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# Comparative Study of Oxidative Stress Response Between Gram-positive and Gram-negative Bacteria

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**Abstract- This study compared the oxidative stress responses of Gram-positive and Gram-negative bacteria exposed to hydrogen peroxide (5–20 mM) and a vitamin C–ferric chloride ($FeCl_3$) system under varying environmental conditions. Using the disc diffusion method, zones of inhibition were measured for *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, and *Salmonella* spp. Gram-negative bacteria were generally more susceptible to hydrogen peroxide, particularly under aerobic conditions, while Gram-positive bacteria showed greater resistance. The vitamin C–$FeCl_3$ system demonstrated consistent broad-spectrum antibacterial activity across all strains. Environmental factors, especially oxygen availability, influenced oxidative susceptibility. Findings highlight structural and physiological differences affecting bacterial oxidative stress tolerance and suggest the potential of vitamin C–$FeCl_3$ as an adjunct antimicrobial strategy.**

**Keywords: oxidative stress, hydrogen peroxide, vitamin C, ferric chloride, Gram-positive bacteria, Gram-negative bacteria**

## I. INTRODUCTION

Oxidative stress is a critical factor influencing bacterial survival, pathogenicity, and antibiotic resistance. Despite advances in understanding individual bacterial responses, current literature lacks comprehensive comparative studies on how Gram-positive and Gram-negative bacteria differentially respond to oxidative stress. This gap limits our ability to develop targeted interventions, thereby increasing the risk of antibiotic resistance and reducing the effectiveness of current treatments.

According to O'Neill and Pozzi (2021) Staphylococcus aureus managed oxidative stress through the use of nitric oxide synthase to counter reactive nitrogen species. Bielecka and Pilecki (2020) highlight Pseudomonas aeruginosa's advanced antioxidant strategies that contribute to its survival in hostile environments. Research by Zheng et al. (2022) demonstrated that Klebsiella pneumoniae relied on regulatory proteins and metabolic pathways to mitigate oxidative damage, further complicating treatment efforts. Wiegand and Osmetti (2018) provided a comparative analysis that underscored significant differences between Gram-positive and Gram-negative bacteria, revealing substantial variations in how these pathogens manage oxidative stress.

However, despite these individual findings, there remained a significant gap in fully understanding how these oxidative stress mechanisms differ between Gram-positive and Gram-negative bacteria, and how these differences impacted bacterial virulence, survival, and antibiotic resistance across species. Without a comprehensive comparative2 analysis, treatment options remained suboptimal, and the development of effective strategies to combat bacterial infections was impeded.

This study aimed to conduct a comparative analysis of oxidative stress responses in Gram-positive and Gram-negative bacteria, focusing on key species like Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa, and Salmonella spp. The research primarily assessed bacterial enzyme activity under oxidative stress conditions, using hydrogen peroxide ($H_2O_2$) as a source of hydrogen peroxide stress and a combination of Vitamin C + $FeCl_3$ to generate superoxide anions.

Additionally, the study investigated how environmental factors such as pH, temperature, and oxygen levels influence the oxidative stress responses of these bacteria.

By evaluating these parameters, the research aimed to better understand how bacteria survive hostile conditions and how their oxidative stress defenses contributed to their pathogenicity.

By evaluating oxidative stress responses under different conditions, this research could contribute to identifying potential weaknesses in bacterial defense mechanisms. Although not directly targeting drug development, the findings could still inform future studies on therapeutic strategies and infection control. The study aimed to bridge a gap in understanding bacterial stress responses and provide foundational insights useful in addressing the growing threat of antibiotic resistance.

## II. MATERIALS AND METHODS

### Study Design and Bacterial Strains

This experimental study was conducted to compare oxidative stress responses between Gram-positive and Gram-negative bacteria. The bacterial strains included Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa, and Salmonella spp. The experiment involved exposing bacterial cultures to controlled oxidative stress conditions and observing cellular responses to determine differences in survival mechanisms, pathogenicity, and resistance patterns.

Bacterial species were selected based on their classification as Gram-positive or Gram-negative organisms and their clinical relevance. This ensured the inclusion of representative strains with documented responses to oxidative stress.

### Materials and Instruments

Mueller–Hinton agar plates were used as the culture medium for bacterial growth. Reactive oxygen species (ROS) solutions included hydrogen peroxide ($H_2O_2$) and superoxide anion ($O_2^-$) generated using Vitamin C and ferric chloride ($FeCl_3$). Sterile filter paper discs were utilized for applying oxidative agents. Additional instruments included an incubator for temperature control, a pH meter for monitoring and adjusting agar pH, a thermometer for verifying environmental temperature, sterile forceps for handling discs, and a ruler for measuring zones of inhibition. These materials were essential for evaluating bacterial responses under oxidative stress conditions.

### Induction of Oxidative Stress

Oxidative stress was induced using hydrogen peroxide at concentrations of 5 mM, 10 mM, and 20 mM. Superoxide anion was generated by combining Vitamin C and ferric chloride solutions. Sterile filter paper discs were soaked in the prepared ROS solutions and placed onto Mueller–Hinton agar plates previously inoculated with bacterial strains. Plates were incubated under controlled pH, temperature, and oxygen conditions to ensure consistent exposure to oxidative stress.

### Preparation of Culture Media

A total of 32 Petri dishes were prepared using Mueller–Hinton agar. The standard preparation consisted of 38 g of agar powder dissolved in 1000 mL of distilled water. For this study, 30.4 g of agar powder was dissolved in 800 mL of sterile distilled water. The mixture was heated until completely dissolved and then transferred into clean containers.

For pH manipulation, portions of the agar solution were adjusted using 1M hydrochloric acid (HCl) to lower the pH and 1M sodium hydroxide (NaOH) to increase the pH. A calibrated pH meter was used to achieve the desired acidic and alkaline levels. All prepared media were sterilized by autoclaving at 121°C for 15–20 minutes. After sterilization, the agar was poured into sterile Petri dishes and allowed to solidify.

### Inoculation and Application of Oxidative Agents

Bacterial strains were inoculated onto agar plates using the swab technique. Hydrogen peroxide solutions were prepared from 3% stock concentrations to obtain final molarities of 5 mM, 10 mM, and 20 mM. Vitamin C (500 mg in 100 mL sterile water) and ferric chloride solutions were prepared under laboratory conditions.

Using a micropipette, one drop of each ROS solution was applied to sterile filter paper discs. For combination treatments, Vitamin C was first applied to the disc followed by ferric chloride. Negative control discs were moistened with sterile distilled water. Prepared discs were placed onto inoculated agar plates.

### Incubation Conditions and Measurement

Plates were incubated under various environmental conditions, including standard incubation at 37°C, room temperature exposure, pH-adjusted conditions, aerobic conditions, microaerophilic conditions using a candle jar method, and anaerobic conditions using an anaerobic chamber.

After 24 hours of incubation, antibacterial activity was evaluated by measuring the diameter of zones of inhibition (ZOI) in centimeters using a transparent ruler. The clear zones surrounding the discs indicated bacterial sensitivity to oxidative stress.

Data analysis was performed by comparing measured zones of inhibition across bacterial strains, oxidative stress concentrations, and environmental conditions. Differences in susceptibility and resistance patterns were interpreted through direct comparison of inhibition diameters, enabling assessment of oxidative stress tolerance between Gram-positive and Gram-negative bacteria.

## III. RESULTS AND DISCUSSION

The experimental findings on the oxidative stress responses of selected Gram-positive and Gram-negative bacteria. The study evaluated bacterial susceptibility to hydrogen peroxide ($H_2O_2$) and a combination of Vitamin C with ferric chloride ($FeCl_3$) under varying oxygen levels, temperatures, and pH conditions by measuring zones of inhibition. Distilled water, used as a negative control, showed no inhibitory effect.

### Baseline Response to Oxidative Agents

The baseline antibacterial activity of Vitamin C with $FeCl_3$ and hydrogen peroxide against four bacterial strains without environmental stress conditions. The data provided a reference for understanding how each strain response to oxidative agents under normal laboratory conditions, allowing comparisons with altered environmental conditions such as varying oxygen levels, temperature, and pH. Both Gram-positive and Gram-negative bacteria were tested using different concentrations of hydrogen peroxide (5 mM, 10 mM, 20 mM), while the Vitamin C-$FeCl_3$ combination was tested at a single concentration.

In comparison, the effects of $H_2O_2$ varied among the strains. Staphylococcus aureus showed complete resistance at all concentrations. This finding was supported by a study from Lineback et al. (2018) which reported that S. aureus biofilms exhibited high tolerance to $H_2O_2$ due to protective enzymatic systems and structural barriers. Meanwhile, S. pyogenes exhibited a measurable response only at 5 mM, suggesting a slightly higher susceptibility under minimal stress levels.

Among the Gram-negative strains, Pseudomonas aeruginosa was the most responsive, showing inhibition zones up to 24 cm at 10 mM. This result agreed with research by Al-Shabib et al. (2017), which found hydrogen peroxide ($H_2O_2$) to be particularly effective against Gram-negative bacteria due to their thinner peptidoglycan layers and higher membrane permeability. Salmonella spp. showed moderate susceptibility, increasing with concentration, which further supports the idea that Gram-negative bacteria were more vulnerable to oxidative agents compared to Gram-positive ones.

The consistent effectiveness of the Vitamin C and $FeCl_3$ mixture also pointed to a potential synergistic mechanism. According to Shivaprasad et al. (2021), Vitamin C could act as a pro-oxidant in the presence of iron, generating reactive oxygen species such as hydroxyl radicals, which cause irreversible damage to bacterial cells. This could explain the robust activity of the combination across all tested bacterial species in this study.

**Effect of Oxygen Levels**

The antibacterial activity of $H_2O_2$ at concentrations of 5 mM, 10 mM, and 20 mM, as well as the combination of Vitamin C with $FeCl_3$, against Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa, and Salmonella spp. under varying oxygen conditions. The tested environments include aerobic, microaerophilic, and anaerobic. The zone of inhibition was used to assess bacterial susceptibility to oxidative stress under each condition.

P. aeruginosa exhibited an unusual pattern, responding only at 10 mM but not at 20 mM, which could suggest possible oxidative adaptation or stress-induced tolerance mechanisms at higher concentrations. The combination of Vitamin C with $FeCl_3$ produced strong and consistent antibacterial effects across all strains, with zones ranging from 18 to 20 cm. This suggested that the vitamin C–iron complex remains highly effective under aerobic conditions, likely due to enhanced ROS production through iron-catalyzed reactions.

These findings supported previous research by Sharma et al. (2020), who demonstrated that ascorbic acid in the presence of ferric ions could enhance oxidative stress by generating hydroxyl radicals through Fenton-like reactions. Furthermore, Al-Shabib et al. (2017) observed that hydrogen peroxide was more effective in aerobic environments where oxygen availability promoted the formation of reactive species, especially in Gram-negative bacteria like P. aeruginosa and Salmonella.

Pseudomonas aeruginosa showed strong inhibition at 20 mM hydrogen peroxide ($H_2O_2$), a response that might be attributed to its oxidative stress response mechanisms. As detailed by da Cruz Nizer et al., 2021 P. aeruginosa adapts to oxidative stress by altering gene expression and eliciting stress responses to survive under such conditions. Salmonella spp. displayed inhibition across all concentrations, though with smaller zones compared to aerobic conditions. This observation was supported by Hébrard et al., 2009, who found that Salmonella possesses multiple hydrogen peroxide-degrading enzymes, contributing to its oxidative stress resistance.

The combination of Vitamin C and $FeCl_3$ consistently showed inhibitory activity across all bacteria, with S. pyogenes exhibiting the highest sensitivity. This suggested that the redox interaction between Vitamin C and $FeCl_3$ might generate reactive species even in low-oxygen environments, maintaining antibacterial efficacy.

These findings underscored the importance of considering both bacterial species and environmental oxygen levels when evaluating oxidative stress responses and the efficacy of antimicrobial agents.

In contrast, the combination of Vitamin C and $FeCl_3$ showed notable inhibitory effects on S. aureus, S. pyogenes, and Salmonella spp., but remained ineffective against P . aeruginosa. This indicated that the redox interaction between Vitamin C and $FeCl_3$ might generate reactive species even in low-oxygen environments. According to Pervaiz et al., (2012) the Fenton-like reaction between ascorbic acid and $FeCl_3$ could lead to the formation of hydroxyl radicals even in reduced oxygen conditions, sustaining its antibacterial activity. Additionally, green synthesis studies by Saif et al. (2016) confirmed that ascorbic-acid-mediated iron complexes showed potent antimicrobial properties against both Gram-positive and Gram-negative bacteria.

## Effect of Temperature

The antibacterial effects of hydrogen peroxide ($H_2O_2$) at varying concentrations and the Vitamin C-$FeCl_3$ combination on four bacterial strains under different temperature conditions: 37°C (body temperature) and room temperature.

Pseudomonas aeruginosa exhibited strong inhibition at 20 mM, in agreement with studies by Deng et al (2014), which demonstrated that P . aeruginosa undergoes major transcriptional shifts under oxidative stress, including increased glycolysis and pentose phosphate pathway activity. Streptococcus pyogenes was resistant at lower $H_2O_2$50 concentrations and only inhibited at 20 mM, consistent with King et al. (2000), who showed that S. pyogenes possessed an inducible peroxide resistance mechanism regulated by the PerR repressor. The combination of Vitamin C and $FeCl_3$ produced inhibition zones ranging from 18 cm to 20 cm across all bacteria tested, suggesting that the redox interaction between ascorbate and ferric ions might lead to the formation of reactive species that remain effective even in reduced oxygen conditions.

Despite the reduced efficacy of hydrogen peroxide ($H_2O_2$) at room temperature, all tested bacteria remained susceptible to the Vitamin C–$FeCl_3$ combination, albeit with slightly diminished inhibition zones ranging from 13 mm to 20 mm. This consistent antibacterial activity suggested that the redox interaction between ascorbic acid and ferric ions generates reactive species capable of exerting bactericidal effects even at lower temperatures. Supporting this, Ali et al. (2021) demonstrated that Vitamin C maintains significant antibacterial activity across various temperatures and pH levels, effectively inhibiting both Gram-positive and Gram-negative bacteria.

## Effect of pH Conditions

All bacterial strains exhibited no sensitivity to hydrogen peroxide ($H_2O_2$) or the Vitamin C–$FeCl_3$ complex under acidic conditions, except for P .52 aeruginosa and Salmonella spp., which showed mild inhibition zones of 8 cm and 6 cm respectively with the Vitamin C combination. These results suggested that highly acidic environments might reduce the antibacterial activity of both oxidative agents. According to Espinoza-Vergara et al. (2021) the enzymatic activity of catalase, which decomposes hydrogen peroxide ($H_2O_2$), exhibits maximum activity at pH 7.5; at lower pH levels, the decomposition of hydrogen peroxide ($H_2O_2$) was less efficient, potentially reducing its antibacterial efficacy.

Exposure to an alkaline environment produced more noticeable effects compared to acidic conditions. S. aureus and S. pyogenes showed weak inhibition with $H_2O_2$ at higher concentrations, and all bacteria showed some level of susceptibility to the Vitamin C–$FeCl_3$ combination, ranging from 4 cm to 10 cm. These findings were supported by Espinoza-Vergara et al. (2021), who reported that the enzymatic activity of catalase was optimal around pH 7.5, and deviations from this pH can affect the decomposition rate of hydrogen peroxide ($H_2O_2$), thereby influencing its antibacterial activity. These findings suggested that oxidative agents were more effective in basic environments than in acidic ones.

The combination of Vitamin C with ferric chloride consistently produced large zones of inhibition across all strains, indicating broad-spectrum effectiveness. In contrast hydrogen peroxide showed selective and environment-dependent activity, with Gram-negative bacteria generally more sensitive under aerobic and microaerophilic conditions. Gram-positive bacteria were more resistant to hydrogen peroxide but responded well to the Vitamin C–$FeCl_3$ combination, particularly under anaerobic and alkaline environments. These results highlighted the differential oxidative stress responses between Gram groups and emphasized the superior and stable performance of the Vitamin C–$FeCl_3$ complex.

**Table 1 - Comparative Zone of Inhibition of Gram-Positive and Gram-Negative Bacteria under Various Environmental Conditions**

| Condition | Oxidative Agent | S. aureus (G+) | S. pyogenes (G+) | P. aeruginosa (G-) | Salmonella spp. (G-) |
|---|---|---|---|---|---|
| **Normal (Control)** | $H_2O_2$ 5 mM | 0 | 8 | 15 | 0 |
| | $H_2O_2$ | 0 | 6 | 24 | 11 |

| | | | | | |
|---|---|---|---|---|---|
| | 10mM | | | | |
| | $H_2O_2$ 20mM | 0 | 0 | 22 | 13 |
| | Vit. C + $FeCl_3$ | 22 | 20 | 20 | 24 |
| **Aerobic (37°C)** | $H_2O_2$ 5 mM | 0 | 0 | 0 | 8 |
| | $H_2O_2$ 10mM | 7 | 0 | 7 | 10 |
| | $H_2O_2$ 20mM | 9 | 10 | 0 | 11 |
| | Vit. C + $FeCl_3$ | 20 | 18 | 19 | 18 |
| **Microaerophilic** | $H_2O_2$ 5 mM | 0 | 0 | 0 | 8 |
| | $H_2O_2$ 10mM | 9 | 0 | 8 | 3 |
| | $H_2O_2$ 20mM | 6 | 0 | 20 | 4 |
| | Vit. C + $FeCl_3$ | 16 | 22 | 14 | 18 |
| **Anaerobic** | $H_2O_2$ 5 mM | 0 | 0 | 0 | 0 |
| | $H_2O_2$ 10mM | 0 | 11 | 0 | 0 |
| | $H_2O_2$ 20mM | 0 | 10 | 0 | 0 |
| | Vit. C + $FeCl_3$ | 19 | 18 | 0 | 19 |
| **Room Temperature** | $H_2O_2$ 5 mM | 0 | 0 | 0 | 0 |
| | $H_2O_2$ 10mM | 0 | 0 | 0 | 0 |
| | $H_2O_2$ 20mM | 6 | 0 | 0 | 0 |
| | Vit. C + $FeCl_3$ | 15 | 13 | 20 | 13 |
| **Acidic pH (HCl)** | $H_2O_2$ (All Conc.) | 0 | 0 | 0 | 0 |
| | Vit. C + $FeCl_3$ | 0 | 0 | 8 | 6 |
| **Alkaline pH (NaOH)** | $H_2O_2$ 5 mM | 0 | 0 | 0 | 0 |
| | $H_2O_2$ 10mM | 0 | 6 | 0 | 0 |
| | $H_2O_2$ 20mM | 6 | 2 | 0 | 0 |
| | Vit. C + $FeCl_3$ | 7 | 10 | 6 | 4 |

Table 1 summarized the comparative antibacterial responses of Gram-positive and Gram-negative bacteria under various oxidative agents and environmental conditions. The combination of Vitamin C with ferric chloride consistently produced large zones of inhibition across all strains, indicating broad-spectrum effectiveness. In contrast, hydrogen peroxide showed selective and environment-dependent activity, with Gram-negative bacteria generally more sensitive under aerobic and microaerophilic conditions. Gram-positive bacteria were more resistant to hydrogen peroxide but responded well to the Vitamin C–$FeCl_3$ combination, particularly under anaerobic and alkaline environments. These results highlighted the differential oxidative stress responses between Gram groups and emphasized the superior and stable performance of the Vitamin C–$FeCl_3$ complex.

Gram-negative bacteria, Pseudomonas aeruginosa and Salmonella spp., demonstrated greater susceptibility to oxidative stress, particularly to $H_2O_2$, under both aerobic and microaerophilic conditions. Salmonella consistently showed the largest zones of inhibition across multiple conditions and concentrations, especially at 37°C and in the presence of Vitamin C with $FeCl_3$. In contrast, P. aeruginosa showed variable responses to hydrogen peroxide ($H_2O_2$), with increased sensitivity observed under microaerophilic and aerobic conditions but minimal inhibition under anaerobic and room temperature environments.

Gram-positive bacteria, Staphylococcus aureus and Streptococcus pyogenes, were generally more resistant to $H_2O_2$, particularly at lower concentrations and in oxygen-deprived settings. However, both species remained susceptible to the combination of Vitamin C and $FeCl_3$ under most tested conditions, including anaerobic and alkaline environments. Notably, S. pyogenes exhibited complete resistance to hydrogen peroxide ($H_2O_2$) under microaerophilic conditions but still responded effectively to the Vitamin C and $FeCl_3$ treatment.

The combination of Vitamin C and $FeCl_3$ consistently exhibited broad-spectrum antibacterial activity across all strains, regardless of oxygen level, temperature, or pH. This suggested that its mechanism of action might involve both oxidative and non-oxidative pathways, allowing efficacy even under environmental stress. The bactericidal effect was especially pronounced at 37°C, highlighting its potential clinical relevance.

These findings suggested that Gram-negative bacteria might be more vulnerable to oxidative damage, likely due to their thinner peptidoglycan layer and more permeable outer membrane. In contrast, Gram-positive bacteria might possess enhanced protective mechanisms, possibly involving thicker cell walls or more efficient antioxidant systems. Nonetheless, the consistent effectiveness of the Vitamin C and $FeCl_3$ combination across both bacterial groups highlighted its potential as a robust antimicrobial agent for a wide range of pathogens under various physiological conditions.

## IV. CONCLUSION

Gram-negative bacteria demonstrated greater susceptibility to hydrogen peroxide, whereas Gram-positive bacteria exhibited higher resistance under specific conditions. The vitamin C–$FeCl_3$ system showed consistent and broad-spectrum antibacterial activity.

These findings highlight the influence of bacterial cell structure and environmental factors on oxidative stress responses and suggest potential roles for oxidative systems in antimicrobial research.

**Ethical Considerations**

The study strictly adhered to ethical standards for handling pathogenic microorganisms. All experimental procedures complied with biosafety guidelines to ensure the safe handling, storage, and disposal of bacterial cultures. Additionally, the study was conducted with approval from the college's institutional review board to maintain ethical integrity.

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