

# Effect of BCP Gene Silencing on Bacterial Survival Under Oxidative Stress Conditions

Sheama Alali<sup>1</sup>, Khalid Alyodawi<sup>2\*</sup>, Mohammed Altwirgai<sup>3</sup>

<sup>1</sup> Department of Microbiology, College of Medicine, University of Wasit, Wasit, Iraq

<sup>2</sup> Department of Anatomy and Biology, College of Medicine, University of Wasit, Wasit, Iraq

<sup>3</sup> Department of Biotechnology, College of Sciences, Taif University, Saudi Arabia

## ABSTRACT

Oxidative stress poses a significant challenge to bacterial survival, with resistance to reactive oxygen species (ROS) being critical for bacterial pathogenesis. The *bcp* gene, encoding a bacterioferritin comigratory protein with thioredoxin-dependent peroxidase activity, is hypothesized to play a key role in oxidative stress defense. This study examines the impact of *bcp* gene silencing on the oxidative stress resistance of *Bartonella henselae*, a pathogen known for its intracellular survival. Gene knockdown experiments utilizing a *bcp*-specific antisense construct revealed significantly increased sensitivity of *B. henselae* to hydrogen peroxide ( $H_2O_2$ ), leading to reduced growth under oxidative stress conditions. Additionally, silencing of *bcp* heightened the bacterium's susceptibility to oxidative stress induced by methyl viologen dichloride hydrate, a potent superoxide generator. These findings underscore the essential role of the *bcp* gene in mediating *B. henselae* resistance to oxidative stress and suggest its involvement in the bacterium's pathogenic mechanisms. Further exploration of the downstream effects of *bcp* silencing may uncover novel therapeutic targets for managing *B. henselae* infections.

**Keywords:** Bcp Gene, Bartonella Henselae, Oxidative Stress Resistance, Gene Silencing

## INTRODUCTION

Bacterioferritin comigratory protein (BCP) is a member of the TSA/AhpC family in *Escherichia coli* and works as thiol peroxidase. BCP is responsible for reduced linoleic acid hydroperoxide rather than  $H_2O_2$  and t-butyl hydroperoxide with the use of thioredoxin as an in vivo immediate electron donor and showed a thioredoxin-dependent thiol peroxidase activity [1].

Bacteria are exposed to a serious risk from ROS during aerobic metabolism. Through normal aerobic metabolism, these compounds are generated and cause oxidative damage to cellular components, leading to cell death through processes such as DNA damage and the peroxidation of membrane lipids, which causes functional and structural integrity loss [2, 3]. In *Bartonella* spp, no catalases or alkylhydro-reductases are generally recognized. However, a *Bcp* [1] encoding gene is well conserved, indicating *Bartonella* has the capacity to degrade peroxides using a *Bcp* enzyme. There are over 40 species of the genus *Bartonella*, with at least 14 species being infectious to humans [4]. Such species include *B. bacilliformis* [5], *B. quintana* [6], and *B. henselae* [7].

*Bartonella* spp. are Gram-negative bacteria in the  $\alpha$ -Proteobacteria class. They are obligate aerobes that are nutritionally fastidious (slow growing in vitro), requiring hemin supplements for enhanced growth and a moist atmosphere with 5-10%  $CO_2$  and a temperature of 28-37 °C [8]. *Bartonella* cells are rods or slightly curved rods. Species of the genus *Bartonella* are some of the most important pathogens in humans. They are responsible for a wide range of diseases: cat-scratch disease, trench fever, Carron's disease, bacteremia with fever, bacillary angiomatosis and peliosis, endocarditis, and neuroretinitis. *Bartonella* spp. are transmitted by specific blood-

sucking arthropods such as lice, fleas, sand flies, and mosquitoes. In mammals, the target cells are endothelial cells and erythrocytes. *Bartonella* has the ability to survive inside endothelial cells and erythrocytes and causes long-lasting intra-erythrocytic bacteremia [9]. They are considered opportunistic pathogens possessing a facultative intracellular lifestyle [10].

## MATERIALS AND METHODS

### Growth of Bartonella Henselae Under Hydrogen Peroxide Stress

*Bartonella henselae* (strain ATCC 49882/DSM 28221/Houston) was cultured in 50 ml Brucella broth supplemented with haemin and L-histidine (BBH-H) in 250 ml shake flasks. Various concentrations of  $H_2O_2$  (0, 25, 500, and 1000  $\mu M$ ) were added to the cultures, with the initial optical density (OD) adjusted to 0.05. Cultures were maintained at 37 °C and 180 rpm for 10 days. Growth and viability of *B. henselae* under hydrogen peroxide stress were monitored, and results were analyzed to determine the effect of  $H_2O_2$  concentrations on bacterial growth and viability (figure 1). *B. henselae* was grown aerobically in 50 ml of BBH-H medium with  $H_2O_2$  at 0, 25, 500 and 1000  $\mu M$  and 37 °C. Data shown are the means  $\pm$  standard error of two independent experiments, each conducted in duplicate, *P*-value 0.017

### Knockdown of Bartonella Henselae bcp Gene

Genomic DNA of *B. henselae* was extracted from cultures grown for 72 hours in brain heart infusion broth. Genomic DNA was isolated using the GeneJET™ Genomic DNA purification kit, and DNA concentration was estimated using a Nanodrop spectrophotometer (figure 2).

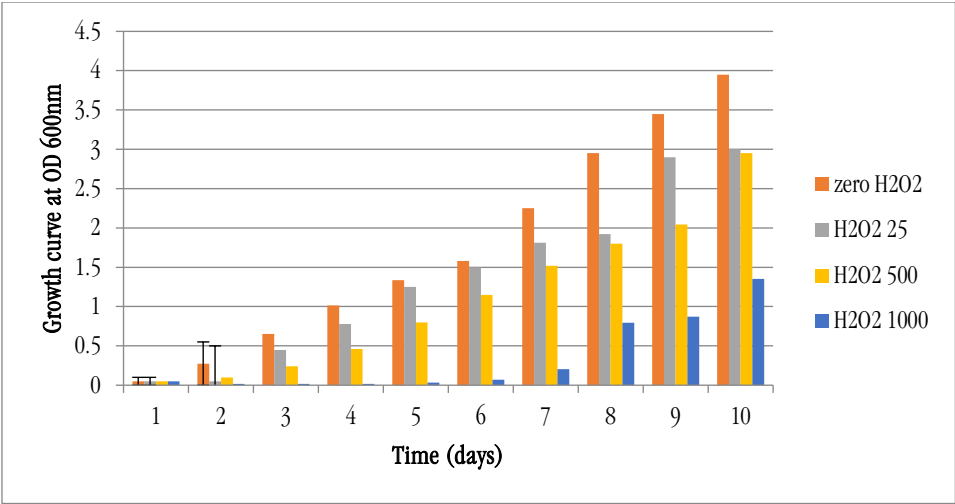


**FIGURE 1:** Effect of Hydrogen Peroxide on *B. henselae* Growth.

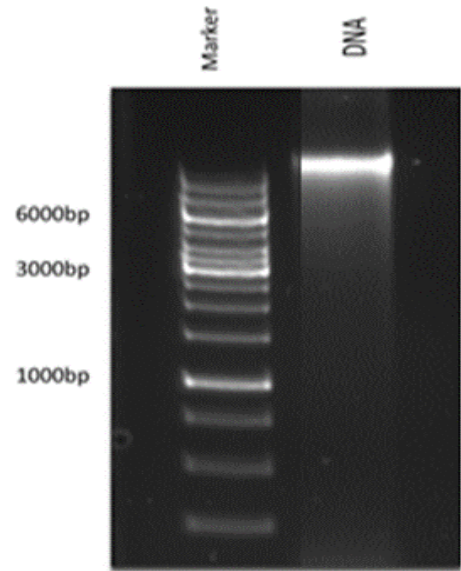


**FIGURE 2:** Electrophoretic Analysis (Agarose 0.7%) Genomic DNA of *B. henselae* Marker, 1 kb GeneRuler (Thermo Fisher Scientific).

The *bcp* gene was amplified from genomic DNA using PCR, with primers containing specific restriction sites (BamHI and XbaI). PCR conditions included denaturation, annealing, and extension steps

(figure 3) Purified PCR products were digested using BamHI and XbaI restriction enzymes, followed by purification using the GeneJET™ Gel Extraction kit (figure 4).

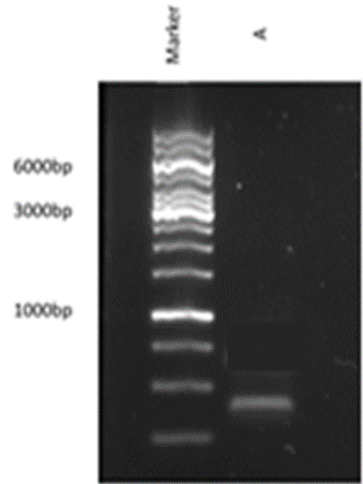


**FIGURE 3:** Electrophoretic Analysis (Agarose 0.7%) of *bcp* PCR Products. Marker, 1 kb GeneRuler (Thermo Fisher Scientific).

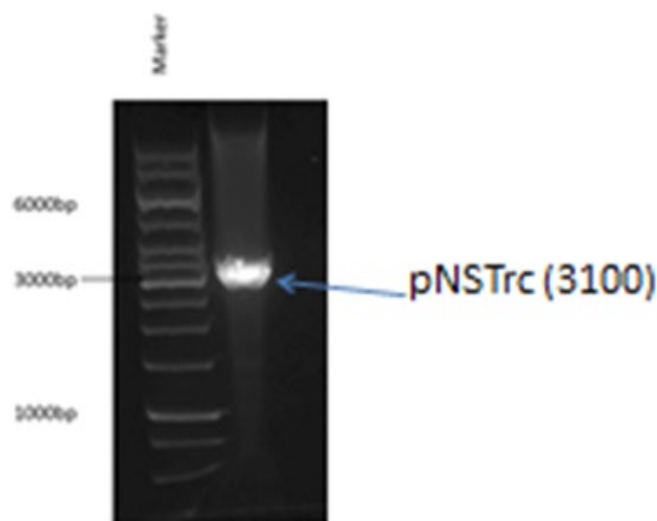


**Figure 4:** Electrophoretic Analysis (Agarose 0.7%) of pNS2Trc After Digestion by BamHI and XbaI and Purification.

Plasmid DNA was extracted from *E. coli* TOP10 cultures harboring the pNS2Trc plasmid using the GeneJET™ Plasmid Miniprep Kit. Ligation reactions were conducted with T4 ligase using vector and insert DNA, followed by transformation into chemically competent *E. coli* TOP10 cells.

Transformants containing kanamycin were selected on L-agar plates. Sequencing was performed to confirm the presence of the desired insert in plasmids (figure 5).

#### Electroporation of *B. Henselae*

*B. henselae* cells were made competent and electroporated with plasmids. Electroporated cells were cultured on selective media containing kanamycin to assess resistance after knockdown. The effect of hydrogen peroxide or Methyl viologen dichloride hydrate on the growth of *B. henselae* (pNS2Trc::bcp and pNS2Trc) was evaluated in BBH-H medium. Data analysis was performed to assess the impact of gene knockdown on bacterial growth under oxidative stress conditions.

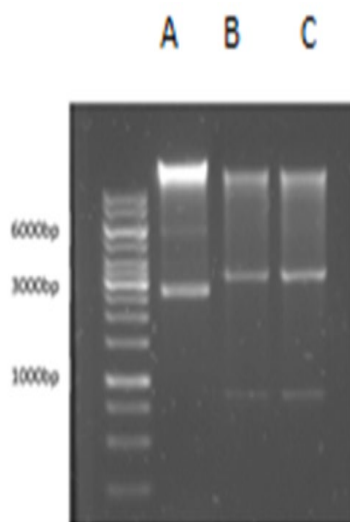


**Figure 5:** Electrophoretic Analysis (Agarose 0.7%) of bcp Knockdown Constructs.

A- pNS2Trc::revsodbcp without digestion, B, C pNS2Trc::revs bcp isolates following digestion by BamHI and XbaI

#### Statistical Analysis

Data were analyzed using GraphPad Prism, comparing growth between strains (pNS2Trc::bcp and pNS2Trc) and conditions (control vs. oxidative stress treatments) at both the 10-day endpoint of the time course and the peak of growth. Statistical significance was determined using a two-tailed unpaired t-test. Data are presented as means  $\pm$  standard error of the mean (SEM) from two independent experiments, each conducted in duplicate.

#### Results

To study effective of hydrogen peroxide as exogenous oxidative stress in *B. henselae* bcp knockdown, *B. henselae* (pNS2Trc::bcp and pNS2Trc) were grown in 50 ml Brucella broth (with haemin and L-histidine; BBH-H) in 250 ml shake flasks and H2O2 (0, 500 and 1000  $\mu$ M). The bacterial cultures were inoculated at a starting OD of 0.05 and grown at 37 °C and 180 rpm for 8 days. The result showed the *B. henselae* (pNS2Trc::bcp) were grown inhibited to 2-fold with 500  $\mu$ M and 3-fold with 1000  $\mu$ M hydrogen peroxide (figure 6).

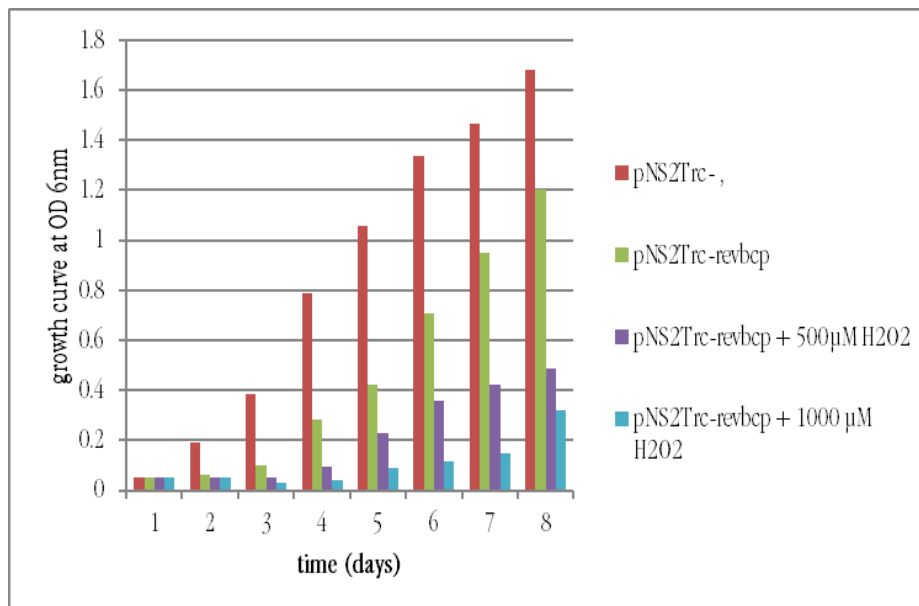


**Figure 6:** Effect of Hydrogen Peroxide on *B. henselae* Growth.

*B. henselae* (pNS2Trc-revbcp and pNS2Trc) were grown Aerobically in 50 ml of BBH-H Medium With Hydrogen Peroxide (0, 500 and 1000 µM) and at 37 °C. Data Shown are the means  $\pm$  Standard Error of two Independent Experiments, Each Conducted in Duplicate. P- Value 0.019

The growth of *B. henselae* strains carrying either pNS2Trc or pNS2Trc-revbcp plasmids was analyzed in the presence of varying concentrations of Methyl viologen dichloride hydrate (paraquat). Both strains exhibited similar growth patterns in BBH-H medium at 37°C, reaching an OD600 of approximately 3.0 by day 10 in the absence of paraquat. The addition of 10 µM paraquat to the pNS2Trc strain resulted in a slight decrease in growth, with a final OD600 of 2.0 at day 10. Interestingly, 100 µM paraquat did not significantly impact the

growth of the pNS2Trc-revbcp strain, which achieved a similar final OD600 as the untreated control. However, the pNS2Trc-revbcp strain exposed to 100 µM paraquat exhibited delayed growth, reaching an OD600 of only 1.1 by day 10. Error bars representing the standard error of the mean from two independent experiments, each conducted in duplicate, are not discernible in the figure, suggesting minimal variation between replicates (figure 7).

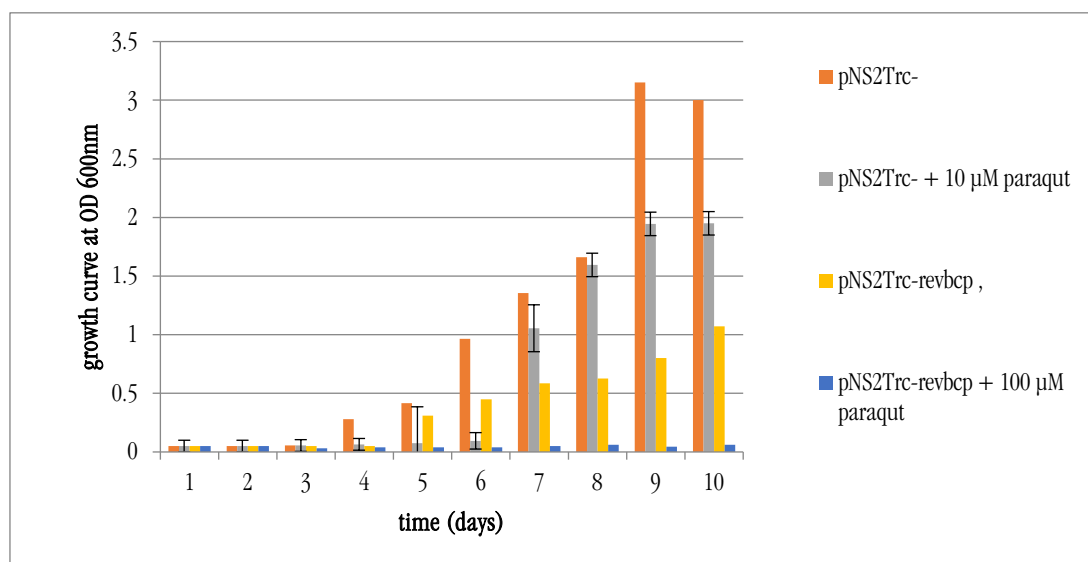


**Figure 7:** Effect of Methyl Viologen Dichloride Hydrate on *B. henselae* Growth.

*B. henselae* (pNS2Trc::bcp, and pNS2Trc) were grown aerobically in 50 ml of BBH-H medium with Methyl viologen dichloride hydrate (0 and 100 µM) and at 37 °C. Data shown are the means  $\pm$  standard error of two independent experiments, each conducted in duplicate. P- Value=0.0006

## Discussion

We have examined the responses of *Bartonella henselae* to oxidative stress by calculating its growth rate and regulating specific genes through knockdown analysis. This helped us to understand the

complex mechanisms used by the bacterium to survive environmental stresses. The simultaneous methodology of the research in which the growth of the microbe under hydrogen peroxide pressure is characterized and the selective disruption of the bcp gene involved in the oxidative stress resistance offer a thorough comprehension of

bacteria's resistance. The hydrogen peroxide resistance strength of *B. henselae* has been found to be significant, as seen in the growth analysis. Given the fact that growth was not affected up to 500  $\mu\text{M}$  peroxide, it suggests that there might be an adaptive response that limits the lethal effect when oxygen is in shortage [11]. Nonetheless, a decrease in growth at 1000  $\mu\text{M}$  means the existence of an upper concentration threshold that results in oxidative stress, which can cause the bacterium to yield, meaning the upper concentration of the oxidative stress for the growth inhibition or cell death [12, 13]. This point accentuates the critical concept that is mainly addressed by other researchers on the microbial treatment of oxidative distress, highlighting the necessity of determining the role of concentrations [14, 15]. The following experiment employed the precise RNAi technology to eliminate the vital *bcp* gene, which is located in the micro metabolism of antioxidant defence [1]. The tendency of transversing the *bcp* gene in the antisense orientation into the plasmid after strict molecular methods were chosen. This tool helped us in laying bare the consequences of lower *bcp* expression on the resilience of oxidative stress. Knockdown of the *bcp* gene via successful electroporation of *B. henselae* has significantly correlated with the bacteria's resistance to oxidative stress in laboratory conditions, observed through growth analysis. The higher peroxide concentration in *bcp* knockdown strains, which look susceptible to oxidative stress, is

another evidence that the *bcp* gene is indispensable in the oxidative stress response [16]. The research also made an exhilarating move, and its area of study broadened for Resistance Studies under Methyl viologen dichloride hydrate-induced oxidative stress. More growth patterns shown under varying concentrations of this oxidative stressor also give a clear explanation behind the mechanism specificity in the *B. henselae* response process that the bacterium is selectively damaged by hydrogen peroxide stress [17]. However, this study has contributed to clarifying the pertinent mechanism of oxidative stress of the organisms to a great extent, and therefore, further research is required in this field. Future research may be directed at exploring the downstream follower effects of *bcp* down-regulation in order to identify the specific pathways whose expressed forms are altered. Furthermore, the scope of the study can be extended to encompass other disease-causing bacteria, especially those with catalase-peroxidase metabolism, which may bear similarities too. The study here, however, is complete and holds the key to the underlying relationship between the bacterium, hydrogen peroxide-induced oxidative stress and the catalase-peroxidase gene. Through the investigation of the details of these interplays, this study develops a general awareness of the mechanisms of the bacterial stress response, with the implication that, in the end, it is useful both for essential microbiology and for the creation of therapeutic strategies targeting pathogenic bacteria.

## REFERENCES

- [1] W. Jeong, M.-K. Cha, and I.-H. Kim, "Thioredoxin-dependent hydroperoxide peroxidase activity of bacterioferritin comigratory protein (BCP) as a new member of the thiol-specific antioxidant protein (TSA)/alkyl hydroperoxide peroxidase C (AhpC) family," *Journal of Biological Chemistry*, vol. 275, no. 4, pp. 2924-2930, 2000.
- [2] S. Kanazawa, S. Sano, T. Koshihara, and T. Ushimaru, "Changes in antioxidative enzymes in cucumber cotyledons during natural senescence: comparison with those during dark-induced senescence," *Physiologia Plantarum*, vol. 109, no. 2, pp. 211-216, 2000.
- [3] K. Shah, R. G. Kumar, S. Verma, and R. S. Dubey, "Effect of cadmium on lipid peroxidation, superoxide anion generation and activities of antioxidant enzymes in growing rice seedlings," *Plant science*, vol. 161, no. 6, pp. 1135-1144, 2001.
- [4] K. Huang *et al.*, "Molecular detection of Bartonella spp. in China and St. Kitts," *Canadian Journal of Infectious Diseases and Medical Microbiology*, vol. 2019, 2019.
- [5] M. F. Minnick, B. E. Anderson, A. Lima, J. M. Battisti, P. G. Lawyer, and R. J. Birtles, "Oroya fever and verruga peruana: bartonellosis unique to South America," *PLoS neglected tropical diseases*, vol. 8, no. 7, p. e2919, 2014.
- [6] M. Arvand, D. Raoult, and E. J. Feil, "Multi-locus sequence typing of a geographically and temporally diverse sample of the highly clonal human pathogen Bartonella quintana," *PLoS one*, vol. 5, no. 3, p. e9765, 2010.
- [7] R. Chaudhry *et al.*, "Bartonella henselae infection in diverse clinical conditions in a tertiary care hospital in north India," *Indian journal of medical research*, vol. 147, no. 2, pp. 189-194, 2018.
- [8] A. Müller, M. Reiter, K. Mantlik, A.-M. Schötta, H. Stockinger, and G. Stanek, "Development of a serum-free liquid medium for Bartonella species," *Folia microbiologica*, vol. 61, pp. 393-398, 2016.
- [9] C. Dehio, "Bartonella interactions with endothelial cells and erythrocytes," *Trends in microbiology*, vol. 9, no. 6, pp. 279-285, 2001.
- [10] U. Okaro, *A Small RNA and DNA Binding Protein Contribute to Biofilm Development in Bartonella henselae*. University of South Florida, 2019.
- [11] A. F. Seixas, A. P. Quendera, J. P. Sousa, A. F. Q. Silva, C. M. Arraiano, and J. M. Andrade, "Bacterial response to oxidative stress and RNA oxidation," *Frontiers in Genetics*, vol. 12, p. 821535, 2022.
- [12] X. Du *et al.*, "Size-dependent antibacterial of carbon dots by selective absorption and differential oxidative stress of bacteria," *Journal of Colloid and Interface Science*, vol. 634, pp. 44-53, 2023.
- [13] P. Rzymiski, P. Klimaszuk, T. Jurczak, and B. Poniedzialek, "Oxidative stress, programmed cell death and microcystin release in Microcystis aeruginosa in response to Daphnia grazers," *Frontiers in Microbiology*, vol. 11, p. 553156, 2020.
- [14] K. Mathivanan, J. U. Chandirika, A. Vinothkanna, H. Yin, X. Liu, and D. Meng, "Bacterial adaptive strategies to cope with metal toxicity in the contaminated environment—A review," *Ecotoxicology and Environmental Safety*, vol. 226, p. 112863, 2021.
- [15] M. Wu, W. Shan, G.-P. Zhao, and L.-D. Lyu, "H2O2 concentration-dependent kinetics of gene expression: linking the intensity of oxidative stress and mycobacterial physiological adaptation," *Emerging Microbes & Infections*, vol. 11, no. 1, pp. 573-584, 2022.
- [16] J. Lu and A. Holmgren, "The thioredoxin antioxidant system," *Free radical biology and medicine*, vol. 66, pp. 75-87, 2014.
- [17] J. Soni, S. Sinha, and R. Pandey, "Understanding bacterial pathogenicity: a closer look at the journey of harmful microbes," *Frontiers in Microbiology*, vol. 15, p. 1370818, 2024.



This is an open-access article distributed under the terms of the Creative Commons Attribution License.