



COMPARATIVE EFFECTS OF PLASMA-ACTIVATED WATER AND HYDROGEN PEROXIDE ON *ESCHERICHIA COLI* GROWTH KINETICS AND EPS CARBOHYDRATE CONTENT

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ABSTRACT

Plasma-activated water (PAW) is a chemically complex antimicrobial liquid whose biological effects cannot be fully predicted from hydrogen peroxide (H_2O_2) concentration by itself. This study compared the effects of surfatron-generated PAW and free H_2O_2 on *Escherichia coli* ATCC 25922 using two exposure strategies: pulse exposure in phosphate-buffered saline (PBS) followed by sodium thiosulfate neutralization and growth in Mueller-Hinton broth (MHB), and direct oxidant addition to MHB before growth monitoring. Abiotic stability assays showed that MHB almost completely depleted detectable H_2O_2 after 24 h. Under pulse exposure, PAW at 50-mg/L H_2O_2 -equivalent suppressed detectable OD_{600} -based growth, whereas free H_2O_2 at the same concentration allowed recovery. Under direct addition, PAW allowed delayed growth while H_2O_2 caused severe growth delay at 50 mg/L. The saccharidic fraction of extracellular polymeric substances (EPS) differed among treatments: PAW-treated groups, especially direct addition at 50-mg/L H_2O_2 -equivalent, showed the strongest carbohydrate accumulation in cell-associated and cuvette-adhered fractions. These results demonstrated that H_2O_2 -equivalence alone is insufficient to predict PAW bioactivity and that exposure matrix strongly modulates bacterial growth recovery and EPS-associated phenotypes.

KEYWORDS: Plasma-Activated Water, Surfatron, *Escherichia coli*, Hydrogen Peroxide, Growth Kinetics, Extracellular Polymeric Substances, Oxidative Stress.

EFEITOS COMPARATIVOS DA ÁGUA ATIVADA POR PLASMA E DO PERÓXIDO DE HIDROGÊNIO SOBRE A CINÉTICA DE CRESCIMENTO E O TEOR DE CARBOIDRATOS DO EPS DE *ESCHERICHIA COLI*

RESUMO

A água ativada por plasma (PAW) é um líquido antimicrobiano quimicamente complexo cujos efeitos biológicos não podem ser previstos apenas pela concentração de peróxido de hidrogênio (H_2O_2). Este estudo comparou os efeitos da PAW gerada por surfatron e do H_2O_2 livre sobre *Escherichia coli* ATCC 25922 utilizando duas estratégias de exposição: exposição em pulso em solução salina tamponada

com fosfato (PBS) seguida de neutralização com tiosulfato de sódio e crescimento em caldo Mueller-Hinton (MHB), e adição direta do oxidante no MHB. O ensaio abiótico demonstrou que o MHB depleta quase completamente o H_2O_2 detectável após 24 h. Sob exposição em pulso, a PAW a 50 mg/L H_2O_2 -equivalente suprimiu o crescimento detectável por OD_{600} , enquanto o H_2O_2 livre permitiu recuperação. Na adição direta, a PAW permitiu crescimento retardado, enquanto o H_2O_2 causou atraso severo a 50 mg/L. A fração sacarídica de substâncias poliméricas extracelulares (EPS) diferiu entre tratamentos: grupos tratados com PAW, especialmente na adição direta a 50 mg/L, apresentaram maior acúmulo de carboidratos nas frações associadas às células e aderidas à cubeta. Esses resultados demonstraram que a equivalência em H_2O_2 é insuficiente para prever a bioatividade da PAW e que a matriz de exposição modula fortemente a recuperação bacteriana e os fenótipos associados ao EPS.

PALAVRAS-CHAVE: Água Ativada por Plasma, Surfatron, *Escherichia coli*, Peróxido de Hidrogênio, Cinética de Crescimento, Substâncias Poliméricas Extracelulares, Estresse Oxidativo.

INTRODUCTION

Hydrogen peroxide (H_2O_2) is one of the most relevant long-lived reactive oxygen species associated with antimicrobial oxidative stress. In bacterial systems, H_2O_2 can impair cellular structures, interfere with redox homeostasis, and affect growth recovery depending on concentration, exposure time, medium composition, and the physiological state of the cells. In *Escherichia coli*, oxidative stress may therefore be reflected not only as immediate inhibition, but also as delayed growth, altered lag phase, reduced final biomass, and impaired recovery after exposure¹⁻³.

Plasma-activated water (PAW) has been widely investigated as an antimicrobial liquid generated through plasma-water interaction. Its biological activity is commonly associated with a complex mixture of reactive oxygen and nitrogen species, including H_2O_2 , nitrate (NO_3^-), nitrite (NO_2^-), peroxyxynitrite-related chemistry, changes in pH, oxidation-reduction potential, and conductivity. Because of this chemical complexity, PAW should not be interpreted as a simple H_2O_2 solution, even when its H_2O_2 concentration is measured and normalized. The antimicrobial action of PAW has been associated with the combined contribution of reactive species and physicochemical changes, rather than with a single isolated component⁴⁻⁹.

This distinction becomes especially important when oxidants are added to complex microbiological media. Mueller-Hinton broth (MHB) contains organic and inorganic components that may interact with reactive species and modify the effective oxidant dose available to bacteria during incubation. The presence of organic matter has been shown to reduce the inactivation efficacy of PAW by scavenging or transforming reactive species, and dynamic peroxide monitoring in Mueller-Hinton systems indicates that peroxide availability can be affected by both the medium and microbial activity^{10,11}. Thus, when H_2O_2 or PAW is added directly to MHB, the nominal initial concentration may not represent continuous exposure throughout the growth curve.

Based on this limitation, the present study compared two experimental exposure strategies. In the first one, *E. coli* ATCC 25922 was grown directly in MHB containing either free H_2O_2 or PAW-surfatron normalized by H_2O_2 concentration at 25 or 50 mg/L. This condition evaluated bacterial growth under a dynamic system in which the oxidant, MHB components, and bacterial cells interact throughout the 24-h incubation. In the second strategy, bacterial suspensions in phosphate-buffered saline (PBS) were exposed for 60 min to the same nominal concentrations of free H_2O_2 or PAW-surfatron-derived H_2O_2 , followed by neutralization with sodium thiosulfate before growth monitoring in MHB. This design separates the effect of a defined oxidative pulse from the residual presence of oxidants during incubation.

The comparison between these strategies allows the distinction between two different biological questions: what happens when *E. coli* is grown in MHB initially supplemented with oxidants, and what happens after bacteria experience a controlled oxidative exposure before being transferred to growth conditions. Growth curves provide information on lag phase, recovery, apparent growth capacity, and final optical density, while quantification of

the saccharidic fraction of extracellular polymeric substances (EPS) adds complementary information on stress-associated extracellular carbohydrate accumulation and bacterial response to oxidative challenge. Concurrently, these endpoints allow a broader interpretation of oxidative stress than growth monitoring by itself, without assuming that the entire EPS matrix was quantified.

Therefore, this study aimed to compare the effects of surfatron-generated plasma-activated water and hydrogen peroxide on the growth kinetics and saccharidic fraction of extracellular polymeric substances in *E. coli* ATCC 25922. The experimental design included direct addition of H₂O₂ or PAW-surfatron to MHB at 25- and 50-mg/L H₂O₂-equivalent concentrations, as well as 60-min exposure in PBS followed by sodium thiosulfate neutralization and subsequent growth monitoring. Physicochemical characterization of PAW, H₂O₂ solutions, and their mixtures with MHB was performed together with growth-curve analysis and carbohydrate quantification of EPS fractions after 24 h. This approach tested whether nominal equivalence in H₂O₂ concentration results in equivalent biological responses or whether PAW-surfatron produces distinct effects due to its broader reactive chemistry and interaction with the culture medium.

METHODS

Bacterial strain and inoculum preparation

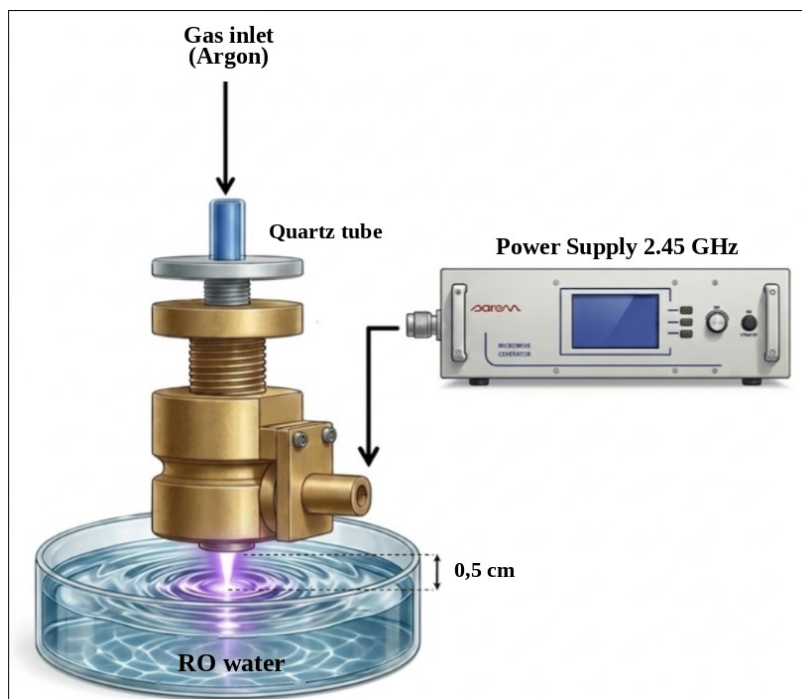
Escherichia coli ATCC 25922 was used as the bacterial model in this study. The strain was reactivated on Luria-Bertani agar plates and incubated at 37°C. For the growth kinetics assays, a single colony was inoculated into MHB and incubated at 37°C. Bacterial suspensions were standardized to obtain a final concentration of approximately 1×10^6 colony-forming unit (CFU)/mL in the growth assays.

For the pulse-exposure experiments, bacterial suspensions were initially prepared in PBS at 9×10^6 CFU/mL. This concentration was defined to compensate for dilution during oxidative exposure, neutralization, and final transfer to MHB, resulting in approximately 1×10^6 CFU/mL in the 2-mL cuvette growth assay.

Plasma-activated water production and characterization

Plasma-activated water was produced using a surfatron launcher reactor driven by a solid-state microwave generator operating at 2.45 GHz^{12,13}. For each activation, 50 mL of reverse-osmosis water were placed in an open 110-mm diameter Petri dish. Argon 3.0, with 99.9% purity, was used as the carrier gas at a constant flow rate of 2.5 L/min. The terminal end of the plasma tube was positioned 5 mm above the water surface, and the plasma plume was maintained in direct contact with the liquid surface during activation. The experimental setup used is shown in Fig. 1. The water was exposed to the surface-wave plasma discharge at 70 W for 40 min. The initial water temperature was 24°C, and the final temperature immediately after activation was 38.7°C. No external stirring was applied during activation; liquid disturbance was limited to the surface motion caused by the gas flow and plasma plume. Plasma-activated water was always used for physicochemical measurements and biological assays within 10 min after the end of activation.

Immediately after activation, physicochemical parameters were measured using a Metrohm 913 meter. The evaluated parameters included pH, oxidation-reduction potential (ORP), electrical conductivity, and total dissolved solids (TDS). H₂O₂, NO₂⁻, and NO₃⁻ concentrations were determined using the Akso Exact Micro20 system. For MHB-containing samples, MHB was used as the matrix blank to account for the culture-medium background, and values were interpreted as residual detectable concentrations within the MHB matrix.



Source: Elaborated by the authors. The initial draft was generated with the assistance of Claude Opus 4.8 (Anthropic) and subsequently reviewed, edited, and finalized by the authors.

Figure 1: Experimental setup used for plasma-activated water production. Argon gas (carrier gas) flows through a quartz tube into a surfatron-driven microwave plasma source operating at 2.45 GHz, generating a surface-wave plasma plume that remains in direct contact with the water surface during activation. The terminal end of the plasma tube was positioned 0.5 cm (5 mm) above the surface of the reverse-osmosis (RO) water contained in an open Petri dish.

Preparation of hydrogen peroxide solutions

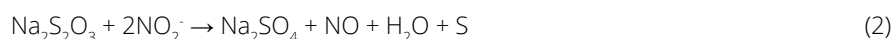
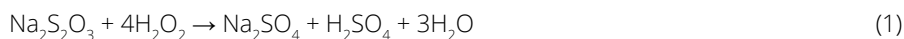
Hydrogen peroxide solutions were prepared from a 35%-H₂O₂ stock solution. Working solutions were freshly prepared in PBS or MHB, depending on the experimental design. For pulse-exposure assays, an intermediate H₂O₂ solution was prepared to allow accurate pipetting and dilution to the target concentrations. The experimental concentrations used in this study were 25- and 50-mg/L H₂O₂.

For PAW treatments, the required volume of PAW was calculated from the H₂O₂ concentration measured after plasma activation, so that PAW treatments corresponded to 25- and 50-mg/L H₂O₂-equivalent concentrations. Therefore, PAW was not treated as a percentage v/v factor in the main experimental design, but as a reactive solution normalized by its measured H₂O₂ content.

Sodium thiosulfate neutralization assay

A preliminary chemical neutralization assay was performed to verify the ability of sodium thiosulfate to quench the main oxidizing species present in PAW before its use in the pulse-exposure experiments. Plasma-activated water was produced under the same surfatron-based conditions used in the biological assays, and the concentrations of H₂O₂, NO₂⁻, and NO₃⁻ were measured before and after sodium thiosulfate addition.

The neutralization assay was based on stoichiometric estimation and experimental verification of residual reactive species after treatment. For H₂O₂ and NO₂⁻, the reactions considered as working stoichiometric approximations for the estimation are shown in Eqs. 1 and 2:



Equations 1 and 2 were used to estimate the sodium thiosulfate concentration required to reduce the main oxidizing fraction of PAW, particularly H_2O_2 and NO_2^- . Sodium thiosulfate was selected because it effectively reduced the oxidizing species of interest while NO_3^- was not fully removed. This behavior was considered suitable for the present experimental design because the goal was to interrupt residual oxidative activity before growth monitoring, rather than completely remove all PAW-derived chemical species.

Based on the preliminary neutralization assay, sodium thiosulfate was used at a final concentration of 2.5 mM. Although chemical quenching was observed within approximately 5 min, the microbiological assays used a 10-min neutralization step as a conservative condition before the transference to MHB for growth monitoring.

Experimental design

Two complementary exposure strategies were used: direct oxidant addition to MHB, and pulse exposure in PBS followed by neutralization and growth in MHB.

The direct-addition assay evaluated bacterial growth when oxidants were added directly to the culture medium. In this condition, *E. coli* ATCC 25922 was grown in MHB containing either free H_2O_2 or PAW-surfatton at final concentrations of 25- and 50-mg/L H_2O_2 -equivalent.

The pulse-exposure assay evaluated bacterial recovery after a defined oxidative challenge. In this condition, bacterial suspensions prepared in PBS were exposed for 60 min to free H_2O_2 or PAW-surfatton at 25- and 50-mg/L H_2O_2 -equivalent. After exposure, residual oxidizing species were neutralized with 2.5 mM sodium thiosulfate for 10 min, and the treated bacterial suspension was then transferred to MHB for growth-curve monitoring.

The untreated growth control consisted of *E. coli* cultivated in MHB without oxidant and was used as the negative control (NC). A procedural control consisting of *E. coli* suspended in PBS, treated with sodium thiosulfate under the same neutralization conditions, and then transferred to MHB was also performed. Because this thiosulfate-control condition did not differ significantly from the negative control in OD_{600} -based growth behavior, it was not displayed as a separate group in the main figures and kinetic table, and NC was retained as the common reference group.

Direct oxidant addition to Mueller-Hinton broth growth kinetics assay

For the direct oxidant addition assay, H_2O_2 or PAW-surfatton was aseptically added to MHB to obtain final concentrations of 25- and 50-mg/L H_2O_2 -equivalent. To maintain nutrient availability constant across all treatments, dehydrated MHB was solubilized considering the total final volume of the mixture, including medium and oxidant solution. This ensured that the final MHB concentration remained equivalent to the manufacturer's recommended formulation.

Because reactive oxygen and nitrogen species are thermosensitive and prone to degradation, PAW- and H_2O_2 -containing media were not autoclaved after preparation. Sterility was maintained by cold filtration through 0.22- μm PTFE syringe filters. PTFE membranes were selected to minimize interactions with reactive species compared with more hydrophilic polymeric membranes. The target H_2O_2 -equivalent concentrations measured at 0h in the abiotic oxidant-MHB mixtures were used as an operational verification that the filtration/preparation procedure did not prevent the treatments from reaching the intended peroxide concentrations.

The bacterial inoculum was added to each treatment to obtain approximately 1×10^6 CFU/mL. Growth kinetics were monitored in 10-mm path-length quartz cuvettes containing a final volume of 2 mL. Measurements were performed using a Thermo Scientific GENESYS 180 ultraviolet-visible (UV-Vis) spectrophotometer equipped with a Peltier-thermostated cell holder maintained at $37 \pm 0.1^\circ\text{C}$. The assays were conducted under continuous magnetic stirring, and optical density at 600 nm was recorded every 10 min for 24 h. At the end of the incubation period, samples were collected for EPS fraction extraction and carbohydrate quantification.

Pulse exposure followed by neutralization and growth assay

For the pulse-exposure assay, bacterial suspensions were prepared in PBS at 9×10^6 CFU/mL. Exposure was performed in microtubes using 450 μ L of bacterial suspension and 450 μ L of oxidant solution, resulting in a total exposure volume of 900 μ L. The oxidant solutions consisted of either free H_2O_2 or PAW-surfatlon adjusted to 25- or 50-mg/L H_2O_2 -equivalent. The exposure was conducted for 60 min at 37°C.

After exposure, sodium thiosulfate was added to each tube to interrupt residual oxidative activity before growth monitoring. Sodium thiosulfate was selected based on the preliminary chemical neutralization assay described above and was used at a final concentration of 2.5 mM. The neutralization step was standardized at 10 min. Sodium thiosulfate was prepared in PBS, protected from light, and used fresh.

A procedural thiosulfate control was performed by subjecting *E. coli* suspended in PBS to the same sodium thiosulfate neutralization step in the absence of oxidants before transfer to MHB. This control showed no significant difference from the negative control, indicating that the thiosulfate carryover under the conditions used did not measurably alter OD_{600} -based growth kinetics.

After neutralization, 500 μ L of the treated bacterial suspension were transferred to 1,500 μ L of MHB in a 2-mL quartz cuvette. This resulted in a final volume of 2 mL and an estimated final bacterial concentration of approximately 1×10^6 CFU/mL. Growth kinetics were then monitored under the same conditions used for the direct-addition assay: $37 \pm 0.1^\circ\text{C}$, continuous magnetic stirring, OD_{600} readings every 10 min by UV-Vis spectrophotometry, and a total monitoring time of 24 h. At the end of incubation, samples were collected for EPS fraction extraction and carbohydrate quantification.

Extracellular polymeric substances extraction and fractionation

Extracellular polymeric substances were extracted from *E. coli* cultures grown under the different oxidative stress conditions after 24 h growth monitoring. Three operational EPS fractions were evaluated: soluble EPS, cell-associated EPS, and cuvette-adhered EPS. The analysis focused specifically on the saccharidic fraction of these EPS fractions; therefore, the procedure should not be interpreted as quantification of total EPS mass or of all EPS components.

For soluble EPS extraction, culture aliquots were centrifuged at $2,000 \times g$ for 20–30 min, and the supernatant was collected. Three volumes of cold absolute ethanol were added to the supernatant, followed by incubation at -20°C for 3 h to precipitate extracellular polymeric material. The precipitated material was recovered by centrifugation, washed with 70% ethanol, and dried at 37 – 60°C for 24 h.

For cell-associated EPS extraction, the cellular pellet obtained after centrifugation was washed with sterile PBS and resuspended in 1 mL of either 0.9% NaCl or 0.1 M EDTA solution. Samples were incubated at 37°C for 30 min to promote EPS detachment from the bacterial surface. The suspension was centrifuged again at $2,000 \times g$ for 20–30 min, and the recovered supernatant was subjected to ethanol precipitation, washing, and drying as described for the soluble EPS fraction.

For cuvette-adhered EPS extraction, the liquid culture was removed from the quartz cuvettes, and the internal surfaces were gently washed with PBS. Material attached to the cuvette walls was mechanically detached using a sterile spatula or wide-bore pipette tip in the presence of PBS. The resulting suspension was transferred to microcentrifuge tubes, centrifuged, and processed by ethanol precipitation, washing, and drying as described for the other fractions.

Quantification of the saccharidic fraction of extracellular polymeric substances by the anthrone method

The saccharidic fraction of the extracted EPS fractions was quantified using the anthrone colorimetric method¹⁴. After extraction and drying, the precipitated material was resuspended in 1 mL of distilled water. Subsequently, 4 mL of freshly prepared cold anthrone reagent, consisting of anthrone dissolved in concentrated sulfuric acid,

were carefully added to each sample. The mixtures were homogenized and incubated in a boiling water bath at 100°C for 10 min.

After incubation, the tubes were immediately cooled in an ice bath for approximately 5 min to stabilize color development. Absorbance was measured at 620 nm using a UV-Vis spectrophotometer. Distilled water subjected to the same reaction conditions was used as the analytical blank.

The saccharidic fraction concentration was estimated from a calibration curve prepared using 500 kDa dextran as the carbohydrate standard. This molecular weight was selected because it approximates the molecular mass range commonly reported for extracellular polysaccharides produced by *E. coli*, particularly colanic acid-rich matrices.

The calibration equation used is Eq. 3:

$$A_{620} = 0.0065x - 0.0178 \quad (3)$$

where: A_{620} : the absorbance at 620 nm; x: the concentration of the saccharidic fraction in µg/mL.

Therefore, the concentration was calculated by Eq. 4:

$$x = (A_{620} + 0.0178) / 0.0065 \quad (4)$$

For normalized EPS analysis, the mean concentration of the saccharidic fraction of each EPS fraction was divided by the corresponding maximum optical density (OD_{max}) obtained from the growth-kinetics assay. This normalization was used to distinguish absolute carbohydrate accumulation from matrix-associated carbohydrate accumulation relative to the OD_{600} -derived biomass signal. The PE 50-mg/L PAW group was not included in the normalized EPS/ OD_{max} analysis because its near-zero OD_{max} made biomass normalization biologically uninterpretable.

This method was used specifically to estimate the carbohydrate-rich fraction of EPS and should not be interpreted as quantification of total EPS biomass.

Physicochemical characterization of oxidant-medium mixtures

Physicochemical characterization was performed for PAW, H_2O_2 solutions, and their mixtures with MHB. The measured parameters included pH, ORP, electrical conductivity, and TDS using the Metrohm 913 meter. H_2O_2 , NO_2^- , and NO_3^- concentrations were determined using the Akso Exact Micro20 system. For MHB-containing samples, MHB was used as a matrix-matched blank to minimize background contribution from the culture medium.

These measurements were used to evaluate how MHB modified the chemical profile of the oxidizing solutions and to verify the relationship between nominal H_2O_2 concentration, medium interaction, and biological response.

Abiotic physicochemical stability of oxidant-Mueller-Hinton broth mixtures

To evaluate whether MHB modified the chemical availability of the oxidizing species independently of bacterial metabolism, an abiotic physicochemical stability assay was performed. MHB alone, MHB supplemented with free H_2O_2 , and MHB supplemented with PAW-surfatone were prepared at the same H_2O_2 -equivalent concentrations used in the biological assays, namely 25 and 50 mg/L. The mixtures were evaluated immediately after preparation and after 24 h.

The measured parameters included pH, ORP, electrical conductivity, TDS, and residual H_2O_2 concentration. NO_2^- and NO_3^- were also determined when applicable. Physicochemical parameters were measured using the Metrohm 913 meter, while H_2O_2 , NO_2^- and NO_3^- concentrations were determined using the Akso Exact Micro20 system. For MHB-containing mixtures, MHB was used as the matrix blank for these determinations. This assay was used to determine whether the MHB matrix alone was able to modify or consume the nominal H_2O_2 concentration before considering the additional effect of bacterial growth. The 0-h H_2O_2 measurements were also used to verify whether the preparation and PTFE filtration steps preserved the intended initial H_2O_2 -equivalent concentrations.

Growth curve analysis and kinetic parameters

Bacterial growth curves were analyzed from optical density measurements at 600 nm (OD_{600}) acquired every 10 min throughout the 24-h incubation period. For each experimental condition, three biological replicates were used, and data were expressed as mean $\pm$ standard deviation (SD). Prior to analysis, the experimental curves were corrected by subtraction of the corresponding MHB blank.

The Logistic 4-Parameter model was selected as the primary descriptive model for growth-curve fitting because it provided stable convergence and consistent performance across the experimental conditions. The model was defined as Eq. 5:

$$OD(t) = D + (A - D) / [1 + (t/C)^B] \quad (5)$$

where: $OD(t)$: the optical density at time t ; A : the upper asymptote; D : the lower asymptote; C : the inflection point; B : the slope parameter associated with growth steepness.

From the fitted Logistic 4P model, the lag phase duration (T_{lag}) was operationally estimated by Eq. 6:

$$T_{lag} = C - 2/B \quad (6)$$

The maximum specific growth rate (μ_{max}) was estimated by Eq. 7:

$$\mu_{max} = B \times (A - D) / 4 \quad (7)$$

The population doubling time (t_d) was then calculated from μ_{max} using Eq. 8:

$$t_d = \ln(2) / \mu_{max} \quad (8)$$

The OD_{max} was defined as the highest OD_{600} value reached during the 24-h experiment. Total OD_{600} -derived biomass signal was estimated by the area under the growth curve (AUC), calculated by trapezoidal numerical integration and expressed as $OD \cdot h$.

Groups exhibiting no-detectable OD_{600} -based growth, defined as $OD_{max} < 0.05$, were classified as non-detectable (ND). For these conditions, T_{lag} , μ_{max} , and t_d were not calculated, although OD_{max} and AUC were retained because they represent biologically relevant strong-growth-inhibition outcomes.

Because t_d was mathematically derived from μ_{max} , statistical interpretation was primarily based on μ_{max} , while t_d was retained as a descriptive parameter to facilitate biological interpretation of growth kinetics.

As a supplementary robustness check, a modified Gompertz model was also fitted to the experimental curves to verify whether the main trends observed with the Logistic 4P model were preserved using an alternative sigmoidal model. Because the kinetic parameters reported and discussed in this study were derived from the Logistic 4P model, Gompertz results were used only as confirmatory support and were not used for the main statistical comparisons.

All nonlinear regressions and kinetic calculations were performed using custom-developed Python scripts. Model fitting was performed by nonlinear least-squares minimization, and the quality of fit was evaluated using the coefficient of determination (R_2). The R_2 values reported in the kinetic table refer to the Logistic 4P fit obtained from the mean growth curve of each condition and were used only as fit-quality indicators, not as replicate-level statistical variables.

Statistical analysis

All statistical analyses were performed using replicate-level kinetic parameters ($n = 3$) obtained from the Logistic 4P-based analysis. For each kinetic parameter, one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test was performed separately within each exposure strategy. Statistical significance was established at $p < 0.05$.

Extracellular polymeric substances datasets were analyzed separately for each operational fraction: soluble EPS, cell-associated EPS, and cuvette-adhered EPS. Absolute EPS values and ODmax-normalized EPS values were analyzed as independent response variables. Results are presented as mean $\pm$ standard deviation. For the normalized analysis, EPS values were divided by the corresponding ODmax values obtained from growth kinetics; the PE 50-mg/L PAW group was excluded from EPS/ODmax analysis due to its near-zero ODmax. Statistical comparisons among treatments were performed using Welch ANOVA followed by the Games-Howell post hoc test. Differences were considered statistically significant at $p < 0.05$. Statistical letters for EPS tables must be interpreted column by column; groups sharing at least one letter are not significantly different.

The statistical notation used in the kinetic table was designed to distinguish two comparison levels. Letters indicate significant differences among treatments within the same experimental methodology. Superscript numbers indicate pairwise comparisons between PE and DA for the same oxidant and H₂O₂-equivalent concentration. The negative control was used as a common reference group across comparisons. ND groups were excluded from statistical comparisons of T_{lag} , μ_{max} , and t_d . Data were presented as mean $\pm$ SD.

RESULTS AND DISCUSSION

Abiotic interactions and physicochemical stability in Mueller-Hinton broth

Before interpreting the bacterial growth curves, the abiotic interaction between the oxidizing solutions and MHB was evaluated over 24 h, and the results are presented in Table 1. This step was essential because the direct-addition assay introduced H₂O₂ or PAW-surfatratron into a complex culture medium, in which reactive species may be consumed or transformed even in the absence of microorganisms.

Table 1: Abiotic physicochemical characterization of Mueller-Hinton broth and oxidant-Mueller-Hinton broth mixtures at 0 and 24 h*.

Time (h)	Condition	pH	ORP (mV)	Conductivity (μ S/cm)	TDS (mg/L)	H ₂ O ₂ (mg/L)	NO ₂ ⁻ (mg/L)	NO ₃ ⁻ (mg/L)
0	PAW	3.719 $\pm$ 0.070	585.113 $\pm$ 14.785	405.001 $\pm$ 19.634	245.514 $\pm$ 11.196	136.98 $\pm$ 0.31	0.56 $\pm$ 0.01	16.61 $\pm$ 0.12
0	MHB	7.310 $\pm$ 0.010	219.333 $\pm$ 4.163	1,414.333 $\pm$ 9.713	707.333 $\pm$ 5.132	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
0	MHB-H ₂ O ₂ 25	7.283 $\pm$ 0.006	248.000 $\pm$ 2.000	1,423.000 $\pm$ 3.606	711.667 $\pm$ 1.528	25.83 $\pm$ 0.012	ND	ND
0	MHB-H ₂ O ₂ 50	7.253 $\pm$ 0.006	281.333 $\pm$ 2.517	1,441.333 $\pm$ 4.509	721.000 $\pm$ 2.000	51.07 $\pm$ 0.011	ND	ND
0	MHB-PAW25	6.833 $\pm$ 0.015	397.000 $\pm$ 4.583	1,584.333 $\pm$ 6.506	792.333 $\pm$ 3.512	26.01 $\pm$ 0.009	ND	ND
0	MHB-PAW50	6.410 $\pm$ 0.020	529.333 $\pm$ 10.599	1,743.333 $\pm$ 15.535	871.667 $\pm$ 7.767	50.94 $\pm$ 0.017	ND	ND
24	PAW	3.838 $\pm$ 0.028	524.216 $\pm$ 6.773	396.937 $\pm$ 19.122	240.792 $\pm$ 11.137	126.98 $\pm$ 0.29	0.30 $\pm$ 0.01	14.78 $\pm$ 0.01
24	MHB	7.233 $\pm$ 0.021	203.333 $\pm$ 5.686	1,430.333 $\pm$ 10.693	715.333 $\pm$ 5.132	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
24	MHB-H ₂ O ₂ 25	7.217 $\pm$ 0.015	223.000 $\pm$ 4.583	1,433.333 $\pm$ 7.095	717.000 $\pm$ 3.606	0.03 $\pm$ 0.003	ND	ND
24	MHB-H ₂ O ₂ 50	7.183 $\pm$ 0.015	251.667 $\pm$ 5.132	1,455.000 $\pm$ 6.557	727.667 $\pm$ 3.512	0.11 $\pm$ 0.001	ND	ND
24	MHB-PAW25	6.783 $\pm$ 0.015	311.667 $\pm$ 6.506	1,617.333 $\pm$ 8.021	809.000 $\pm$ 4.000	0.00 $\pm$ 0.001	ND	ND
24	MHB-PAW50	6.353 $\pm$ 0.032	422.667 $\pm$ 13.796	1,785.667 $\pm$ 15.695	893.000 $\pm$ 7.937	0.00 $\pm$ 0.006	ND	ND

*Values are expressed as mean $\pm$ standard deviation; MHB: pure Mueller-Hinton broth; PAW: pure plasma-activated water; MHB-H₂O₂25: MHB supplemented with free H₂O₂ at 25 mg/L; MHB-H₂O₂50: MHB supplemented with free H₂O₂ at 50 mg/L; MHB-PAW25: MHB supplemented with PAW-surfatratron normalized to 25-mg/L H₂O₂-equivalent; MHB-PAW50: MHB supplemented with PAW-surfatratron normalized to 50-mg/L H₂O₂-equivalent; ORP: oxidation-reduction potential; TDS: total dissolved solids; H₂O₂, NO₂⁻, and NO₃⁻ were determined using the Akso Exact Micro20 system; for MHB-containing samples, MHB was used as the matrix blank; ND: not detected, below the limit of detection of the analytical method. Source: Elaborated by the authors.

At 0 h, the H_2O_2 concentrations measured in MHB were consistent with the intended nominal concentrations. MHB supplemented with free H_2O_2 and MHB supplemented with PAW-surfatratron reached the target 25- and 50-mg/L H_2O_2 -equivalent conditions immediately after preparation. Since the oxidant-MHB mixtures were prepared using cold filtration through PTFE membranes, these 0-h values also indicate that the preparation and filtration procedure did not prevent the treatments from reaching the intended peroxide concentrations.

After 24 h, the detectable H_2O_2 fraction was almost completely depleted in all oxidant-containing MHB mixtures. Residual H_2O_2 decreased from approximately 25 mg/L to trace levels in MHB- H_2O_2 25 and from approximately 50 mg/L to trace levels in MHB- H_2O_2 50. In PAW-containing MHB mixtures, H_2O_2 was no longer detected after 24 h. These results confirm that MHB strongly modifies peroxide availability over time, even in the absence of bacterial cells. Therefore, the direct-addition growth assay should not be interpreted as continuous exposure to a stable H_2O_2 concentration for 24 h, but rather as exposure to a dynamic oxidant-medium system whose chemical profile changes during incubation.

The physicochemical profile also differed between free H_2O_2 and PAW-surfatratron mixtures. Free H_2O_2 caused relatively modest changes in the MHB physicochemical profile compared with PAW-surfatratron. In contrast, PAW-surfatratron produced a stronger shift in the medium, especially in ORP and pH. After 24 h, the PAW-containing media still retained a distinct physicochemical signature compared with MHB alone and MHB supplemented with free H_2O_2 . This indicates that, although H_2O_2 disappeared or was reduced to trace levels in the detectable fraction, PAW-surfatratron left persistent physicochemical changes in MHB.

This result is central to the interpretation of the growth kinetics. In the direct-addition condition, bacteria were not exposed to a fixed concentration of H_2O_2 throughout the 24-h assay. Instead, they were exposed to a medium whose oxidative profile changed over time. For free H_2O_2 , the main oxidizing component was reduced to residual trace levels after 24 h. For PAW-surfatratron, H_2O_2 was no longer detected, but changes in physicochemical parameters persisted. Therefore, the biological differences observed between H_2O_2 and PAW-surfatratron cannot be attributed only to the nominal H_2O_2 concentration; it must be interpreted in relation to the broader and more persistent physicochemical changes induced by PAW.

***Escherichia coli* growth kinetics under pulse exposure and chemical neutralization**

Based on the abiotic stability assay, the growth experiments were interpreted as two complementary biological scenarios: direct addition of an oxidant to a reactive culture medium, and controlled oxidative pulse exposure in PBS followed by chemical neutralization before growth monitoring.

The first biological scenario evaluated the effect of a defined oxidative pulse before growth monitoring. In this condition, *E. coli* ATCC 25922 was exposed in PBS for 60 min to either free H_2O_2 or surfatratron-generated PAW adjusted to 25- or 50-mg/L H_2O_2 -equivalent, followed by neutralization with 2.5 mM sodium thiosulfate for 10 min and subsequent growth monitoring in MHB. Parameters were extracted from OD_{600} growth curves recorded every 10 min for 24 h.

The kinetic parameters extracted from the OD_{600} curves are summarized in Table 2, including AUC, lag phase duration, maximum specific growth rate, doubling time, OD_{max} , and Logistic 4P fitting quality. This table provides the quantitative basis for interpreting the growth profiles shown in Figs. 2–4.

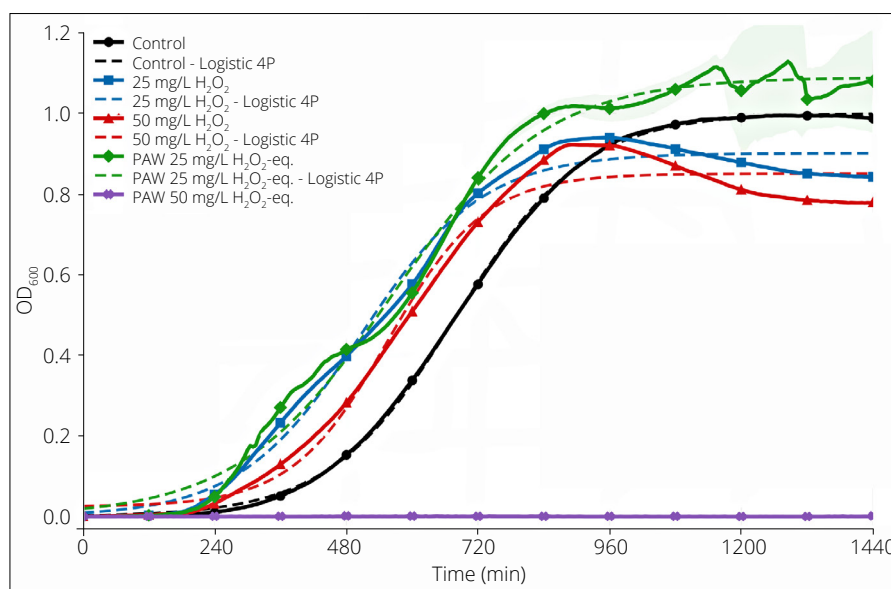
Bacterial suspensions were exposed in PBS for 60 min to free H_2O_2 or PAW-surfatratron at 25- or 50-mg/L H_2O_2 -equivalent, neutralized with 2.5 mM sodium thiosulfate for 10 min, and then transferred to MHB for 24-h growth monitoring. Optical density was measured at 600 nm every 10 min. Lines represent mean OD_{600} values, and shaded areas represent SD. Plasma-activated water-surfatratron 50-mg/L H_2O_2 -equivalent remained near baseline throughout the assay, indicating suppression of detectable OD_{600} -based growth after pulse exposure and neutralization.

As shown in Fig. 2, all treatments except PAW-surfatratron 50-mg/L H_2O_2 -equivalent showed detectable OD_{600} -based growth after pulse exposure followed by neutralization. The untreated control reached a final OD_{600} close to 1 after 24 h. Exposure to free H_2O_2 at both 25 and 50 mg/L allowed subsequent optical growth, indicating that the 60-min oxidative pulse, when chemically interrupted before incubation in MHB, did not prevent OD_{600} -based recovery under these conditions.

Table 2: Kinetic parameters of *Escherichia coli* ATCC 25922 growth under pulse exposure and direct oxidant addition to Mueller-Hinton broth*.

Sample	Treatment	OD _{max}	AUC (OD·h)	T _{lag} (h)	μ _{max} (h ⁻¹)	t _d (min)	R ² Logistic 4P
NC	-	0.996 ± 0.002 ^c	12.590 ± 0.024 ^a	7.222 ± 0.048 ^b	0.750 ± 0.030 ^a	55.530 ± 2.266 ^a	0.99981
PE	25 mg/L H ₂ O ₂	0.939 ± 0.001 ^{b2}	13.956 ± 0.037 ^{c1}	4.667 ± 0.000 ^{c1}	1.282 ± 0.066 ^{c2}	32.506 ± 1.689 ^{c2}	0.98977
PE	50 mg/L H ₂ O ₂	0.922 ± 0.002 ^{b1}	12.694 ± 0.020 ^{b2}	5.528 ± 0.048 ^{b3}	1.107 ± 0.020 ^{b1}	37.569 ± 0.689 ^{b1}	0.98487
PE	25 mg/L PAW	1.211 ± 0.067 ^{c2}	15.797 ± 0.490 ^{d2}	4.555 ± 0.048 ^{d2}	1.502 ± 0.077 ^{d2}	27.742 ± 1.464 ^{d2}	0.99213
PE	50 mg/L PAW	0.002 ± 0.001 ^{a1}	0.007 ± 0.009 ^{a1}	ND	ND	ND	ND
DA	25 mg/L H ₂ O ₂	0.840 ± 0.017 ^{a1}	7.746 ± 5.845 ^{a1}	12.833 ± 7.928 ^{a1}	1.616 ± 0.111 ^{b3}	25.816 ± 1.704 ^{b3}	0.95077
DA	50 mg/L H ₂ O ₂	0.927 ± 0.020 ^{b1}	5.005 ± 1.214 ^{a1}	12.833 ± 1.110 ^{a1}	1.792 ± 0.466 ^{b2}	24.213 ± 5.867 ^{b2}	0.99674
DA	25 mg/L PAW	0.968 ± 0.038 ^{b1}	11.006 ± 3.134 ^{a1}	7.778 ± 1.170 ^{a1}	0.633 ± 0.374 ^{a1}	84.200 ± 49.956 ^{a1}	0.99393
DA	50 mg/L PAW	0.917 ± 0.009 ^{b2}	9.154 ± 1.824 ^{a1}	10.888 ± 1.347 ^{a1}	0.816 ± 0.115 ^{a1}	51.604 ± 6.775 ^{a1}	0.99701

*Treatments are organized according to methodology; NC: negative control; PE: pulse exposure in phosphate buffered saline (PBS) followed by chemical neutralization and growth in Mueller-Hinton broth (MHB); DA: direct oxidant addition to MHB; PAW: pure plasma-activated water; PAW treatments were normalized to 25- or 50-mg/L H₂O₂-equivalent; OD_{max}: maximum optical density; AUC: area under the growth curve; T_{lag}: lag phase duration; ND: non-detectable; T_{lag}, μ_{max}, and t_d were derived from the Logistic 4-Parameter model, while OD_{max} and AUC were calculated directly from the experimental OD₆₀₀ data. ND indicates non-detectable OD₆₀₀-based growth or non-determined kinetic parameter due to absence of a detectable growth phase. Statistical notation: letters indicate significant differences among treatments within the same methodology; superscript numbers indicate pairwise comparisons between PE and DA for the same oxidant and H₂O₂-equivalent concentration. NC was used as a common reference group across comparisons. ND groups were excluded from statistical comparisons of T_{lag}, μ_{max}, and t_d. Because t_d was mathematically derived from μ_{max}, statistical interpretation was primarily based on μ_{max}, while t_d was retained as a descriptive parameter. R² values refer to the Logistic 4P fit obtained from the mean growth curve of each condition. Data are presented as mean ± standard deviation. Source: Elaborated by the authors.



PAW: plasma-activated water. Source: Elaborated by the authors.

Figure 2: Growth kinetics of *Escherichia coli* ATCC 25922 after pulse exposure followed by neutralization. Curves represent the mean OD₆₀₀ values obtained from independent biological replicates, while the shaded bands indicate the point-by-point experimental variability along each growth curve, expressed as mean ± standard deviation at each measured time point.

Plasma-activated water-surfatton at 25-mg/L H₂O₂-equivalent also allowed growth after neutralization, with a higher OD₆₀₀-derived biomass signal than the untreated control. This response may indicate altered OD₆₀₀-based recovery after sublethal PAW exposure. However, because OD₆₀₀ does not distinguish viable biomass from optical changes associated with cell morphology, aggregation, or extracellular material, this observation should be interpreted cautiously and requires CFU enumeration or flow cytometry confirmation, as OD₆₀₀ does not distinguish viable biomass from extracellular material, cell debris, or morphological changes induced by oxidative stress.

In contrast, PAW-surfatton at 50-mg/L H₂O₂-equivalent suppressed detectable OD₆₀₀-based growth within 24 h after the 60-min exposure and neutralization step. This concentration-dependent contrast suggests that PAW-surfatton was not biologically equivalent to free H₂O₂, even when both were normalized to the same nominal H₂O₂ concentration.

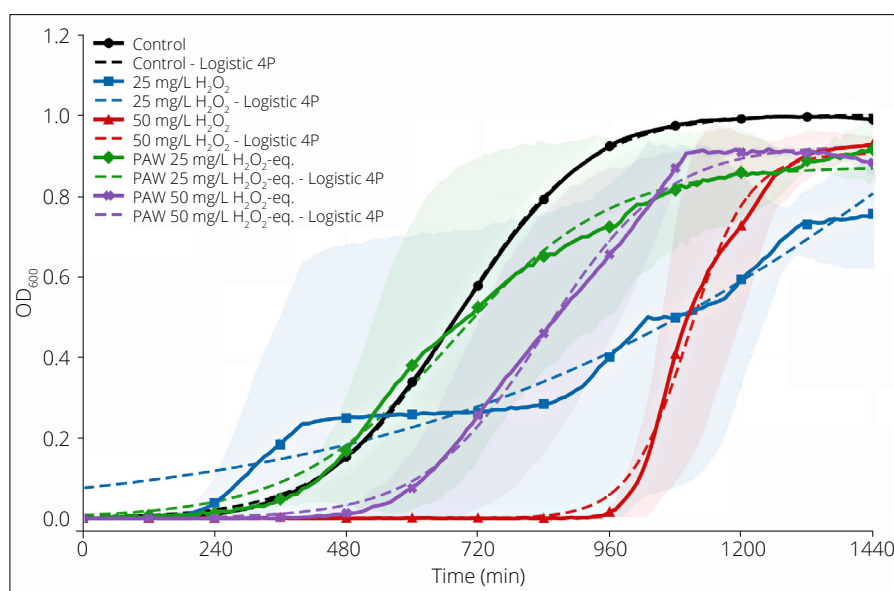
The absence of detectable growth after PAW-surfatron 50-mg/L H_2O_2 -equivalent pulse exposure is consistent with the broader interpretation of PAW as a chemically complex antimicrobial liquid. Under PBS exposure, the bacterial suspension was challenged in a low-organic-load matrix, allowing PAW-derived reactive species to interact more directly with the cells before neutralization. Since free H_2O_2 at 50 mg/L still allowed OD_{600} -based recovery after the same exposure and neutralization procedure, the biological effect of PAW-surfatron cannot be attributed to H_2O_2 concentration alone. Instead, it likely reflects the combined contribution of PAW-derived reactive oxygen and nitrogen species, altered redox properties, and other physicochemical features associated with plasma-treated liquids^{4-7,9}.

Growth dynamics under direct oxidant addition to Mueller-Hinton broth

The second biological scenario evaluated bacterial growth when oxidants were added directly to MHB before incubation. This approach represents a dynamic system in which the oxidant, the medium components, and bacterial cells interact throughout the 24-h monitoring period. For this reason, this condition should be described as direct oxidant addition to MHB, rather than constant exposure, because the oxidant concentration is not assumed to remain unchanged during incubation.

Free H_2O_2 or PAW-surfatron was added directly to MHB at 25- or 50-mg/L H_2O_2 -equivalent before inoculation, and bacterial growth was monitored for 24 h by OD_{600} measurements every 10 min. Lines represent mean OD_{600} values, and shaded areas represent standard deviation. Curves represent the mean OD_{600} values obtained from independent biological replicates, while the shaded bands indicate the point-by-point experimental variability along each growth curve, expressed as mean $\pm$ standard deviation at each measured time point. Therefore, the width of the shaded region reflects the dispersion/error of the OD_{600} measurements over time and should not be interpreted as a model-fitting confidence interval. This condition represents direct oxidant addition to a complex culture medium, not constant exposure to a stable oxidant concentration. Direct H_2O_2 at 50 mg/L produced a prolonged lag phase and delayed recovery rather than a complete absence of optical growth.

As shown in Fig. 3, the response pattern differed substantially from that observed after pulse exposure and neutralization. Direct addition of H_2O_2 at 25 mg/L allowed detectable growth, although with delayed kinetics and high variability. Direct addition of H_2O_2 at 50 mg/L did not completely prevent growth, but it produced the most prolonged lag phase among the detectable-growth conditions, indicating severe delay in recovery rather than full suppression of optical growth.



PAW: plasma-activated water. Source: Elaborated by the authors.

Figure 3: Growth kinetics of *Escherichia coli* ATCC 25922 under direct oxidant addition to MHB.

The direct-addition groups, especially DA 25-mg/L H_2O_2 and DA 25-mg/L PAW, showed higher replicate-to-replicate variability, suggesting heterogeneous recovery behavior under oxidant-medium interaction conditions.

Plasma-activated water-surfatron behaved differently from free H_2O_2 under direct addition to MHB. Both PAW-surfatron 25- and 50-mg/L H_2O_2 -equivalent allowed detectable growth, although with delayed and reduced kinetics compared with the untreated control. The 50-mg/L PAW-surfatron condition showed a longer delay than the 25-mg/L condition, but it did not suppress growth completely.

These results reinforce that free H_2O_2 and PAW-surfatron are not biologically equivalent under direct addition to MHB. H_2O_2 50 mg/L caused severe growth delay after direct addition to MHB, whereas PAW-surfatron 50-mg/L H_2O_2 -equivalent allowed delayed recovery under the same nominal H_2O_2 -equivalent condition. At first glance, this behavior contrasts with the pulse-exposure assay, in which PAW-surfatron 50-mg/L H_2O_2 -equivalent suppressed detectable growth while free H_2O_2 did not. This inversion is one of the most relevant findings of the study and indicates that the exposure matrix strongly modulates the effective biological activity of each oxidant.

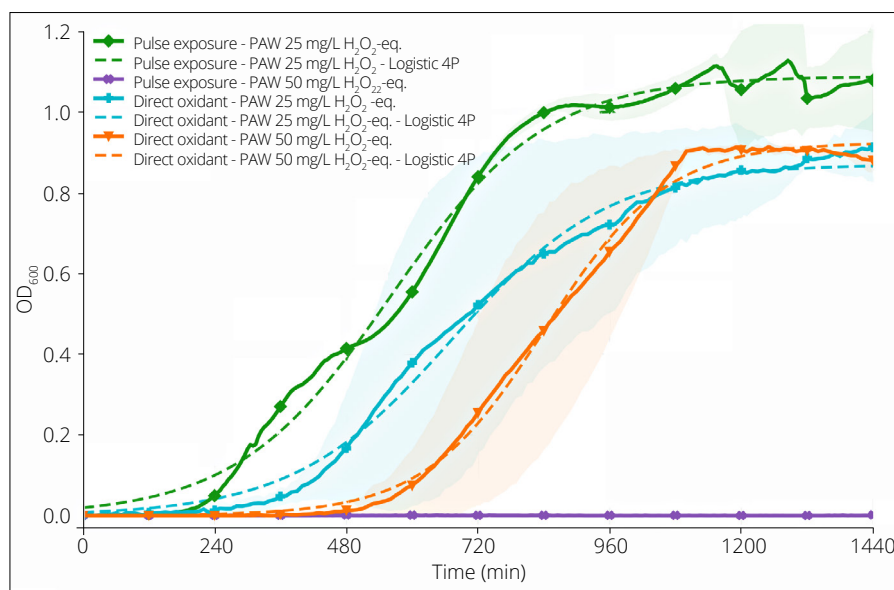
The direct-addition assay must be interpreted considering the abiotic stability results. MHB almost completely depleted the detectable H_2O_2 fraction over 24 h even without bacterial metabolism, showing that the medium itself is chemically reactive toward peroxide. However, the biological outcome differed between free H_2O_2 and PAW-surfatron. This suggests that the interaction between PAW-surfatron and MHB modifies the effective oxidative challenge differently from a simple H_2O_2 solution.

The presence of organic matter has been shown to reduce PAW inactivation efficacy, and peroxide availability in Mueller-Hinton systems can be affected by medium composition and microbial activity^{10,11}. Therefore, the direct-addition condition reflects the biological outcome of an oxidant-medium-cell interaction, in which the initial H_2O_2 -equivalent concentration is only the starting point. In this context, the persistence of a broader physicochemical signature in PAW-containing MHB may contribute to delayed growth, while the early interaction of free H_2O_2 with cells and medium may be sufficient to cause severe growth delay at 50 mg/L.

The stronger apparent inhibition observed for direct H_2O_2 addition, particularly at 50 mg/L, should therefore be interpreted as the outcome of early interaction between peroxide, cells, and MHB components rather than as a proof of stable peroxide exposure throughout the full incubation period. In contrast, the weaker inhibition observed for direct PAW-surfatron addition suggests that the rich medium may rapidly scavenge or transform part of the plasma-derived reactive chemistry, reducing the intensity of the oxidative burst that would otherwise occur in a simpler matrix such as PBS.

Plasma-activated water-surfatron was normalized to 25- or 50-mg/L H_2O_2 -equivalent and applied either as a 60-min pulse exposure in PBS followed by neutralization with 2.5 mM sodium thiosulfate for 10 min, or by direct addition to MHB before growth monitoring. Growth was monitored by OD₆₀₀ every 10 min for 24 h. Lines represent mean OD₆₀₀ values, and shaded areas represent standard deviation. Bacterial growth was monitored for 24 h by OD₆₀₀ measurements every 10 min. Curves represent the mean OD₆₀₀ values obtained from independent biological replicates, while the shaded bands indicate the point-by-point experimental variability along each growth curve, expressed as mean $\pm$ standard deviation at each measured time point. Thus, the width of each shaded region reflects the dispersion/error of the OD₆₀₀ measurements over time and should not be interpreted as a model-fitting confidence interval. The comparison shows that PAW-surfatron 50-mg/L H_2O_2 -equivalent suppressed detectable OD₆₀₀-based growth after pulse exposure, whereas the same nominal H_2O_2 -equivalent concentration allowed delayed recovery when added directly to MHB. This figure highlights the dependence of PAW biological activity on exposure matrix and experimental strategy.

Figure 4 directly summarizes the exposure-strategy effect for PAW-surfatron. At 25-mg/L H_2O_2 -equivalent, PAW-surfatron allowed detectable growth in both strategies, but pulse exposure followed by neutralization resulted in faster recovery and higher OD₆₀₀ values than direct addition to MHB. At 50-mg/L H_2O_2 -equivalent, the response diverged sharply: PAW-surfatron suppressed detectable OD₆₀₀-based growth after pulse exposure in PBS, whereas direct addition to MHB allowed delayed recovery. This contrast indicates that PAW-surfatron activity depends not only on the nominal H_2O_2 -equivalent concentration, but also on the exposure matrix, timing, and chemical interaction with the growth medium.



PAW: plasma-activated water. Source: Elaborated by the authors.

Figure 4: Effect of exposure strategy on the growth kinetics of *Escherichia coli* ATCC 25922 treated with PAW-surfatrin.

Overall, the PE and DA datasets indicate that nominal H_2O_2 equivalence is insufficient to predict the biological effect of PAW. Instead, the biological outcome is matrix-dependent: PAW-surfatrin produced stronger suppression of detectable optical growth under pulse-exposure conditions, whereas direct addition to MHB attenuated its inhibitory effect, likely due to rapid interaction between plasma-derived reactive chemistry and medium components.

Oxidative stress-induced changes in the saccharidic fraction of extracellular polymeric substances

Quantification of the saccharidic fraction of EPS provided a second biological layer for interpreting the response of *E. coli* to oxidative challenge. While OD_{600} growth curves describe population-level optical growth dynamics, carbohydrate accumulation in EPS fractions can indicate whether oxidative exposure promotes extracellular saccharidic material during stress adaptation or recovery, since exopolysaccharide production has been described as a protective response induced by oxidative and envelope stress in gram-negative bacteria^{15,16}. In this study, EPS was operationally fractionated into soluble EPS, cell-associated EPS, and cuvette-adhered EPS, and the anthrone assay¹⁴ was used specifically to estimate carbohydrate-rich material rather than total EPS biomass. This quantification is presented in Table 3.

Table 3: Saccharidic fraction of extracellular polymeric substances produced by *Escherichia coli* ATCC 25922 after oxidative challenge*.

Sample	Soluble EPS (μg dextran-equivalent/mL)	Cell-associated EPS (μg dextran-equivalent/mL)	Cuvette-adhered EPS (μg dextran-equivalent/mL)
NC	36.400 ± 1.700^b	67.167 ± 3.400^c	22.733 ± 1.850^c
PE 25 mg/L H_2O_2	46.233 ± 2.650^b	87.433 ± 3.853^b	34.100 ± 2.300^c
PE 50 mg/L H_2O_2	52.033 ± 2.801^a	99.933 ± 4.601^b	40.200 ± 2.651^{ab}
PE 25 mg/L PAW	63.433 ± 3.811^a	126.933 ± 7.753^a	53.767 ± 4.352^a
PE 50 mg/L PAW	7.433 ± 0.651^c	6.567 ± 0.651^d	5.233 ± 0.451^d
DA 25 mg/L H_2O_2	54.500 ± 7.155^{ab}	103.900 ± 14.352^{abc}	42.333 ± 7.104^{abcd}
DA 50 mg/L H_2O_2	57.167 ± 3.450^a	111.567 ± 6.857^{ab}	43.567 ± 3.800^{ab}
DA 25 mg/L PAW	61.433 ± 5.850^{ab}	119.833 ± 11.752^{abc}	50.400 ± 6.355^{abc}
DA 50 mg/L PAW	66.467 ± 4.350^a	132.367 ± 7.406^a	56.200 ± 4.551^a

*Values are presented as mean $\pm$ standard deviation and expressed as μg dextran-equivalent/mL; NC: negative control; PAW: plasma-activated water; PE: pulse exposure in phosphate buffered saline (PBS) followed by chemical neutralization and growth in Mueller-Hinton broth; DA: direct oxidant addition to MHB; EPS: extracellular polymeric substances; EPS fractions were analyzed separately using Welch analysis of variance followed by the Games-Howell post hoc test ($p < 0.05$). Statistical letters must be interpreted column by column; groups sharing at least one letter are not significantly different. Source: Elaborated by the authors.

As shown in Table 3, the saccharidic fraction of EPS differed among treatments in all evaluated fractions. The NC showed the lowest carbohydrate accumulation among growth-positive groups, with 36.400 ± 1.700 µg/mL in soluble EPS, 67.167 ± 3.400 µg/mL in cell-associated EPS, and 22.733 ± 1.850 µg/mL in cuvette-adhered EPS. In contrast, PAW-treated groups generally showed higher EPS carbohydrate accumulation, particularly in the cell-associated and cuvette-adhered fractions.

Under pulse exposure, PE 25 mg/L PAW produced the highest absolute EPS values among PE groups with detectable growth, reaching 63.433 ± 3.811 µg/mL in soluble EPS, 126.933 ± 7.753 µg/mL in cell-associated EPS, and 53.767 ± 4.352 µg/mL in cuvette-adhered EPS. By contrast, PE 50 mg/L PAW showed only residual EPS carbohydrate levels, consistent with the near absence of detectable OD₆₀₀-based growth after this treatment. Therefore, the low absolute EPS values in PE 50 mg/L PAW should be interpreted primarily as a consequence of strong growth suppression rather than as evidence of reduced EPS production by an actively growing population.

Under direct oxidant addition to MHB, the highest absolute EPS values were observed for DA 50 mg/L PAW, which reached 66.467 ± 4.350 µg/mL in soluble EPS, 132.367 ± 7.406 µg/mL in cell-associated EPS, and 56.200 ± 4.551 µg/mL in cuvette-adhered EPS. H₂O₂ treatments also increased EPS carbohydrate accumulation relative to the NC, but the strongest matrix-associated response was associated with PAW, especially at 50 mg/L H₂O₂-equivalent under direct addition. This result supports the interpretation that PAW does not behave as a simple H₂O₂-equivalent solution, because its broader physicochemical profile appears to influence extracellular carbohydrate accumulation and surface-associated phenotypes.

Normalization by OD_{max} helped distinguish absolute EPS accumulation from EPS carbohydrate accumulation relative to the OD₆₀₀-derived biomass signal. As shown in Table 4, DA 50-mg/L PAW remained the highest response after normalization, with 72.483 ± 4.743 for soluble EPS/OD_{max}, 144.348 ± 8.076 for cell-associated EPS/OD_{max}, and 61.287 ± 4.963 for cuvette-adhered EPS/OD_{max}. This indicates that the higher EPS response in this condition was not merely a consequence of higher optical biomass but reflected a disproportionate matrix-associated carbohydrate phenotype relative to growth.

Table 4: OD_{max}-normalized saccharidic fraction of extracellular polymeric substances produced by *Escherichia coli* ATCC 25922 after oxidative challenge*.

Sample	Soluble EPS/OD _{max} (µg dextran-equivalent/mL)	Cell-associated EPS/OD _{max} (µg dextran-equivalent/mL)	Cuvette-adhered EPS/OD _{max} (µg dextran-equivalent/mL)
NC	36.547 ± 1.707^c	67.437 ± 3.414^c	22.824 ± 1.858^c
PE 25 mg/L H ₂ O ₂	49.236 ± 2.822^b	93.113 ± 4.104^b	36.315 ± 2.449^b
PE 50 mg/L H ₂ O ₂	56.435 ± 3.038^{ab}	108.387 ± 4.990^b	43.601 ± 2.875^{ab}
PE 25 mg/L PAW	52.381 ± 3.147^b	104.817 ± 6.402^b	44.399 ± 3.594^{ab}
PE 50 mg/L PAW	ND	ND	ND
DA 25 mg/L H ₂ O ₂	64.881 ± 8.518^{abc}	123.690 ± 17.086^{abc}	50.396 ± 8.457^{abc}
DA 50 mg/L H ₂ O ₂	61.669 ± 3.721^{ab}	120.352 ± 7.397^{ab}	46.998 ± 4.099^{ab}
DA 25 mg/L PAW	63.464 ± 6.044^{abc}	123.794 ± 12.141^{abc}	52.066 ± 6.565^{abc}
DA 50 mg/L PAW	72.483 ± 4.743^a	144.348 ± 8.076^a	61.287 ± 4.963^a

*Values are presented as mean $\pm$ standard deviation; NC: negative control; PAW: plasma-activated water; PE: pulse exposure in phosphate buffered saline (PBS) followed by chemical neutralization and growth in Mueller-Hinton broth; DA: direct oxidant addition to MHB; EPS: extracellular polymeric substances; OD_{max}: maximum optical density; ND: non-detectable; EPS/OD_{max} was calculated by dividing the EPS carbohydrate concentration by the corresponding OD_{max} from the growth-kinetics assay. The PE 50-mg/L PAW group was classified as ND because its near-zero OD_{max} made normalization biologically uninterpretable. Statistical comparisons were performed separately for each EPS fraction using Welch analysis of variance followed by the Games-Howell post hoc test ($p < 0.05$). Statistical letters must be interpreted column by column; groups sharing at least one letter are not significantly different. Source: Elaborated by the authors.

The normalized analysis also shows that H₂O₂ exposure increased EPS carbohydrate accumulation per OD_{max} relative to the NC, especially under direct addition. However, PAW-treated groups, particularly DA 50-mg/L PAW, remained among the highest responses across the cell-associated and cuvette-adhered fractions. These fractions

are biologically more informative for matrix-associated and surface-associated responses than soluble EPS by itself, because they reflect carbohydrate-rich material associated with the cell surface and with attachment to the growth vessel, consistent with the operational distinction between soluble and bound EPS fractions described for microbial aggregates¹⁷.

The soluble EPS fraction should be interpreted more cautiously. Although soluble carbohydrate increased under several oxidative conditions, this fraction may include extracellular carbohydrates released into the medium, products from damaged or stressed cells, and soluble components that are less directly related to cell-surface matrix reinforcement, in line with the recognized compositional and functional differences between soluble and cell-bound EPS fractions¹⁷. Therefore, soluble EPS alone should not be treated as evidence of biofilm formation. The more robust interpretation comes from the combined increase in cell-associated EPS and cuvette-adhered EPS, which suggests a shift toward extracellular carbohydrate accumulation associated with protection, persistence, and surface interaction, consistent with the role of exopolysaccharide matrices in protecting bacterial cells under stress conditions¹⁵.

Together, the EPS data complement the growth-kinetics results. Plasma-activated water 50-mg/L H₂O₂-equivalent suppressed detectable OD₆₀₀-based growth under pulse exposure, but when added directly to MHB it allowed delayed recovery accompanied by the highest absolute and normalized EPS carbohydrate responses. This reinforces the central conclusion that nominal H₂O₂ equivalence is insufficient to predict the biological effect of PAW. Instead, the biological outcome depends on the combined action of plasma-derived reactive species, redox imbalance, pH shifts, medium interactions, and the timing of exposure relative to growth conditions.

Several limitations of this study should be acknowledged. First, bacterial viability was assessed exclusively by OD₆₀₀, without confirmation by CFU enumeration. Optical density cannot distinguish viable biomass from cell debris, aggregates, or extracellular material; this is particularly relevant for the PE 25-mg/L PAW group, in which OD₆₀₀ exceeded the untreated control, and conclusions regarding actual biomass increase require CFU or flow cytometry confirmation. Second, the saccharidic fraction of EPS was normalized by OD_{max} as a biomass proxy; future studies should incorporate total protein content (e.g., BCA or Bradford assay) or dry weight measurements as independent co-variables to decouple carbohydrate accumulation from optical biomass variation. Third, each biological replicate of the growth-kinetics and EPS assays was performed with an independently generated PAW activation, so that the three biological replicates correspond to three independent plasma activations. The physicochemical parameters reported in Table 1 (pH, ORP, conductivity, TDS, H₂O₂, NO₂⁻, NO₃⁻) represent the mean ± SD of these three independent activations, meaning that inter-activation variability is already captured in the reported data. This is a methodological strength rather than a limitation, as it demonstrates that the surfatron system produced reproducible PAW across independent runs under the conditions used. Finally, the high replicate-to-replicate variability observed in the DA 25-mg/L H₂O₂ and DA 25-mg/L PAW groups (coefficient of variation > 50% for AUC and T_{lag}) reflects genuine biological heterogeneity in growth recovery under oxidant-medium interaction conditions, consistent with the phenotypic heterogeneity described among genetically identical bacterial cells exposed to fluctuating or stressful environments¹⁸. This variability should be characterized further and interpreted as an intrinsic feature of direct-addition assays in complex media rather than as experimental noise.

CONCLUSION

This study demonstrates that surfatron-generated PAW and free H₂O₂ produce distinct biological responses in *E. coli* ATCC 25922, even when treatments are normalized to the same H₂O₂-equivalent concentration. The abiotic stability assay showed that MHB strongly modifies peroxide availability over time, confirming that direct oxidant addition to MHB represents a dynamic oxidant-medium-cell system rather than a constant exposure to a stable H₂O₂ concentration.

The exposure strategy was decisive. Under pulse exposure in PBS followed by thiosulfate neutralization, PAW at 50-mg/L H₂O₂-equivalent suppressed detectable OD₆₀₀-based growth, whereas free H₂O₂ at the same nominal

concentration allowed recovery. Under direct addition to MHB, PAW inhibitory activity was attenuated and delayed growth recovery occurred, while free H₂O₂ produced severe growth delay at 50 mg/L. These contrasting outcomes indicate that PAW bioactivity depends not only on nominal H₂O₂-equivalence, but also on the exposure matrix, timing of exposure, and interaction with medium components.

Extracellular polymeric substances carbohydrate analysis added a complementary phenotypic layer to the growth kinetics. Plasma-activated water-treated groups, particularly direct addition at 50-mg/L H₂O₂-equivalent, showed the strongest accumulation of the saccharidic fraction of EPS, especially in the cell-associated and cuvette-adhered fractions. After OD_{max} normalization, this condition remained among the highest responses, suggesting a disproportionate matrix-associated carbohydrate phenotype rather than EPS accumulation merely proportional to optical biomass.

Overall, the results support the conclusion that PAW-surfatron should not be treated as a simple H₂O₂ solution. Its biological effects arise from broader physicochemical reactivity involving reactive oxygen and nitrogen species, redox changes, pH shifts, and medium-dependent transformations. This reinforces the need to evaluate PAW bioactivity under exposure conditions that reflect the intended biological application rather than relying only on H₂O₂-equivalent normalization.

CONFLICT OF INTEREST

Nothing to declare.

AUTHOR CONTRIBUTIONS

Conceptualization: Martins EF, Santos MCR, Augustroze JHC and Martins AL. **Formal Analysis:** Martins EF, Santos MCR, Martins AL and Sant'Anna Junior AB. **Investigation:** Martins EF, Santos MCR, Augustroze JHC and Martins AL. **Resources:** Brito MS, Sibelino SK, Silva Sobrinho AS and Pessoa RS. **Supervision:** Sibelino SK, Silva Sobrinho AS and Pessoa RS. **Validation:** Brito MS, Sibelino SK, Silva Sobrinho AS and Pessoa RS. **Data curation:** Martins AL and Sant'Anna Junior AB. **Writing – original draft:** Martins EF and Santos MCR. **Writing – review and editing:** Martins EF and Santos MCR. **Final approval:** Pessoa RS.

AVAILABILITY OF DATA AND MATERIALS

All datasets were generated or analyzed in the current study.

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DECLARATION OF USE OF INTELLIGENCE ARTIFICIAL TOOLS

During the preparation of this work, the authors used ChatGPT (OpenAI) and Claude (Anthropic) to assist with language editing, including spelling and grammar review, improvement of textual clarity, and verification of consistency throughout the manuscript. These tools were also used to support the creation and refinement of explanatory schematic figures intended exclusively to illustrate scientific concepts, processes, and relationships.

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