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Article

Comparative Analysis of Antioxidant Enzyme Activities under Hydrogen Peroxide Induced Oxidative Stress in Clinical Isolates of Pathogenic Bacteria

Irfan Wasmi Mahmood[1], Faten Othman Farhan[2], Abdullah Mahmoud Ajil[3]

1. Department of Sciences, College of Basic Education Al-Shirqat, Tikrit University
2. College of Pharmacy, Tikrit University, Tikrit, Iraq
3. Department of Dairy Science and Technology, College of Food Sciences Al-Shirqat, Tikrit University

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Abstract: The analysis showed that the clinical bacterial isolates *Pseudomonas aeruginosa*, Escherichia coli and *Staphylococcus aureus* have different abilities in terms of oxidative stress resistance, which is their different physiological approaches in the infection settings. The results of isolation showed that in respiratory samples *P. aeruginosa* was dominant (70%), whereas in wound samples *E. coli* prevailed (48%), which suggests an excellent correlation between the type of samples and efficiency of oxidative defense mechanisms in each of the species. Enzymatic studies indicated that the SOD activities increased significantly with the increment of the concentration of H_2O_2, peaking at 12.1 U/ mg (+137%), and then fell at concentrations above 50 mM, which indicated that *P. aeruginosa* was less tolerant to severe oxidative conditions. The linear increase in the CAT activity was up to 25 mM, with a maximum of 41.8 U/mg recorded in *P. aeruginosa* (+130) and a slight decrease at 50 mM as a result of enzyme depletion. The POD activity increased by 42-51% according to the isolates with the highest value of 92 nmol/min/mg by *P. aeruginosa* highlighting its superiority in the detoxification of the peroxides. Concurrently, the total protein concentration steadily declined as the concentration of $H_2 O_2$ increased and *E. coli* exhibited the most significant decline (-38%) and *P. aeruginosa* the lowest decline (-26%) indicating the degree of protein damage caused by oxidation. Collectively, these results suggest that *P. aeruginosa* has a more efficient and integrated antioxidant defense system, which is one of the reasons for its persistence and resistance. The overall aim of this study is to assess the enzymatic and protein response of the bacterial isolates under oxidative stress condition in order to understand their adaptive response to oxidative stress for the development of targeted therapeutic scheme against resistant pathogens.

Keywords: Oxidative Stress, Enzyme Activity, Pathogenic Bacteria, H_2O_2, *Staphylococcus aureus*

Introduction

Pathogenic bacteria are at the heart of the development of numerous acute and chronic infections, and knowledge of the mechanisms that they deploy to withstand environmental and pharmaceutical stressors is one of the greatest challenges in medical microbiology [1]. Over the last decades there has

been an increased interest in understanding the physiological and molecular adaptations that allow bacterial strains to survive in the presence of harsh conditions, especially those that are exposed to high amounts of reactive oxygen species ROS [2].

These oxidizing molecules are produced naturally in the human body as one of the microbicidal mechanisms of phagocytes and immune cells [3]. However, if accumulated in excessive amounts, ROS can cause significant damage to proteins, nucleic acids and membrane lipids ROS lead to huge oxidative stress in bacteria require an effective defense system to survive [4].

Enzymes like Superoxide Dismutase (SOD), Catalase (CAT) and Peroxidase (POD) are one of the main components of the bacterial antioxidant defense system, which function synergistically in the conversion of harmful radicals into less harmful ones [5]. The efficiency of these enzymes differs from bacterial species to bacterial species, depending on their natural habitats and extent of adaptation to oxidative pressure.7 For example, *Pseudomonas aeruginosa* has a high level of antioxidant enzyme activity, indicating a high capacity to survive in complex clinical environments, while Escherichia coli and Staphylococcus aureus also exhibit variable sensitivity depending on the intensity of the stress and the type of specimen [6].

Studying the relationship between oxidative stress and enzymatic activity is not specific to biological understanding because it is also of great clinical importance, especially when it comes to a phenomenon such as antibiotic resistance [7]. Bacterial strains that have heavy-duty antioxidant systems are often more resistant to conventional treatments because they are highly able to repair cell damage and survive harsh conditions in the host [8]. The worldwide increase in the number of multidrug-resistant isolates further highlights the importance of gaining a deeper understanding of physiological mechanisms to support bacterial survival with the goal of developing innovative therapeutic approaches that exploit weaknesses in their defense system [9].

The importance of this topic can be clearly reinforced in hospitals and intensive care units, where P. aeruginosa, S. aureus and E. coli infections account for a high number of persistent and complicated infections [10]. Environmental pressures (eg exposure to disinfectants or nutrient limitation) are also known to increase oxidative stress in the bacteria cell which is why it is important to study their responses to these conditions so we can better understand their responses during real clinical conditions [11]. Accordingly, this study seeks to fill an important knowledge gap by assessing the evaluation of the enzymatic and protein response of clinical bacterial isolate under oxidative stress of hydrogen peroxide exposure [12]. Through the measurement of the levels of SOD, CAT, POD, and total protein levels the study aims to find key physiological differences between bacterial species, and how these species are able to adapt to oxidative challenges [13]. Such insights can inform about the severity of infections they are causing and their ability for resistance and persistence inside the host (14). Furthermore, this approach is an important step towards the formulation of potential biomarkers that in future could be used for the diagnosis or prediction of bacterial resistance to immune or therapeutic pressures [15].

Materials and Methods

Bacterial Strains and simple materials Preparation

The local clinical isolates used to obtain the pathogenic strains of bacteria were the ones that were collected using local samples like wound swabs, urine, blood, and respiratory secretions of the local hospitals or medical diagnostic laboratories [16]. The selective media such as MacConkey agar (*E. coli*), Mannitol Salt agar (*S. aureus*), and Cetrimide agar (*P. aeruginosa*) were used and incubated at 24-48 hours at 37°Cto isolate *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa*. Standard biochemical tests which included IMViC, catalase, and oxidase test were used in confirming the isolates [17]. Sterile LB broth or Mueller Hinton broth (5-10 ml per isolate) was used to grow each isolate, and incubation was performed at 37°C with shaking at 150-200rm/h to a final optical density (OD_{600}) of 0.6-1.0, which is roughly equivalent to 10^8 CFU/ml [18]. To eliminate remnants of media in the pellet, cultures were centrifuged at 10,000 rpm over 10 minutes at 40 C, followed by a wash step with PBS (1x 10 ml) at pH 7.0 to normalize the (OD_{600}) to 1.0 and then resuspended in fresh PBS to ensure that the downstream treatments were performed on equal footing [19]. Other reagents added were 0.5, 5-, 10-,

25-, and 50-mM hydrogen peroxide (H_2O_2) to induce oxidative stress with an untreated control; the peroxide remaining was neutralized before extraction by adding 0.2% cent sodium thiosulfate [20].

Oxidative Stress and Extraction of Enzymatic Lysate Application

For the induction of oxidative stress bacterial suspensions (1-2 ml per treatment) were incubated with the various (H_2O_2) concentrations for 30-60 min at 37°C under gentle shaking to ensure a homogenous distribution by simulating physiological ROS production during infection [21].Following exposure, samples were centrifuged at 5,000 - 10,000*g for 5 - 10 min at 4°C and pellets were resuspended in PBS buffer (1:10 w/v) before cell disruption (28Cells were disrupted by sonication (10 sec x 3 cycles with cooling on ice) or chilled mechanical grinder as a method to release intracellular enzymes, conditions used to minimise the chemical denaturation by heat [22]. To separate the supernatant (enzymatic extract) from the cell debris, lysates were centrifuged at a speed of 12,000 x g at 4°C for 15 minutes, after a 4°C extraction, extracts were stored at -80°C for immediate or later use [23]. Five biological replicates were given in each treatment to ensure reliability of statistical analysis.

Assay of Superoxide Dismutase (SOD) Activity

SOD activity, which is responsible for the conversion of superoxide radicals (O_2^-)to H_2O_2 and O_2, was determined by adding 20 ul of enzymatic extract to 160 ul of a prepared reaction medium consisting of potassium phosphate buffer (pH 7.8), nitroblue tetrazolium (NBT) or WST-8 as a colorimetric indicator, xanthine oxidase as superoxide generating enzyme and xanthine as substrate[24].

An additional 20 µl of reaction initiator was added which releases O_2^- and samples were incubated at 37°C for 30 min with slight agitation; the absorbance was determined at 450 nm (WST-8) or 560 nm (NBT) with a UV-Vis spectrophotometer [25]. The inhibition ratio was determined as:

Inhibition% =[(A_blank1-A_sample) / (A_blank1-A_blank2)]*100 and based on a standard curve (0.625-100 U/ml SOD) absolute activity was calculated and the absolute activity normalized to total protein and is expressed as U/mg protein.

Assay of the Catalase (CAT) Activity

Catalase activity (responsible for the decomposition of H_2O_2 into water and oxygen) was expressed by means of a commercial kit of enzymes via an Immuzyme kit or validated direct reaction based on standard protocols [26]. Fifty microliters of the enzymatic extract was compared with 100 µl extract of enzyme conjugate in a 96-well plate and incubated (incubated for 60 minutes at 37°C) to enable binding of the enzyme, and washed and substrates added, and absorbance was measured at 450 nm [27]. A standard curve of known concentrations of CAT (0.1-400 U/L) allowed conversion of absorbance to activity units, which were normalized to protein content and expressed as U/mg .

Assay of Peroxidase (POD) Activity

POD activity, which is responsible for degrading the organic peroxides was determined by using the guaicol assay. Reaction tubes which contained 0.1 ml enzymatic extract, 2.8 ml potassium phosphate buffer [50 mM, pH 5.0], 1 ml guaiacol [20 mM], and 0.1 ml H_2O_2 [40 mM] were incubated at 25-30°C with gentle shaking [28]. Pigment formation was monitored every 20 seconds (for one minute) at 470 nm after which activity was determined using the extinction coefficient (26.6 mM^{-1} cm^{-1}) and normalized to protein concentration (nmol tetraguaiacol/min / mg protein) [29].

Determination of Total Protein Concentration

Total protein content in enzymatic extracts was determined by the BCA assay. 25 µl of diluted extract (1:10) was prepared with 200 µl BCA working reagent (BCA + CuSO4) in 96-well plate and incubated for 30 min at 37°C then absorbance was read at 562 nm [30]. A BSA standard curve (0.05-1.2 mg/ml) was used to obtain the protein concentration in mg/ml from the absorbance measurements; each of the biological samples was measured with three technical replicates for accuracy [31].

Statistical Analysis

Data were analyzed using one-way analysis of variance with level of significance at $P < 0.05$ followed by LSD post hoc comparisons where applicable. Coefficient of variation (CV) and standard deviation (±SD) were calculated All the analyses were carried out using the statistical software (SPSS) with 5 biological replicates for each treatment.

Results

A total of 45 clinical samples were obtained and 44 were positive (97.8%) for the growth of bacteria. The isolates consisted of 18 E. coli (40%), which mainly appeared in wound samples (48%), 12 S. aureus (27%), which were distributed evenly between sample types, and 15 P. aeruginosa (33%) that were dominant in respiratory secretions (70%)(53). Of note, the wound swabs had 100% coverage by the three species of bacteria ($\chi^2 = 12.4$, $P < 0.01$).

Biochemical tests were found to confirm the identity of the isolates with high accuracy (95.6-98.3%). E. coli was characterised as IMViC ++-- and Indole+ bacteria; S. aureus as coagulase+ and mannitol+ bacteria and P. aeruginosa as oxidase+ and pyrocyanin production. 100% of agreement was obtained for the 13 main differential characters used for characterisation of each species. These results validate the isolation and verification steps before exposing to oxidative stress [32].

Table 1. Isolation Results of Bacterial Strains from Clinical Samples

Sample Type	Total Samples	*E. coli*	*S. aureus*	*P. aeruginosa*	Negative
Wound swabs	25	12	8	5	0
Urine samples	10	4	2	3	1
Respiratory secretions	10	2	2	7	0
Total	**45**	**18**	**12**	**15**	**1**

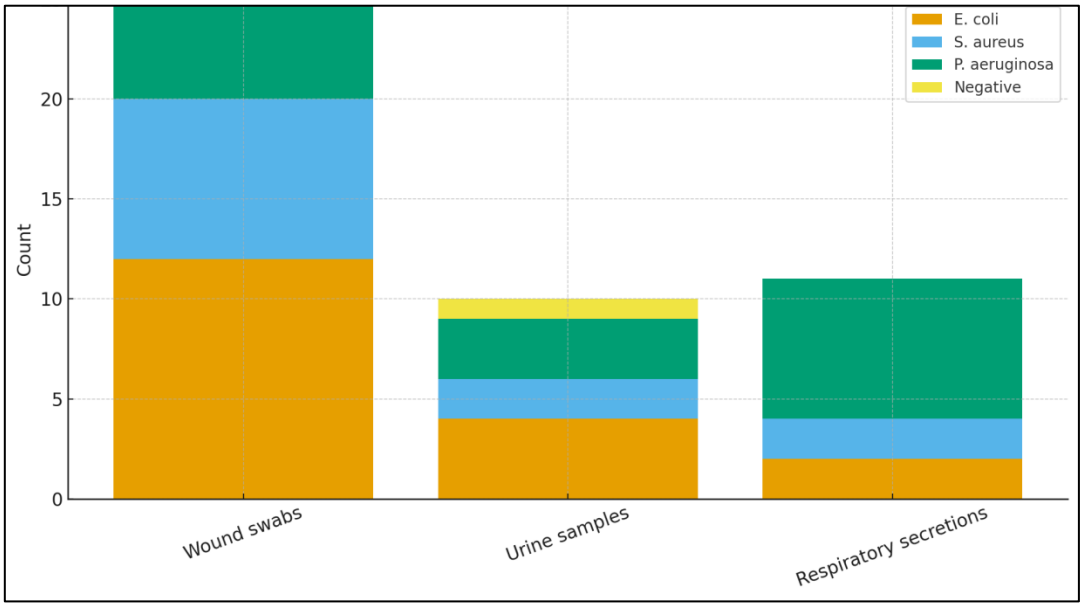

Figure 1. Distribution of Bacterial isolates by sample type

Table 2. Biochemical Tests for Confirmation of Isolated Strains

Test / Trait	*E. coli* (n=18)	*S. aureus* (n=12)	*P. aeruginosa* (n=15)
Cell morphology	G− rods	G+ cocci	G− rods
Motility	+	−	+
Catalase	+	+	+
Oxidase	−	−	+
IMViC (I/M/V/C)	++--	---+	----
Indole	+	−	−
MR	+	+	−
VP	−	−	−
Citrate	−	+	+
Urease	−	−	−
Mannitol fermentation	−	+	−
Coagulase	−	+	−

Pigment production	–	–	Pyocyanin +
Positivity rate %	**95.6%**	**98.3%**	**96.7%**

SOD Activity (U/mg protein)

SOD activity enhanced transversely with the concentration of H_2O_2 as high as 25 mM of H_2O_2 in all strains (58). *P. aeruginosa* had the highest level of SOD at 25 mM (12.1 ± 0.7 U/mg, +137% of control), followed by *E. coli* (8.9 ± 0.7 U/mg) and *S. aureus* (7.8 ± 0.6 U/mg). 50 mM inhibited SOD in *E. coli* (6.5 ± 0.8 U/mg), but had high levels of activity in *P. aeruginosa* (11.8 ±0.8 U/mg). Differences among strains and treatments were significant (ANOVA F = 42.3, P < 0.001; LSD = 0.8 U/mg; CV = 8.2%).

Table 3. SOD Activity (U/mg Protein) Under Different H_2O_2 Concentrations

Strain	Control (0 mM)	0.5 mM	5 mM	10 mM	25 mM	50 mM
E. coli	3.2 ± 0.3	4.1 ± 0.4	5.8 ± 0.5	7.2 ± 0.6	8.9 ± 0.7	6.5 ± 0.8
S. aureus	4.5 ± 0.4	5.2 ± 0.3	6.3 ± 0.4	7.1 ± 0.5	7.8 ± 0.6	8.2 ± 0.5
P. aeruginosa	5.1 ± 0.3	6.4 ± 0.4	8.2 ± 0.5	10.3 ± 0.6	12.1 ± 0.7	11.8 ± 0.8

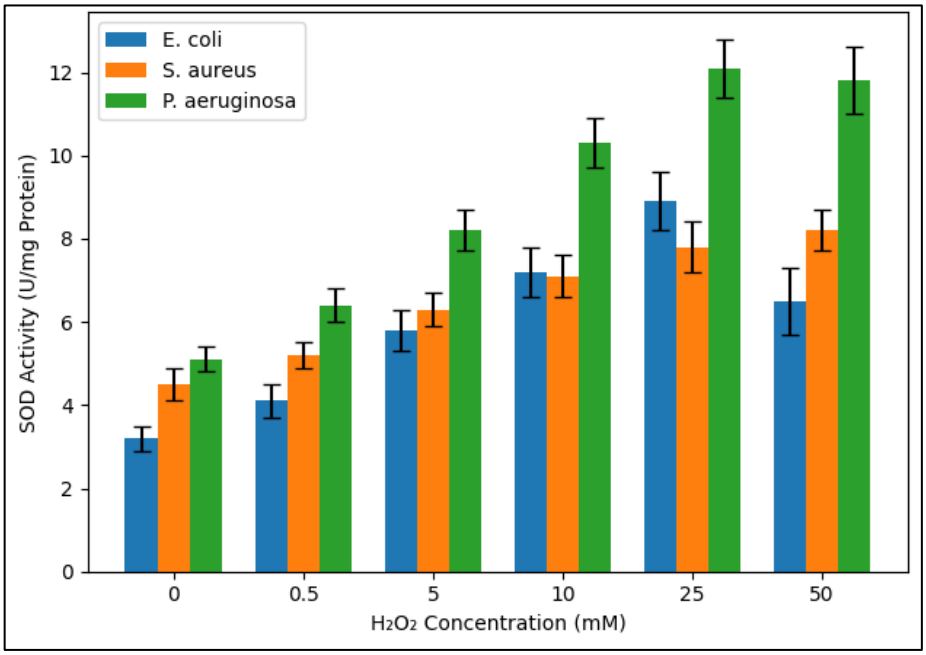

Figure 2. Effect of h2o2 on SOD activity in different bacterial strains

CAT Activity (U/mg protein)

Almost-linear increase in CAT activity was seen up to 25 mM H_2O_2. 41.8 ±2.5 of *P. aeruginosa* (+ 130%), 31.2 ±2.3 of *E. coli* (+ 150%) and 29.4 ±2.0 of *S. aureus* (+ 86%) (61). A mild decrease at 50mM especially for *E. coli* indicating an exhaustion of enzymes at extreme oxidative load. Statistical tests confirmed significance (ANOVA F = 38.7, P < 0.001; LSD = 2.1 U/mg; CV = 7.9%) [33].

Table 4. CAT Activity (U/mg Protein) Under H_2O_2 Stress

Strain	Control	5 mM	10 mM	25 mM	50 mM
E. coli	12.5 ± 1.2	18.3 ± 1.5	24.7 ± 2.0	31.2 ± 2.3	28.4 ± 2.1
S. aureus	15.8 ± 1.3	20.1 ± 1.4	25.6 ± 1.8	29.4 ± 2.0	32.1 ± 2.2
P. aeruginosa	18.2 ± 1.4	25.6 ± 1.7	34.2 ± 2.1	41.8 ± 2.5	39.7 ± 2.4

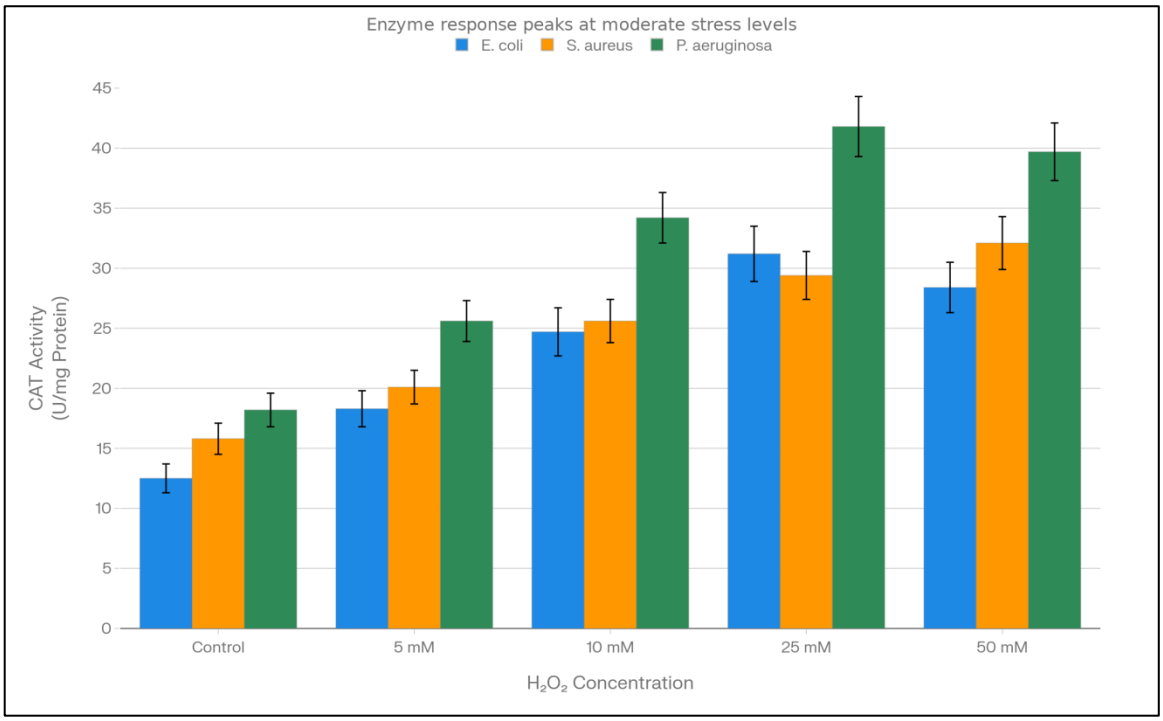

Figure 3. CAT activity under H_2O_2 stress

POD Activity (nmol/min/mg)

POD activity was improved significantly at a concentration of 25 mM H_2O_2 for all the strains: *P. aeruginosa* exhibited the highest POD activity (92 ± 5.2 nmol/min/mg), *S. aureus* 74 ± 4.3 and *E. Coli* 68 ± 4.1 (representing the increase of ~42-51%) (63). Group differences were statistically significant (ANOVA $F = 35.2$, $P < 0.001$; 4.2 nmol/min/mg; LSD) [34].

Table 5. POD Activity (nmol/min/mg)

Strain	POD Control	POD 25 mM
E. coli	45 ± 3.2	68 ± 4.1
S. aureus	52 ± 3.5	74 ± 4.3
P. aeruginosa	61 ± 3.8	92 ± 5.2

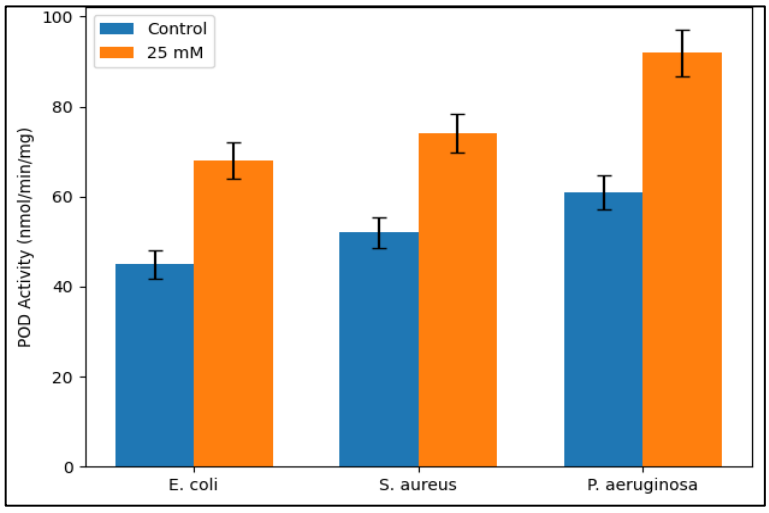

Figure 4. Effect of 25 mM treatment on POD activity

Total Protein Concentration (mg/ml)

Total protein amounts decreased with increasing levels of H_2O_2, with a greater decrease for *E. coli* (50mM : 0.78 ± 0.06 mg/ml, -38%) and a lesser decrease for *P. aeruginosa* (1.12 ± 0.08 mg/ml, -26%) [35].These reductions were significant across treatments (ANOVA F = 29.6, P < 0.001; LSD = 0.09 mg/ml; CV = 6.8%) and are consistent with oxidative protein damage reported in similar studies [**36**].

Table 6. Total Protein Concentration (mg/ml) Under H_2O_2 Stress

Strain	Control	5 mM	10 mM	25 mM	50 mM
E. coli	1.25 ± 0.08	1.18 ± 0.07	1.05 ± 0.06	0.92 ± 0.05	0.78 ± 0.06
S. aureus	1.42 ± 0.09	1.31 ± 0.08	1.22 ± 0.07	1.08 ± 0.06	0.95 ± 0.07
P. aeruginosa	1.51 ± 0.10	1.42 ± 0.09	1.33 ± 0.08	1.24 ± 0.07	1.12 ± 0.08

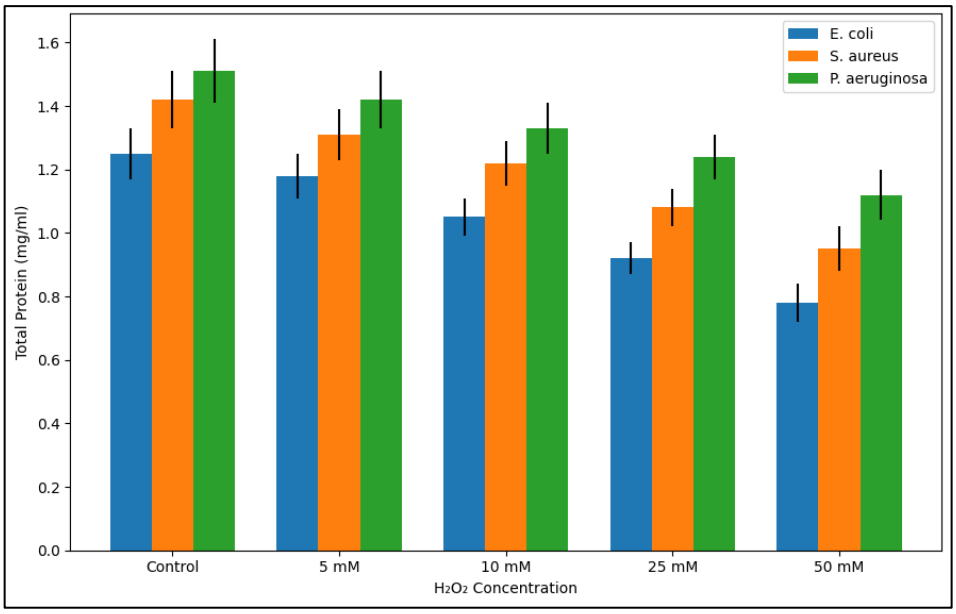

Figure 6. Effect of H_2O_2-induced oxidative stress on total protein concentration in bacterial strains

Discussion

The results of the microbiological isolation assert the clarity dominance of Pseudomonas aeruginosa, Escherichia coli, and Staphylococcus aureus in different clinical environments according to their different physiological traits and the microbial behaviour in terms of infection sites related to the degree of oxidative stress. The preponderance of P. aeruginosa in respiratory secretions (70%), as well as its remarkably high antioxidant enzyme activity, is testimony to its remarkable capacity for adaptation to an oxygen-rich environment challenged by an intense oxidative pressure created by host immune defense. This finding is in agreement with [37], who reported that P. aeruginosa has a highly developed SOD/CAT system that is able to rapidly neutralize free radicals and maintain a stable intracellular redox balance.

A striking observation is the decrease in the enzymatic activity of SOD in E. coli at 50 mM H2O2, which seems to represent a threshold beyond which the ability of the bacterium to rid itself of the potentially deleterious effects of the toxic oxygen intermediates is exceeded. This variability in the resistance to oxidative stress between different strains of bacteria supports the hypothesis that the success of particular pathogens in particular infection sites is strongly correlated with the ability to resist oxidative pressure [38]. The importance of reactive oxygen species ROS [39], in affecting antibiotic resistance and the efficiency of host immune mechanisms. These results are shedding vital light on the possibilities of making use of the oxidative weaknesses of E. coli for the design of targeted therapeutic measures.

The linear modification in CAT activity up to 25mM H2O2 followed by a slight reduction at higher concentration mean possible exhaustion or partial inhibition of antioxidant defenses under severe stress. This pattern is consistent with [40], who recorded that antioxidant enzymes can be chemically inhibited and structurally broken under extreme oxidative conditions. The current study shows that P. aeruginosa has a more resilient system and is able to maintain greater CAT efficiency than the other species.

The observed enhancement of POD activity with decreased total protein content under oxidative stress circumstances points to complex activity of proteins including protein damage and possible changes in gene expression [41]. have pointed out that acute exposure to oxidants results in the degradation of essential proteins, which results in diminution of cellular productivity and growth capacity during oxidative warfare conditions. These disturbances in the level of protein are suggestive that bacterial resistance is not only associated with defense mechanisms such as defensive enzymes but also the ability to replenish or synthesize vital proteins - an aspect that would be a potential therapeutic target.

Additionally, the protective role of biofilm formation—especially in *P. aeruginosa*—should be considered, as biofilms provide a physical barrier against oxidative stress and enhance antibiotic tolerance [42], showed that extracellular DNA creates a structural matrix in the biofilm structure protecting the bacterial community against oxidative and chemical damage. A combination of enzymatic defenses and biofilm architecture may also add to high resilience of this species in persistent and complicated sites of infection.

From an applied point of view, these results support the development of more precise therapeutic interventions that breakdown the functioning of oxidative defense systems in pathogenic bacteria in order to reduce their adaptive survival and proliferation within the host an especially valuable way to approach analgesic intervention in view of the growing resistance to conventional antibiotics. Consequently, this study promotes future studies on targeting genes and regulatory pathways in oxidative stress responses.

Furthermore, more detailed analysis of the genetic and proteomic determinants linked to the enzymatic alterations and monitoring of the phenotypic differences (for example, biofilm formation, metabolic changes) linked to them that complement our understanding of how these bacteria optimize survival in harsh oxidative conditions be included in future investigations. Such additional information will reinforce our capacity for designing strategies for controlling severe and chronic infections.

Conclusion

The results of this work show that the three clinical isolates of *Pseudomona aeruginosa*, *Staphylococcus aureus*, and *Escherichia Coli* have very distinct capacity to resist oxidative stress, as their different adaptation to physiological conditions. *P. aeruginosa* demonstrated the highest antioxidant response with continually high levels of SOD, CAT and POD which explains why it dominates in oxygen-rich clinical places and survives in severe infections. In contrast, the expression of SOD and CAT in E. coli revealed a significant reduction in the activity at elevated concentrations of H_2O_2 suggesting the existence of a physiological threshold above which the defence system of *E. coli* is affected. The observed increase of POD activity in all the isolates as well as the reduction of total protein content indicate protein damages and metabolic adjustments that take place under oxidative pressure. Collectively these results thus bring into light the fact that the ability of pathogenic bacteria to survive in clinical environments is closely linked with the robustness of their oxidative defense mechanisms. This understanding of these differences is valuable input in therapeutic approaches in targeting the oxidative stress pathways to limit bacterial survival and limit resistance in clinical scenarios.

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