightcycler96 fluorescent quantitative PCR instrument, Roche, Germany). The 16s was used as the reference gene. The sequences of primers used in this study are shown Table 1. Three biological repeats were performed for each experiment.

### Statistical analysis

Statistical analysis was performed using the SPSS software, Version 26.0. Continuous data were expressed as mean  $\pm$  SD. Differences between groups were analyzed using the *t*-test, while multiple groups were compared using the single factor variance analysis.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Effect of Lla-Met on *H. pylori* morphology

The impact of the Lla-Met compound on the morphology of *H. pylori* was observed using scanning electron microscopy. *H. pylori* in the control group was found to have a smooth and homogenous cell surface (Figures 1A and D). The surface of *H. pylori* in the treatment group (4 µg/mL and 8 µg/mL) was rough and swollen, and the cell damage worsened with the Lla-Met concentration (Figures 1B, C, E, and F).

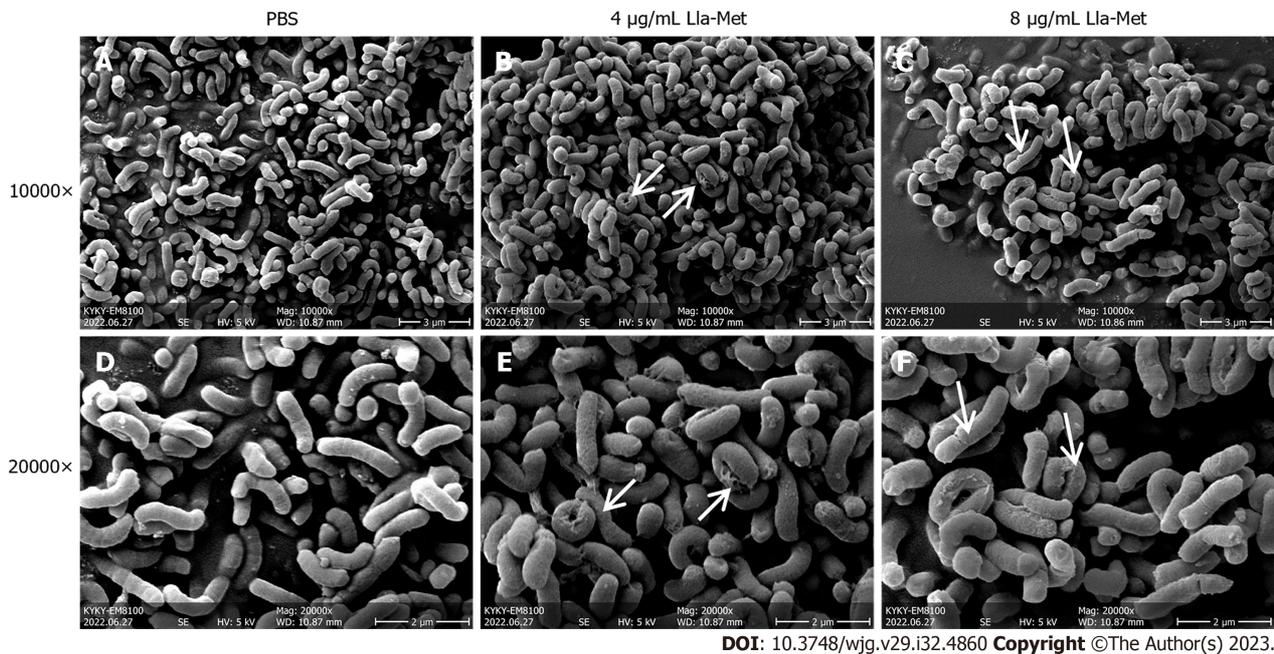
### Lla-Met impact of cell membrane integrity

PI penetrates through a damaged cell membrane, where it binds and stains the DNA. Therefore, an influx of intracellular PI represents the integrity of the bacterial cell membrane. The fluorescence intensity of PI in the G27 treatment group (16 µg/mL Lla-Met) was weaker than in the control group, though statistically insignificant (Figure 2A).

FITC-labelled glucans of different pore sizes (FD4 and FD10) were detected using a multifunctional microplate detector to examine damage to the *H. pylori* cell membrane after treatment with Lla-Met. Linolenic acid treatment had no significant effect on the permeability of *H. pylori* cells (Figures 2B and C).

**Table 1** List of primers used in this study

Name	Forward primers	Reverse primers
16s	AGGATCAAGGTTTAAGGATT	CTGGAGACTAAGCCCTCC
<i>MdaB</i>	AGGCTATGAACACGCTCAAGAAGTG	TTTCACAATCCAAGGCTCTCCCATC
<i>SodB</i>	AAGCGACTGCCTTAAGCGATGAG	TCCAGCCAGAGCCAAACAAAGTG



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**Figure 1** The effect of linolenic acid-metronidazole on *Helicobacter pylori* morphology. A: The control group shows the cell morphology at  $\times 10000$  magnification; B: The appearance of *Helicobacter pylori* (*H. pylori*) in linolenic acid-metronidazole concentrations of 4  $\mu\text{g}/\text{mL}$  shows the cell morphology at  $\times 10000$  magnification; C: The appearance of *H. pylori* in linolenic acid-metronidazole concentrations of 8  $\mu\text{g}/\text{mL}$  shows the cell morphology at  $\times 10000$  magnification; D: The control group shows the cell morphology at  $\times 20000$  magnification; E: The appearance of *H. pylori* in linolenic acid-metronidazole concentrations of 4  $\mu\text{g}/\text{mL}$  shows the cell morphology at  $\times 20000$  magnification; F: The appearance of *H. pylori* in linolenic acid-metronidazole concentrations of 8  $\mu\text{g}/\text{mL}$  shows the cell morphology at  $\times 20000$  magnification. The arrow points to the cell damage. Roughness, swelling, breakages on the cell surface, etc., are shown. PBS: Phosphate buffered saline; Lla-Met: Linolenic acid-metronidazole.

### Lla-Met impact on *H. pylori* ion channels

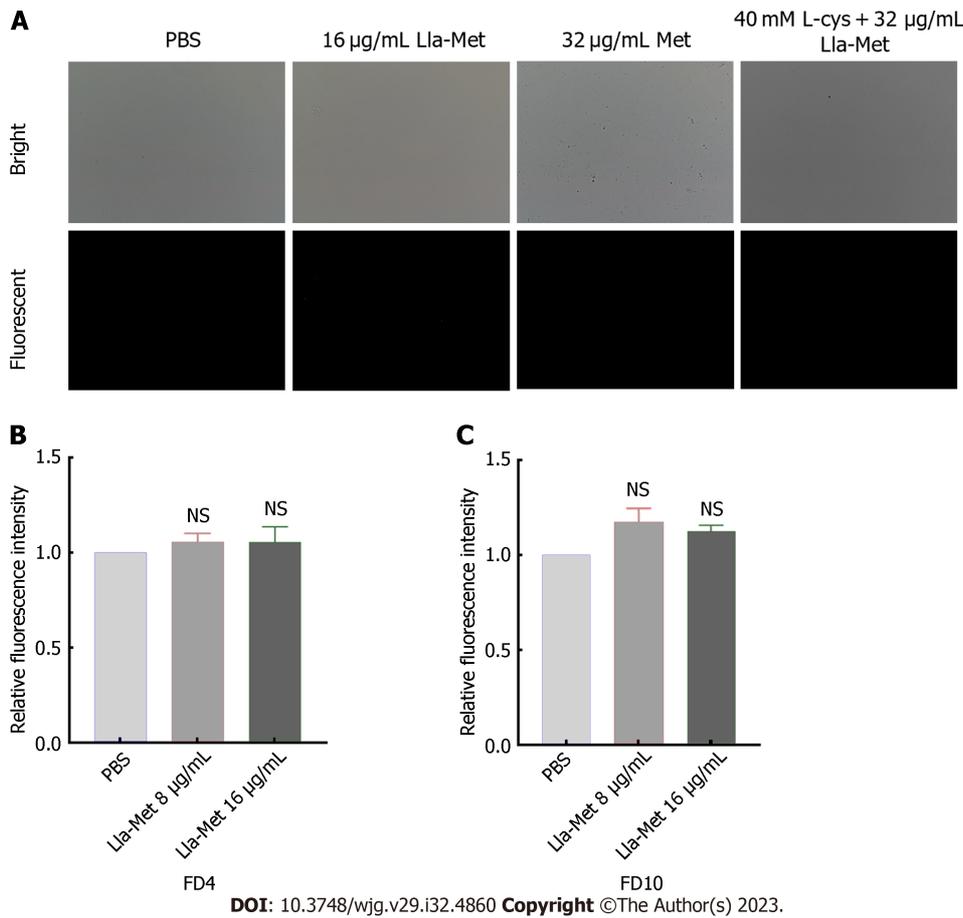
To further investigate whether Lla-Met compound penetrated the cells *via* ion channels, the concentrations of  $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{Cl}^-$ , and  $\text{Ca}^{2+}$  ions in the supernatant were measured after treating *H. pylori* with Lla-Met. There was no statistically significant difference in the concentrations of the aforementioned ions between the treatment and the control group after G27 was applied with 16  $\mu\text{g}/\text{mL}$  compound (Figures 3A and B).

### Lla-Met impact on the intracellular ROS

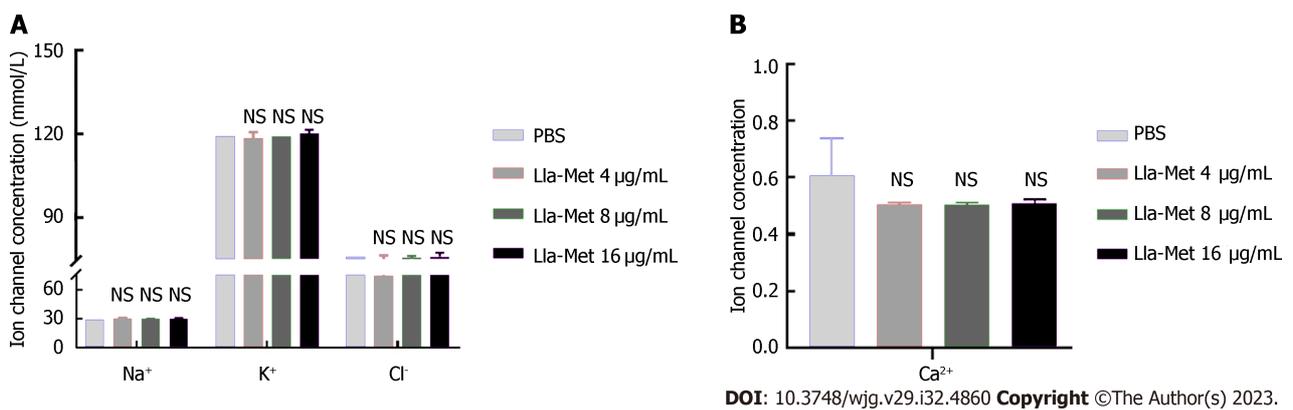
DC-FDA fluorescent probes can be used to detect whether Lla-Met compounds can accelerate intracellular oxidation reactions. Compared with the control group, 8  $\mu\text{g}/\text{mL}$  and 16  $\mu\text{g}/\text{mL}$  Lla-Met compound increased the intracellular oxidation in *H. pylori* cell. Moreover, 8  $\mu\text{g}/\text{mL}$  Lla-Met was more potent than 16  $\mu\text{g}/\text{mL}$  metronidazole (Figure 4A); The relative fluorescence intensity of *H. pylori* treated with 16  $\mu\text{g}/\text{mL}$  Lla-Met was stronger than that treated with 40  $\mu\text{g}/\text{mL}$  polymyxin B ( $P < 0.01$ , Figure 4D). However, L-cys treatment abrogated the effect of Lla-Met (Figures 4B and D,  $P < 0.01$ ), implying that L-cys abolished ROS generated by Lla-Met in *H. pylori*. In addition, L-cys pretreatment increased the cell viability from 20.5% to 57.7% (Figure 4C).

### Lla-Met induced DNA fragmentation and apoptosis

In the early stage of apoptosis, PS translocates to the cell surface, where it could be bound by  $\text{Ca}^{2+}$ -dependent phospholipid-binding protein. Thus, the phospholipid-binding protein could be used for analyzing cell apoptosis in prokaryotes. In the present study, we found that the fluorescence intensity of *H. pylori* treated with 16  $\mu\text{g}/\text{mL}$  Lla-Met was higher than that of the untreated group and the L-cys pretreatment group (Figure 5A). The multifunctional microplate labelling instrument results showed that the relative fluorescence intensity of Lla-Met-compound treatment group was significantly stronger than that of the PBS group and L-cys pretreatment group (Figure 5C,  $P < 0.0001$ ). These results indicated that linolenic-acid-metronidazole caused PS eversion, but L-cys pretreatment inhibited this



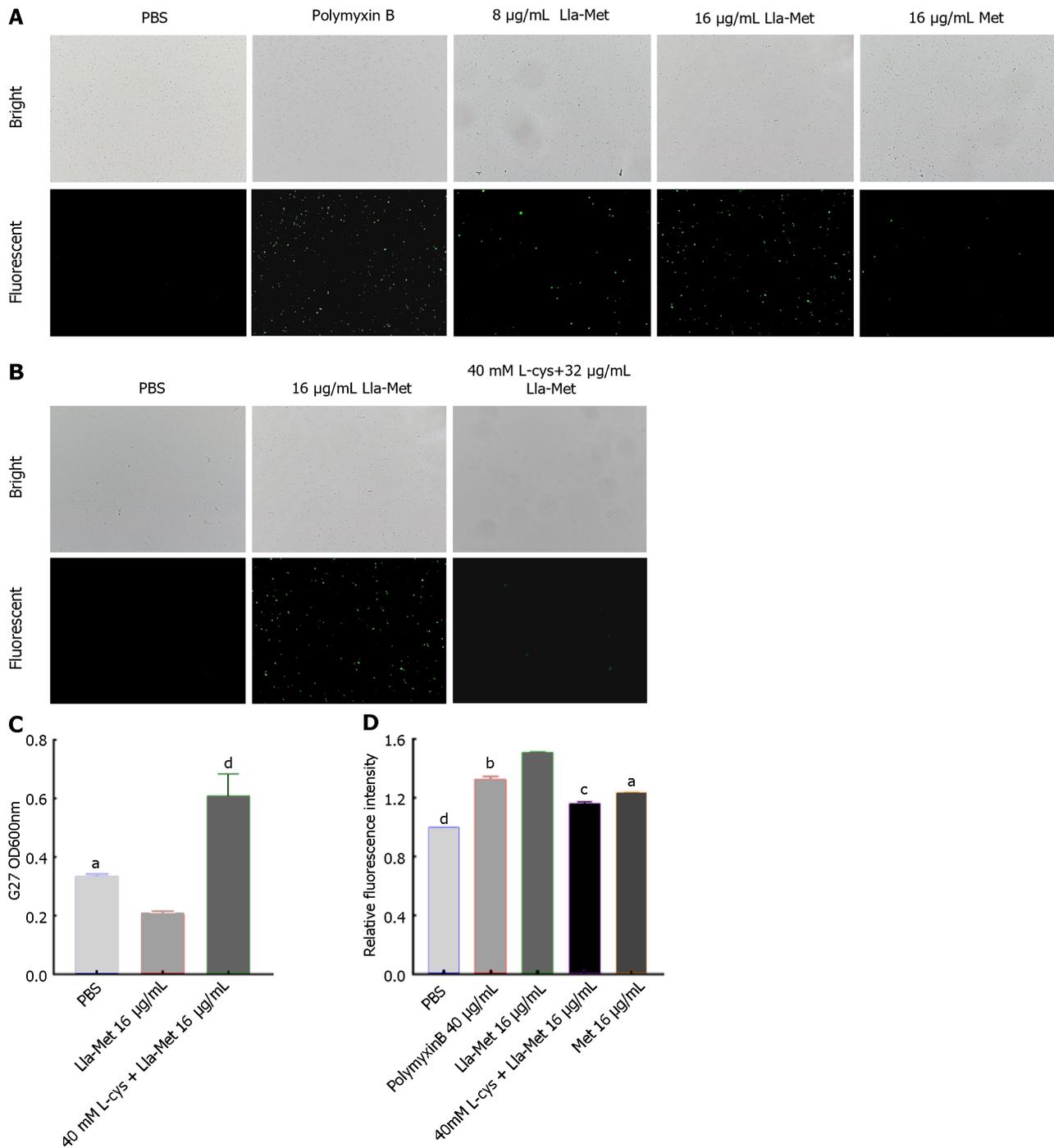
**Figure 2** The effect of linolenic acid-metronidazole on cell membrane permeability. A: Linolenic acid-metronidazole (Lla-met) induced membrane damages of *Helicobacter pylori* (*H. pylori*) using PI staining; B: Lla-met induced membrane pore size damage of *H. pylori* using FD4; C: Lla-met induced membrane pore size damage of *H. pylori* using FD10. PI, FD4 and FD10 can't pass through the intact cell membrane. NS: Not significant; PBS: Phosphate buffered saline; Lla-Met: Linolenic acid-metronidazole.



**Figure 3** The effect of the linolenic acid-metronidazole compound on *Helicobacter pylori* ion channels. A: K<sup>+</sup>, Na<sup>+</sup>, Cl<sup>-</sup>, and Ca<sup>2+</sup> ion channels; B: Ca<sup>2+</sup> ion channels. NS: Not significant; PBS: Phosphate buffered saline; Lla-Met: Linolenic acid-metronidazole.

phenomenon.

Subsequently, cellular DNA fragmentation serves as a marker of late apoptosis. The DAPI staining evaluated whether Lla-Met compound could cause the fragmentation of *H. pylori* DNA. The results showed that 16 µg/mL Lla-Met caused the fragmentation of bacterial *H. pylori* DNA (Figure 5B red circles represent the fragmented DNA). However, DNA fragmentation was inhibited in the L-cys treatment group (Figure 5D,  $P < 0.0001$ ). These findings suggested that Lla-Met caused the fragmentation of *H. pylori* DNA by inducing the accumulation of ROS.

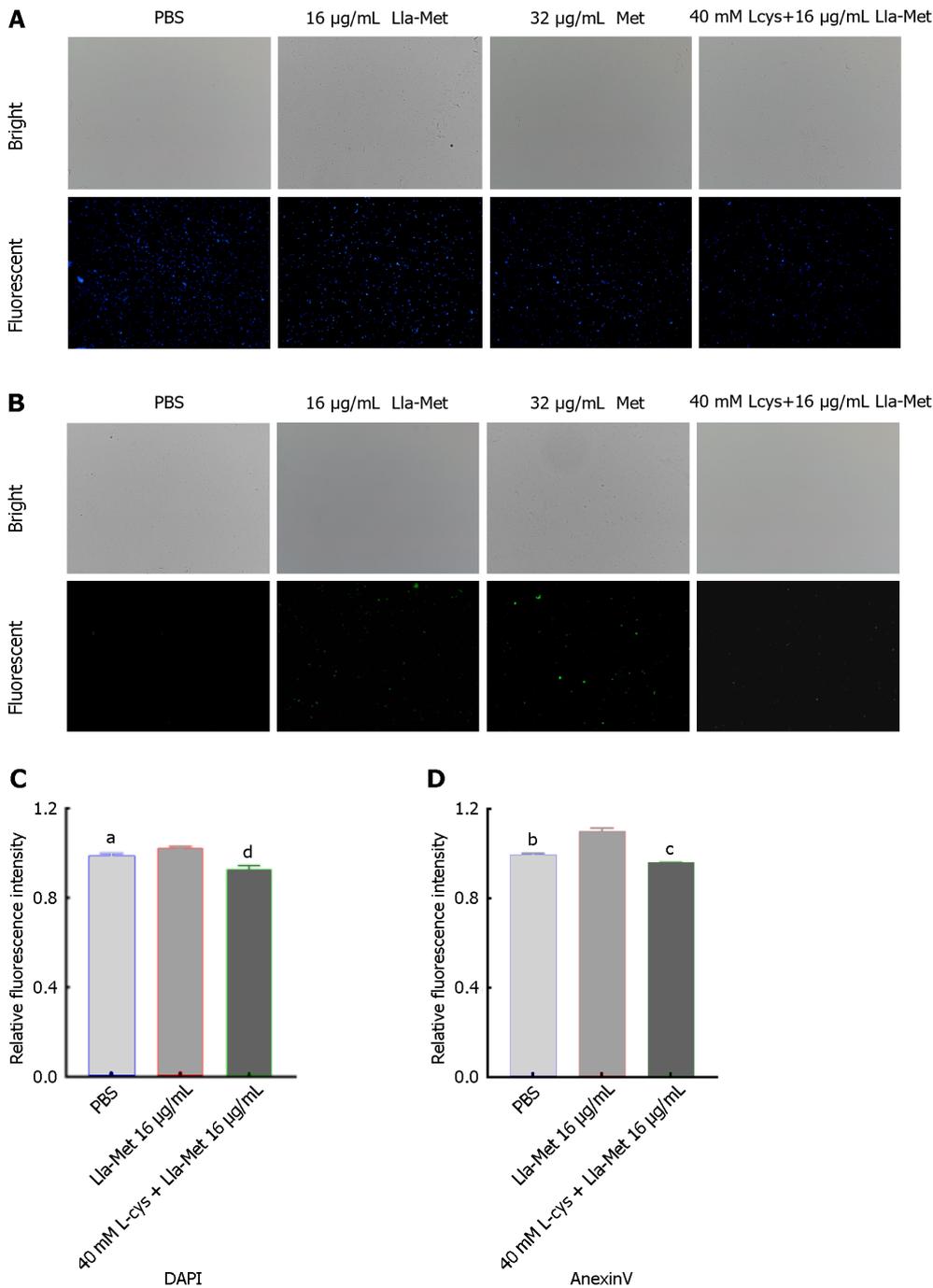


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**Figure 4** The effect of linolenic acid-metronidazole on the intracellular reactive oxygen species content in *Helicobacter pylori*. A: Fluorescent microscopy for the effect of linolenic acid-metronidazole on the production of reactive oxygen species (ROS); B: The effect of L-cysteine (L-Cys) on intracellular production of ROS; C: The effect of L-cys on cell viability; D: Effect of L-cys on ROS. <sup>a</sup>*P* < 0.05; <sup>b</sup>*P* < 0.01; <sup>c</sup>*P* < 0.001; <sup>d</sup>*P* < 0.0001. PBS: Phosphate buffered saline; Lla-Met: Linolenic acid-metronidazole; L-Cys: L-cysteine.

### Lla-Met upregulates MdaB, SodB expression

The half inhibitory concentration of Lla-Met was used for the oxidation analysis (Figure 6A). The OD values were unchanged after *H. pylori* was dosed with 8 µg/mL Lla-Met compound for 0, 4, and 8 h. The RNA-seq data for *H. pylori* in different treatment groups (Figure 6B). The closer the Pearson correlation coefficient approaches 1, the higher the similarity of events. The PCA is in Figure 6C. The difference and distance between samples are illustrated. The closer the similarity between samples, the closer the distance in the PCA diagram. The Venn diagram, which shows the DEGs in each group. A total of 1130 DEGs were detected between A\_M\_1 and A\_M\_2, of which 575 were up-regulated and 555 were down-regulated. A total of 1016 DEGs were detected between A\_M\_1 and A\_M\_3, of which 488 were up-regulated, and 528 were down-regulated. A total of 533 DEGs were detected between A\_M\_2 and A\_M\_3, including 265 up-regulated genes and 268 down-regulated genes. Among them, 344 genes were co-expressed in A\_M\_1, A\_M\_2, and



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**Figure 5** Detection of phosphatidylserine ectropion and DNA fragmentation of *Helicobacter pylori*. A and C: Fluorescence microscope detection of phosphatidylserine ectropion; B and D: Multifunctional enzyme labeler detection of bacterial DNA fragmentation. <sup>a</sup>*P* < 0.05; <sup>b</sup>*P* < 0.01; <sup>c</sup>*P* < 0.001; <sup>d</sup>*P* < 0.0001. PBS: Phosphate buffered saline; Lla-Met: Linolenic acid-metronidazole; L-Cys: L-cysteine.

A\_M\_3 (Figure 6D). The gene set enrichment analysis and Gene Ontology of the DEGs. The DEGs were divided into three main categories: Those that regulate biological processes, secretion of cellular components, and molecular function. The differential genes between the groups are primarily concentrated in tRedox pathways, metabolic processes and other pathways. Gene set enrichment analysis revealed that the DEGs regulated the REDOX and the metabolism pathway (Figure 6E). Lla-Met is inducing production of ROS in *H. pylori*, and therefore an increased expression of MdaB and SodB, both of which are associated with protection against the oxidative stress (Table 2). RT-qPCR and transcriptome sequence analyses revealed comparable findings (Figure 6F).

**Table 2 Gene affected by linolenic acid-metronidazole treatment**

Gene	Name	Log <sub>2</sub> fold change	Description	Enrichment pathway
<i>MdaB</i>	HPG27_RS03065	4.962	Flavodoxin family protein	Oxidoreductase activity
<i>SodB</i>	HPG27_RS05265	4.2287	Superoxide dismutase	Oxidoreductase activity

## DISCUSSION

Lla-Met compound is synthesized from linolenic acid and metronidazole. Linolenic acid is an essential fatty acid with broad-spectrum antibacterial spectrum and antioxidant activities and capability to overcome *H. pylori* resistance to antibiotic treatment. In addition, with more functional groups, it can react with various substances to form related derivatives, which are widely used in the anti-infection treatment. Obonyo *et al*[25] suggested that the antibacterial mechanism of linolenic acid liposome against *H. pylori* is mainly to cause damage to the bacterial cell membrane. Huang *et al*[26] used linolenic acid and zinc to synthesize zinc linolenic acid with an MIC of 4-8 µg/mL to drug-resistant *H. pylori* strains. Its antibacterial mechanism is mainly to destroy cell membrane and cause accumulation of ROS, which finally leads to the death of bacteria. In this experiment, the damage caused by Lla-Met compound to the cell membrane was detected by PI, FD4, FD10 and lactate dehydrogenase activity determination. There was no change in cell membrane permeability after *H. pylori* was treated with 16 µg/mL Lla-Met compound. This result suggested that Lla-Met compound did not inhibit *H. pylori* by damaging the cell membrane.

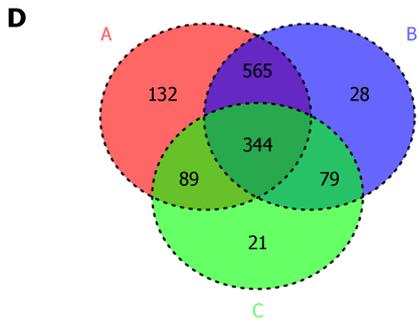
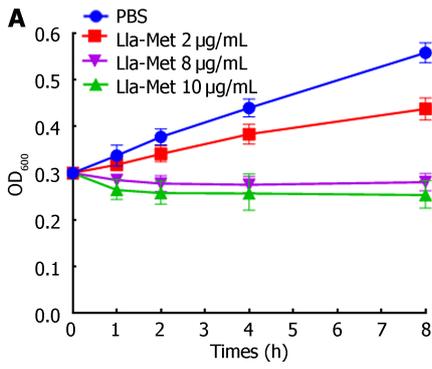
The accumulation of intracellular ROS activates eukaryotic cell apoptosis[27]. This process produces dying cells with typical morphological features, including cell shrinkage, membrane blistering, chromatin condensation, DNA fragmentation, and PS ectropion[28]. Studies have shown that apoptosis also occurs in prokaryotic cells, and is characterized with similar morphological characteristics as those in eukaryotes, such as the destruction of bacterial membrane integrity, DNA fragmentation, and PS ectropion[22,29]. Therefore, in the present study, we investigated whether Lla-Met compound could inhibit *H. pylori* growth by causing oxidative damage. In the experiment, 8 µg/mL Lla-Met compound was found to produce a stronger fluorescence signal compared with the control group. In addition, at a higher dose of 16 µg/mL Lla-Met, *H. pylori* produced a stronger fluorescence signal compared with the positive control group. Indicated that Lla-Met compound could increase accumulation of ROS in *H. pylori* in a dose-and time-dependent manner. This experiment also investigated whether intracellular ROS accumulation could affect *H. pylori* viability. The results showed that excessive accumulation of ROS could affect viability of *H. pylori* by reducing it to 20.5%, which increased to 57.7% when *H. pylori* was treated with 40 mmol/L L-cys. Interestingly, the accumulation of intracellular ROS was also found to significantly decrease after *H. pylori* was treated with L-cys. This result suggested that Lla-Met compound could cause excessive accumulation of intracellular ROS, leading to a decrease in cell viability, and that ROS accumulation could be reversed by L-cys treatment. In addition, after treatment with Lla-Met compound at different concentrations for 24 h, *H. pylori* surface became rough and swollen compared with the control group. As previously demonstrated, Lla-Met compound caused no damage to the cell membrane of *H. pylori*. This suggested that the death of *H. pylori* was due to the accumulation of intracellular ROS caused by Lla-Met compound.

Oxidation can cause both prokaryotic and eukaryotic cell death. In the present study, treatment with 16 µg/mL Lla-Met for 2 h was found to cause *H. pylori* DNA fragmentation and PS ectropion. Meanwhile, these effects were found to be reversed after *H. pylori* was treated with L-cys. DNA damage and membrane depolarization are characteristic changes in eukaryotic cell apoptosis[30]. Our experimental results showed that *H. pylori* cell death is similar to eukaryotic apoptosis, and ROS accumulation could induce prokaryotic cell-like death. However, compared with other studies, significant damage to the cell membrane was not found in the present study. This result indicated that damage to the integrity of the cell membrane might not be as necessary in the apoptosis of prokaryotic cells as slight DNA fragmentation and PS ectropion.

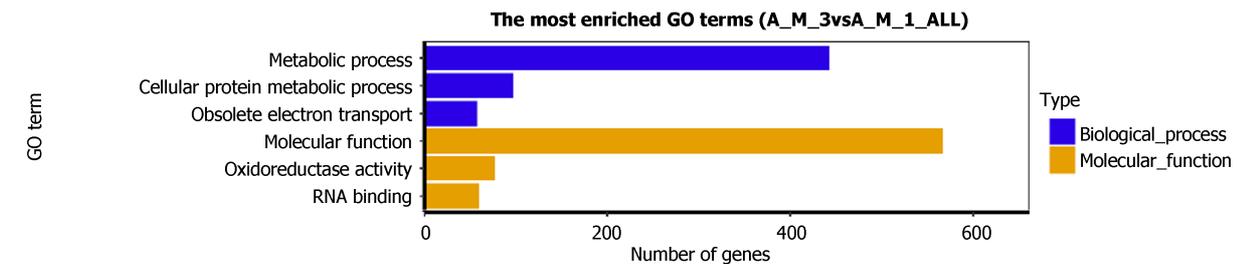
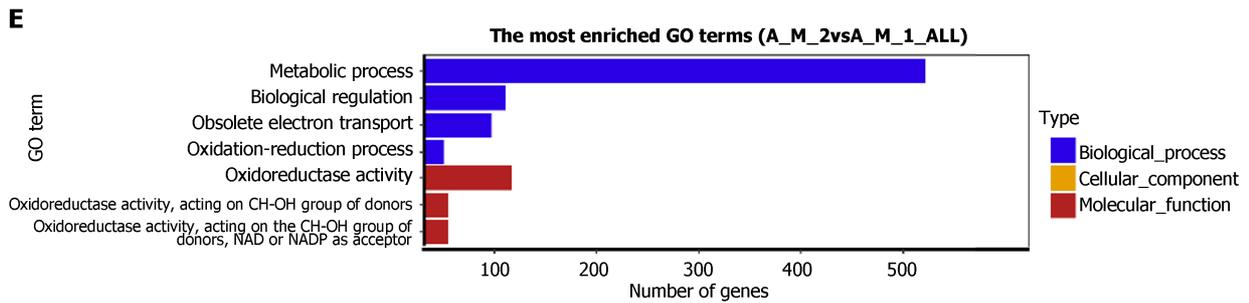
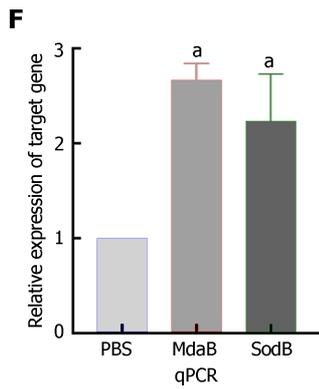
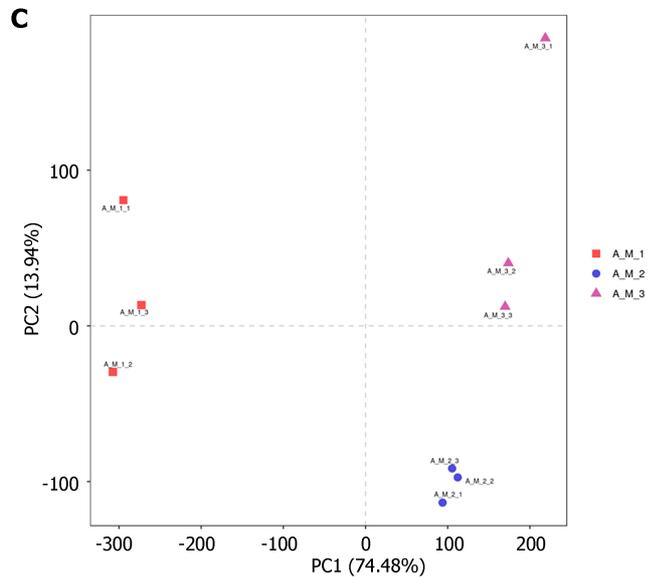
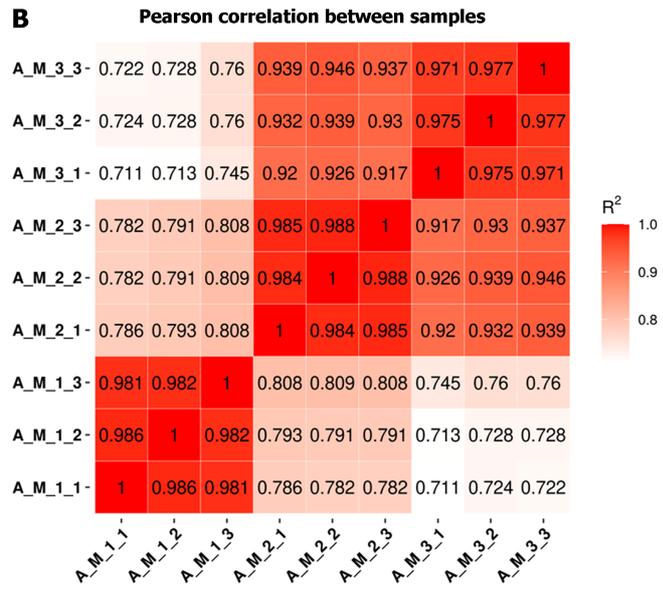
These results indicated that Lla-Met compound can promote intracellular ROS-generation reaction in *H. pylori* and effectively inhibit its growth. However, since several enzymes are involved in ROS-generating reaction, we used RT-qPCR to identify and verify key enzymes involved in this process and detect transcriptome changes. The results revealed that superoxide dismutase *MdaB* and *SodB* genes were found to play an important role. Under normal circumstances, the intracellular oxidative system and antioxidant system are in a dynamic balance. However, after treatment of with linolenic acid-metronidazole compound, superoxide dismutase *MdaB* and *SodB* genes were found to be highly expressed in *H. pylori*, and intracellular ROS was found to accumulate excessively, thereby damaging DNA and causing PS ectropion.

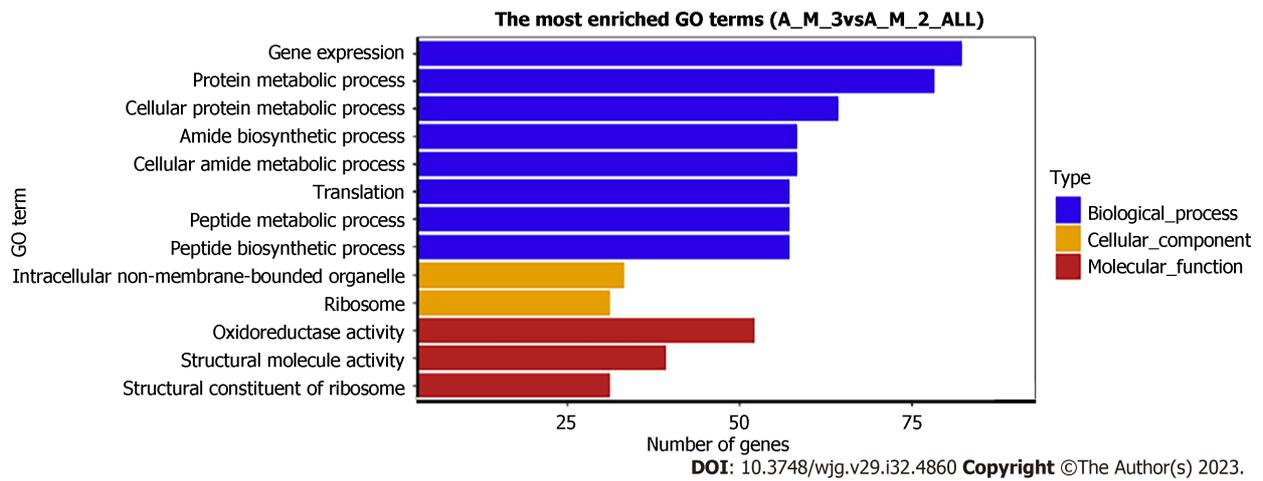
## CONCLUSION

In this paper, the mechanism of linoleic-metronidazole compound was demonstrated to involve inhibiting *H. pylori* growth by inducing excessive ROS accumulation, resulting in excessive superoxide dismutase *MdaB* and *SodB* genes expression (Figure 7). Besides, this study further proves the antibacterial effect of Lla-Met on *H. pylori* at the molecular level, providing theoretical support for further research and development of Lla-Met as an anti-*H. pylori* drug to help overcome *H. pylori* resistance to current antibiotic drugs.

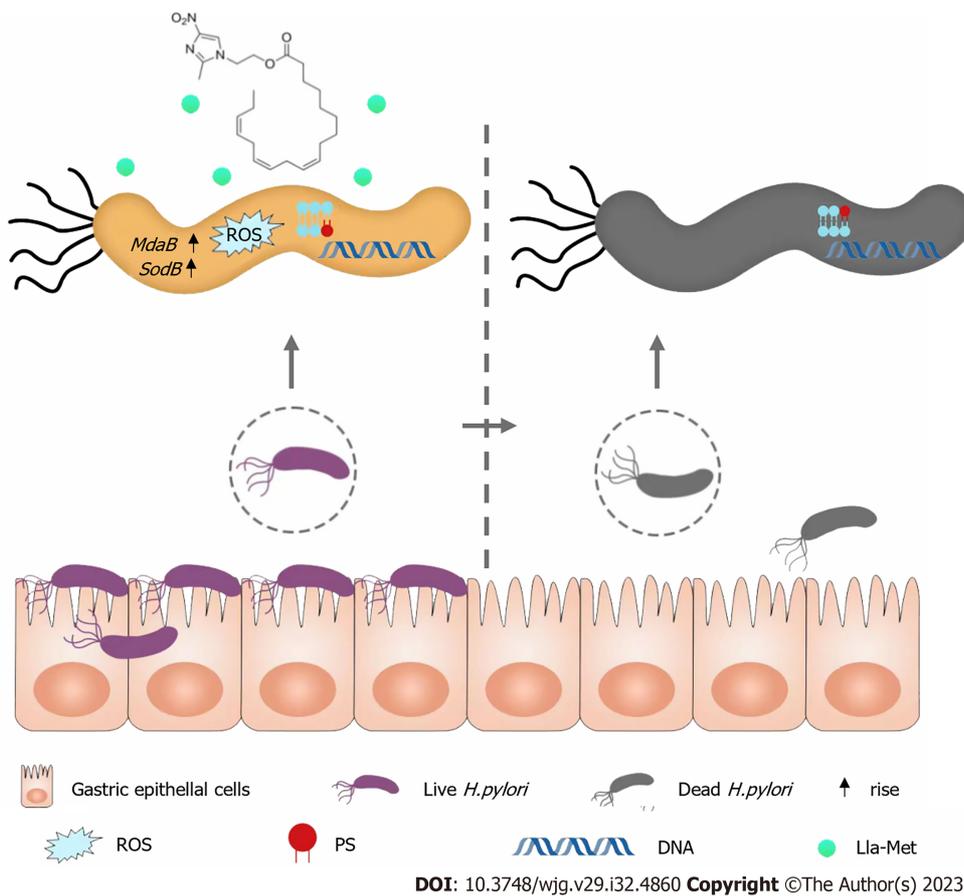


A: A\_M\_2vsA\_M\_1 B: A\_M\_3vsA\_M\_1 C: A\_M\_3vsA\_M\_2





**Figure 6** Linolenic acid-metronidazole up-regulates the expression of superoxide dismutase. A: The half inhibitory concentration of linolenic acid-metronidazole; B: RNA-seq quality data; C: The principal component analysis; D: Venn diagram; E: Gene set enrichment analysis; F: Up-regulates the expression of genes. <sup>a</sup>*P* < 0.05. PBS: Phosphate buffered saline; Lla-Met: Linolenic acid-metronidazole; qPCR: Quantitative polymerase chain reaction; GO: Gene Ontology.



**Figure 7** Hypothesized model of the mechanism of linolenic acid-metronidazole against *Helicobacter pylori*. ROS: Reactive oxygen species; PS: Phosphatidylserine; Lla-Met: Linolenic acid-metronidazole; *H. pylori*: *Helicobacter pylori*.

## ARTICLE HIGHLIGHTS

### Research background

*Helicobacter pylori* (*H. pylori*) is recognized as an important human pathogen associated with superficial gastritis, atrophic gastritis, gastric cancer, *etc.*, each of which has become a serious threat to human health and survival. The rate of drug resistance is increasing due to the wide use of antibiotics and high rates of resistance to clarithromycin, metronidazole, and levofloxacin are associated with the failure of *H. pylori* eradication. At present, the mechanism of antibiotic resistance

of *H. pylori* is not completely understood. It is very difficult to prevent drug resistance and improve the rate of eradication of the target, thus warranting exploration of the mechanism of drug resistance to *H. pylori*, and provision of an experimental basis for the prevention and treatment of drug resistance.

### Research motivation

Currently, there is a serious drug resistance situation in *H. pylori* and new antibiotics are urgently needed; however, antibiotic research and development are very difficult. If we can understand the antibacterial mechanism of linolenic acid-metronidazole (Lla-Met), we can better apply it to antimicrobial treatment and solve the problem of antibiotic resistance.

### Research objectives

The objectives of this study were to confirm the antibacterial effect of Lla-Met on *H. pylori*, and to provide theoretical support for further research and development of Lla-Met as an anti-*H. pylori* drug, and to help overcome the resistance of *H. pylori* to existing antibiotic drugs.

### Research methods

*H. pylori* cells were treated with the Lla-Met compound, and the effect of the compound on the cell morphology, cell membrane permeability, and oxidation of the bacteria cell was assessed by scanning electron microscope, propidium iodide staining, FIFC-FD, detection of ion channels, detection of intracellular reactive oxygen species, and detection of phosphatidylserine ectropion. Meanwhile, the differently expressed genes in *H. pylori* in response to Lla-Met treatment were identified by transcriptome sequencing and quantitative real-time polymerase chain reaction.

### Research results

The expression of both *SodB* and *MdaB* genes was up-regulated after treatment with Lla-Met, and both genes are associated with antioxidants. Lla-Met inhibits the growth of *H. pylori* through oxidation.

### Research conclusions

The mechanism of linoleic-metronidazole compound was demonstrated to involve inhibiting *H. pylori* growth by inducing excessive reactive oxygen species accumulation, resulting in excessive superoxide dismutase *MdaB* and *SodB* genes expression.

### Research perspectives

This study proves the antibacterial effect of Lla-Met on *H. pylori* at the molecular level, providing theoretical support for further research and development of Lla-Met as an anti-*H. pylori* drug to help overcome *H. pylori* resistance to current antibiotic drugs.

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## FOOTNOTES

**Author contributions:** Zhou WT and Dai YY consulted literature, performed experiments, collected and analyzed data and wrote the first draft, making equal contributions to this work; Liao LJ, Yang SX, and Chen H corrected it; Zhao LJ, Huang YQ, and Huang L designed, checked, modified, and completed the manuscript, making equal contributions to this work as co-corresponding authors; Huang L is the first corresponding author; and all authors approved the final version of the article.

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**Country/Territory of origin:** China

**ORCID number:** Wen-Ting Zhou 0009-0004-0575-1126; Yuan-Yuan Dai 0000-0002-5522-4154; Li-Juan Liao 0000-0003-0547-2026; Shi-Xian Yang 0009-0001-4377-4562; Hao Chen 0000-0003-0760-3552; Juan-Li Zhao 0000-0003-0485-0179; Yan-Qiang Huang 0000-0002-0867-0178; Liang Huang 0000-0001-7937-8150.

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