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Adaptation of *Enterobacter* sp. to herbicides is correlated with distinct patterns of quorum sensing molecules

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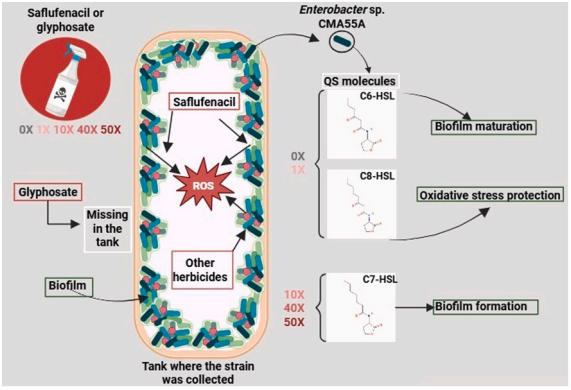
HIGHLIGHTS

- Bacteria can adapt to herbicide contaminated environment through biofilm formation.
- Quorum sensing coordinates responses to herbicides within the bacterial community.
- Bacterial responses to herbicides occur by the release of signaling molecules.
- C6-HSL, C7-HSL, and C8-HSL are not involved in bacterial glyphosate responses.
- *Enterobacter* can produce specific quorum sensing molecules for herbicides responses.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, we employed a model that examined bacterial response systems in an artificial environment with wash water from the packaging of several pesticides, and no nutrient supplementation, allowing for the investigation of quorum sensing (QS) signaling molecules produced by a *Enterobacter* strain isolated from this environment. The herbicide saflufenacil, which was present at the isolation site, and glyphosate, which was absent, were utilized as stress agents in an in vitro system to evaluate the biofilm formation. These adaptive mechanisms are regulated by QS, which orchestrates collective behaviors within bacterial communities and initiates stress responses through the release of autoinducer molecules. The QS signaling molecules C6-HSL, C7-HSL, and C8-

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HSL were analyzed using liquid chromatography coupled with mass spectrometry; these molecules are primarily involved in biofilm maturation, biofilm formation, and protection against oxidative stress, respectively. Notably, the biofilm formation exhibited two distinct strategies in *Enterobacter* sp. CMA55A. One strategy, likely coordinated by the three QS molecules, was associated with the presence of saflufenacil in the culture medium, at concentrations presumably similar to those at the strain's isolation site. The second strategy, without these QS coordination, was observed in response to glyphosate and the highest concentration of saflufenacil, conditions not present at the isolation site. Future comparative genomic and transcriptomic studies may provide insights into these plastic responses and facilitate the exploration of potential applications in the bioremediation of various xenobiotics at differing concentrations in contaminated ecosystems.

1. Introduction

Agriculture is expanding its food production to accommodate the increasing human population [9]. However, a portion of this production is lost due to competition from non-agricultural plants for space and nutrients, leading to the use of pesticides as a solution to mitigate adverse effects on farmers' economy [7].

Herbicides have the potential to disrupt the balance between the production and elimination of ROS, leading to metabolic disorders in bacterial cells. Such disruptions can adversely affect soil microbial diversity, which is crucial for maintaining soil fertility, facilitating nutrient cycling, and promoting nitrogen fixation [28]. The herbicides Heat and Roundup Transorb R contain saflufenacil and glyphosate as their active ingredients, respectively. Saflufenacil functions by inhibiting protoporphyrinogen oxidase (PROTOX), an enzyme that is not exclusive to plants, as it is also present in certain bacteria and cyanobacteria, thereby rendering saflufenacil detrimental to microorganisms as well [39]. Glyphosate-based herbicides have the potential to negatively impact the metabolism of plants, animals, and microorganisms. Consequently, soil-dwelling bacteria may, under specific conditions, adopt alternative survival strategies and/or develop mechanisms to degrade this herbicide [11]. Glyphosate exerts its effects by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, which is found in both plants and bacteria, thus posing a threat to microbial life [3].

One of the survival strategies employed by microorganisms is QS, a communication system mediated by signaling molecules and specific genes [6]. This mechanism is crucial for various processes, including the regulation of virulence factors, the production of extracellular polymeric substances (EPS), and the formation of biofilms [8]. QS modulates these bacterial behaviors through signaling molecules such as acylhomoserine lactones (AHLs), peptides, and AI-2. In Gram-negative bacteria, QS is primarily mediated by AHLs and AI-2, which can also be present in Gram-positive bacteria. Many AHLs exhibit chirality and can exist as enantiomers. Bacterial receptors possess the ability to differentiate between the two forms and preferentially bind to one enantiomer over the other. AHLs are characterized by a conserved homoserine lactone (HSL) ring linked to a variable acyl chain. Factors such as chain length and the presence of functional groups on the acyl chain are critical determinants of receptor binding and signal specificity [6].

This study is based on the hypothesis that adaptation systems in bacteria, which are associated with the presence of herbicides in their environment—such as biofilm formation and tolerance to oxidative stress—should also demonstrate expression control mechanisms mediated by quorum sensing signaling molecules linked to these pesticides. To evaluate this hypothesis, the objective of this research was to investigate whether quorum sensing signaling molecules, which are associated with adaptive responses to stressful environmental conditions (including biofilm formation and reproductive capacity under such conditions), could be correlated with specific quorum sensing molecules. Given that the environment selected for bacterial isolation was characterized by variations in the composition and concentration of pesticides, we also aimed to assess the effects of different concentrations of herbicides that were either present or absent at the isolation site.

2. Material and methods

The experimental focus of this study revolved around investigating the response of the *Enterobacter* sp. strain to elevated concentrations of saflufenacil and glyphosate, exceeding typical levels of agricultural use. This investigation involved performing tolerance tests, growth curve analysis, cell viability assessment, biofilm formation assessment, and extraction and analysis of QS signaling molecules using LC-MS/MS (Fig. 1).

2.1. Experimental design

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2.2. Bacterial strain

The bacterial strain utilized in this study was *Enterobacter* sp. CMA55A (NCBI - OL598345 nucleotide database), which was isolated from the storage tanks of the Capão da Onça School Farm, State University of Ponta Grossa. This environment was characterized by the presence of water used for washing pesticide packaging, as well as the presence of chlorine, fluorine, and fluctuations in pH and temperature. Water contaminated with 30 different pesticides created unfavorable conditions for the survival of microorganisms. The volume of water decreased due to evaporation, and the washing of pesticide containers was conducted repeatedly. Consequently, the concentration of herbicides in the water exhibited variability. Among the genera identified in this location, *Enterobacter* possesses the potential to harbor genes that encode traits conferring phenotypic plasticity, such as xenobiotic degradation and biofilm formation and structure, potentially mediated through QS [19]. The bacterial strain is preserved in a 50 % glycerol solution at −80 ºC within the Environmental Microorganisms Collection of the Environmental Microbiology Laboratory at the State University of Ponta Grossa, Brazil.

2.3. Herbicides

The herbicide Heat (BASF – Ludwigshafen, Rhein, Germany), at a concentration of 70 %, which includes the active compound saflufenacil (N′-{2-chloro-4-fluor-5-[1,2,3,6-tetrahydro-3-methyl-2,6-dioxe-4-(trifluoromethyl)pyrimidine-1-yl]benzoyl}-N-isopropyl-N-methyl-sulfamide), and the herbicide Roundup Transorb R (Agro Bayer – Leverusen, Germany), at a concentration of 58.8 %, which contains the active compound glyphosate (Potassium salt of (N-(phosphonomethyl) glycine were used).

2.4. Growing media and growing conditions

The pre-inoculum of the strain was prepared in Luria-Bertani broth (LB: NaCl 10 g/L, Tryptone 10 g/L and Yeast Extract 5 g/L), as well as on Luria-Bertani agar (LA: LB supplemented with agar 20 g/L). The experiments were conducted in triplicate, with standardized bacterial growth conditions at 30 ºC under agitation at 120 rpm for 24 h.

2.5. Treatments

The dosages selected for the current study were informed by the significant level of contamination present in the water from which the strain was isolated, as well as the findings from prior research conducted by the Environmental Microbiology laboratory. Freitas et al. [8] administered the herbicides glyphosate and saflufenacil at a concentration of 1x. In contrast, Rovida et al. [32] applied saflufenacil at concentrations of 15x and 45x. Additionally, Silva et al. [36] utilized saflufenacil at concentrations of 1x, 10x, and 50x. These investigations have elucidated bacterial responses to varying concentrations of herbicides. For herbicide treatments, LB medium was supplemented with saflufenacil and glyphosate at concentrations equivalent to 1x, 10x, 40x, and 50x those typically applied in agriculture. Concentrations were specifically calculated as follows: 0.0 mM (S0); 0.49 mM (S1); 4.9 mM (S10); 19.6 mM (S40); and 24.5 mM (S50) for saflufenacil and 0.0 mM (G0), 0.23 mM (G1); 2.3 mM (G10); 9.2 mM (G40); and 11.5 mM (G50) for glyphosate. The control group was standardized as S0 and G0 for saflufenacil and glyphosate, respectively.

2.6. Tolerance test

The tolerance test was conducted in accordance with the methodology outlined by Silva et al. [36], with certain modifications implemented. For the tolerance test, growth conditions identical to those detailed in Section 2.3 were employed. The initial optical density (DO) was measured at 0.05 absorbance (abs) at 600 nm. After 24 h of bacterial culture growth in control medium and LB with saflufenacil or glyphosate treatments at the concentrations specified in the previous section, 1 mL aliquots were extracted for growth analysis using a spectrophotometer adjusted to 600 nm. The tolerance test was conducted exclusively with the selected strain to assess its capacity to withstand varying concentrations of the herbicides saflufenacil and glyphosate prior to initiating the bacterial growth curve.

2.7. Bacterial growth curve

The bacterial growth curve was conducted in accordance with the methodology outlined by Silva et al. [36], with several modifications implemented. The culture was incubated according to the procedure described in Section 2.3. The inoculum was standardized to an initial optical density (OD) of 0.05 abs at 600 nm. Growth was monitored in the treatments every 1 h at 600 nm using a spectrophotometer, and the resulting data was used to construct the growth curve.

2.8. Cell viability

Cell viability was assessed using the methodology outlined by Silva et al. [36], with certain modifications implemented. To assess cell viability, identical pre-inoculum conditions were employed as described in Section 2.3. Volumes of 100 µL were extracted during the stationary phases, as identified in the growth curve. These samples were diluted in autoclaved saline (0.9 % NaCl) and then spread on LA plates for inoculation. Enumeration of viable cells in the samples was performed by counting colony forming units (CFU).

2.9. Biofilm formation

Biofilm formation was quantified following the methodology outlined by Tang et al. [38] and Wang et al. [43], with some adaptations. The growing conditions are described in detail in Section 2.3. Specifically, 200 µL of bacterial culture was dispensed in microplates and submitted to incubation in a shaker for 15 h at 30 ºC. Subsequently, the culture medium was aspirated and 200 µL of 0.1 % crystal violet dye was introduced into the cells adhered to the surface, allowing an incubation period of 30 min for the reaction. Afterwards, the dye was aspirated and the wells were rinsed three times with distilled water. Then, 200 µL of 95 % ethanol was added and the sample was evaluated using a microplate reader at 570 nm (Elx808™, BioTek, Winooski, VT, USA). A negative control was employed concurrently to account for the crystal violet staining in relation to the OD of the other treatments.

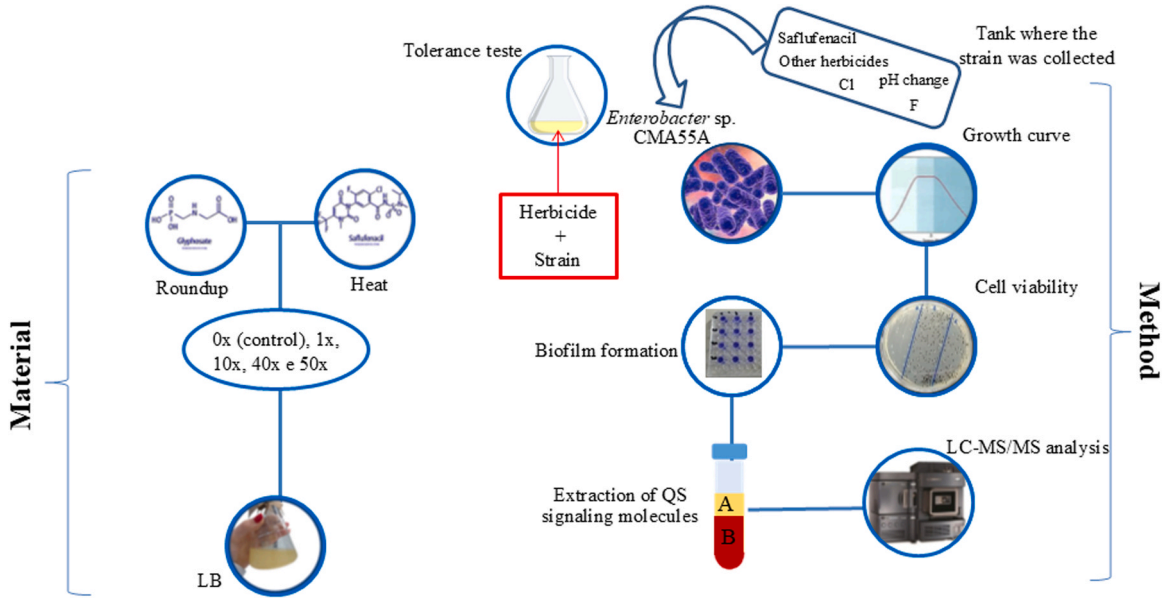

Fig. 1. The bacterial strain *Enterobacter* sp. CMA55A was subjected to several treatments involving the growth medium Luria Bertani (LB) together with the herbicides Heat (containing the active compound saflufenacil) and Roundup (containing the active compound glyphosate) at concentrations of 0x, 1x, 10x, 40x and 50x. The experiments performed included a tolerance test to assess the strain's ability to survive different concentrations of xenobiotics, a growth curve analysis to understand the xenobiotic-induced stress response at varying concentrations and to determine the optimal time for the collection of QS molecules, a cell viability assessment to assess the impact of xenobiotics on the bacteria's ability to divide cell, biofilm formation analysis to study a specific response to xenobiotics and an examination of QS signaling molecules (A – organic phase and B – aqueous phase) using LC-MS/MS to focus on molecules that are more reactive to xenobiotic treatments.

2.10. Extraction of quorum sensing molecule

The extraction of QS molecules was performed following the method described by Ortori et al. [30], with some modifications. The extraction process involved the liquid-liquid method and was carried out over a period of 15 h during bacterial growth. After bacterial culture growth under the conditions specified in Section 2.3, 100 mL of the culture was extracted and centrifuged at 8000 g for 5 min. After centrifugation, 10 mL of the supernatant was collected and 20 μL of the internal standard N-heptanoy-L-homoserine lactone (C7-HSL) was added. The solution was then centrifuged at 8000 g for 5 min. Subsequently, 10 mL of ethyl acetate acidified with 0.01 % acetic acid was introduced into the supernatant. The resulting solution was centrifuged at 8000 g for 5 min, and the organic phase was isolated. The samples were then dried in an oven at 30 ºC and stored at −20 ºC. Prior to LC-MS/MS analysis, 100 μL of methanol (MeOH) were added and agitated for one min.

2.11. Analysis in LC-MS/MS

Using the method described by Ortori et al. [30], with modifications, the LC-MS/MS analyses were performed. The equipment used was the Quatro Premier XE triple quadrupole model (Waters Corporation, Milford, Massachusetts, USA) and the MassLynx software (Waters Corporation, Milford, Massachusetts, USA). The column used was C18 3.5μm, (4.6 ×50 mm). 10 μL of the extracts prepared according to item 2.9 were injected. The mobile phase consisted of 70 % acetonitrile, 30 % ultrapure water, and 0.1 % formic acid, at a flow rate of 0.3 mL min^{-1}. The LC-MS/MS was made in positive mode with a collision energy of 15 V, a dissolution temperature of 400 ºC, a source temperature of 110 ºC, a dissolvation gas flow of 300 L h^{-1} and a collision gas of 0.14 mL min^{-1}. The optimization of the analytical parameters in MS/MS was performed by the infusion of the internal standard N-heaptanoyl-L-homoserine lactone (C7-HSL) and the analytical standards N-hexanoyl-L-homoserine lactone (C6-HSL) and N-octanoyl-L-homoserine lactone (C8-HSL).

2.12. Statistical analysis

Statistical analyses concerning the growth curve, cell viability, and biofilm formation were subjected to normality tests, assessments of homogeneity of variance, analysis of variance (ANOVA), and the Scott-Knott test. QS analyses were conducted using principal component analysis (PCA). The PCA was executed with six quantitative variables: production of C6-HSL, C7-HSL, and C8-HSL, as well as tolerance, cell viability, and biofilm formation. For these analyses, the following R software packages were utilized: agricolae, expDes.pt, ScottKnott, ggplot2, ggord, and vegan, in version 4.4.0.

3. Results and discussion

3.1. Capacity of Enterobacter sp. CMA55A to tolerate herbicides

Certain bacterial strains possess the capability to endure the effects of biocides, even in the absence of prior exposure. For instance, *Escherichia coli* DH5-α exhibited resilience to mesotrione, the primary component of the herbicide Callisto. This resistance can be ascribed to its accelerated metabolic processes and its capacity to degrade this xenobiotic compound [27].

Exposure to toxic environments, such as the presence of herbicides in their growth habitats, can induce both structural and physiological alterations in microorganisms. For instance, an increase in biofilm production has been documented [19,8]. These alterations may encompass modifications in membrane lipid saturation, which can lead to reduced permeability. This adjustment functions as a selective barrier against herbicides, thereby enhancing the organism's tolerance to xenobiotics [31]. Environments treated with toxic substances impose stressful and selective conditions on microbiota, necessitating rapid metabolic adaptations for survival [29]. Consequently, we have selected a storage tank used for washing packaging of various pesticides as a model environment subjected to significant selective pressure. This setting enables the investigation of bacterial characteristics associated with adaptive responses to such conditions.

Enterobacter sp. CMA55A was chosen for its tolerance to treatments involving the herbicides saflufenacil and glyphosate. This selection was informed by the bacterial growth recorded after 24 h of incubation in LB medium under various treatment conditions, as depicted in Fig. 2.

The application of all concentrations of the glyphosate herbicide did not result in a statistically significant reduction in bacterial growth when compared to the control group. In the case of the saflufenacil herbicide treatment, no significant differences in bacterial growth were observed at doses S10 and S50 relative to the control. However, a significant reduction in bacterial growth was noted at doses S10 and S40 when compared to both the control and the other treatment groups. Detailed data regarding the statistical differences can be found in Supplementary Material 1.

The herbicide saflufenacil displayed greater toxicity to the bacterial strain, as indicated by the reduced bacterial growth in this treatment group. The strain had prior exposure to this herbicide, which may promote the selection of strains with enhanced survivability in challenging and stressful environments. A compelling direction for future research is to explore whether distinct mechanisms develop independently in response to the presence of xenobiotics at the site of isolation.

No significant differences in bacterial growth were observed across the various concentrations of the herbicide glyphosate. Furthermore, glyphosate was not detected at the isolation site of the *Enterobacter* sp. strain CMA55A. This absence may suggest that the strain possesses the capacity to tolerate glyphosate through generalist mechanisms, irrespective of any prior exposure to the herbicide. Previous studies have demonstrated that glyphosate can induce the production of reactive oxygen species (ROS) in non-target organisms [13], reduce bacterial growth in a manner analogous to paraquat [24], and contribute to biodiversity loss, which can negatively impact soil functions [37].

The findings suggest that the strain demonstrated significantly higher bacterial grow thin comparison to the control group when exposed to both herbicides, even in the absence of the isolation environment. Future research involving this bacterium should prioritize the investigation of its ability to degrade these herbicides, as well as an examination of whether the presence of these substances at the isolation site had a substantial impact on the observed results.

3.2. Bacterial growth in response to glyphosate and saflufenacil

Fig. 3 depicts the growth curve in response to the herbicide saflufenacil, whereas Fig. 4 presents the growth curve influenced by the herbicide glyphosate. Both figures emphasize the initiation of the log and late logarithmic of growth. The lag phase of bacterial growth was not observed, likely due to the strain's prior adaptation to the culture medium and its production of enzymes conducive to the initiation of the log phase. Consequently, the postponement of herbicide application did not affect this condition.

In the treatment involving the herbicide saflufenacil, the initial growth phase commenced within one h, followed by a gradual transition from the logarithmic phase to the stationary phase over a period of approximately twelve h. Similarly, in the treatment with the herbicide glyphosate, the initial growth phase also began within one h; however, the transition from the logarithmic phase to the stationary phase occurred in approximately ten h. The growth curve was employed to ascertain the onset of the bacterial growth phases, which was essential for the analysis of cell viability and for standardizing the extraction time of the QS signaling molecule. As noted by Ling et al. [20], the production of autoinducer molecules exhibited a proportional increase corresponding to cell density, peaking during the late logarithmic phase. AHL autoinducers displayed diminished activity at low cell concentrations,

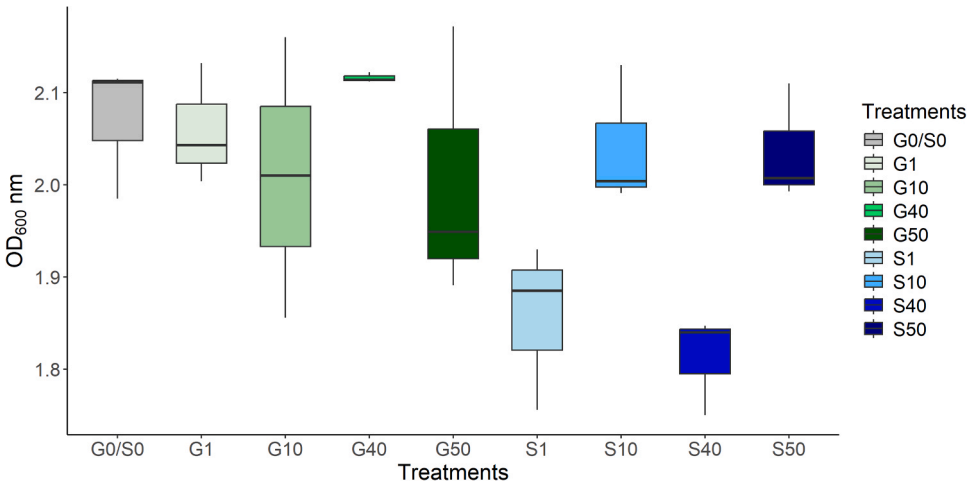

Fig. 2. The tolerance test conducted on *Enterobacter* sp. strain CMA55A, which was performed following 24 h of incubation in LB medium supplemented with varying concentrations of the herbicide glyphosate (G0, G1, G10, G40, and G50) and saflufenacil (S0, S1, S10, S40, and S50).

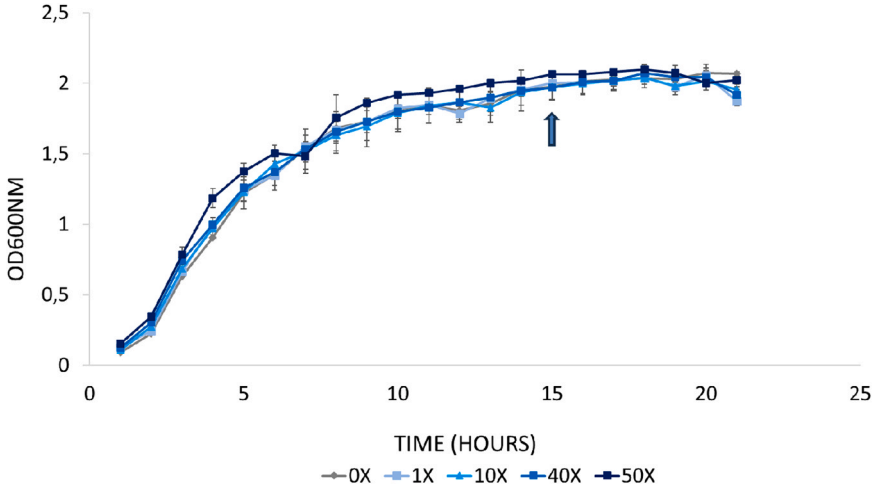

Fig. 3. The growth curve of *Enterobacter* sp. CMA55A was analyzed in the LB control and in the treatments containing the herbicide saflufenacil at concentrations of 0X, 1X, 10X, 40X and 50x. Statistical analysis was performed using Duncan's test with a confidence level of 95 %, and the error bars represent the standard error of the means. The arrow indicates the specific period selected for the extraction of the QS signaling molecules.

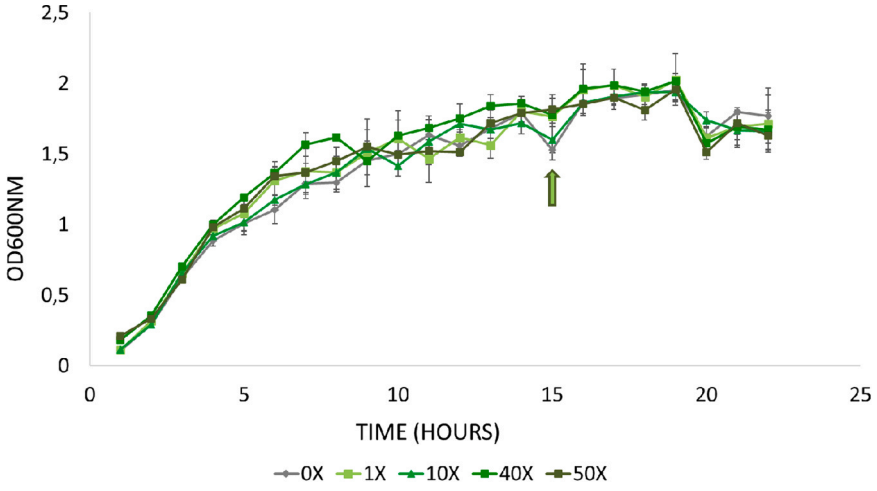

Fig. 4. The growth curve of *Enterobacter* sp. CMA55A was analyzed in the LB control and in the treatments containing the herbicide glyphosate, at concentrations of 0X, 1X, 10X, 40X and 50X. Statistical analysis was performed using Duncan's test with a confidence level of 95 %, and the error bars represent the standard error of the means. The arrow indicates the specific period selected for the extraction of the QS signaling molecules.

while their activity was significantly enhanced at higher cell concentrations, which is characteristic of a cell density-dependent QS system. Consequently, a time point of 15 h was chosen to evaluate the growth of the isolates in relation to their viability and the recovery of QS signaling molecules.

3.3. Cell viability of the CMA55A strain of Enterobacter sp

Fig. 5 presents the results of cell viability for the strain when subjected to the herbicide saflufenacil over a duration of 15 h. Notably, the highest viability value was recorded at the S0 concentration, while no statistically significant differences were detected among the S1, S10, and S40 concentrations. Conversely, the lowest cell viability was observed at the S50 concentration, indicating the herbicide's potential toxicity to the strain. These findings suggest that the concentration of saflufenacil may have influenced the efficacy of the toxicity response system in the *Enterobacter* sp. strain CMA55A.

The existing literature provides limited information regarding the herbicide saflufenacil. Nevertheless, Lima et al. [19] proposed that following prior exposure to herbicides, bacteria may enhance their adaptive capacities through QS response mechanisms. This adaptation could enable them to withstand elevated concentrations of xenobiotics and, as a result, flourish in challenging environments.

Fig. 6 presents the results of cell viability following 15 h of bacterial growth in the presence of the herbicide glyphosate. The analysis indicated that there were no statistically significant differences in the mean concentrations associated with this treatment.

In our investigation, we examined glyphosate concentrations of 0.23 mM, 2.3 mM, 9.2 mM, and 11.5 mM. Although *Enterobacter* sp. CMA55A exhibited a reduction in its bacterial growth, as visualized in the Fig. 6, a consistent decline in the bacterial population was not observed. This finding contrasts with the results reported by Mazhari et al. [24], who employed lower glyphosate concentrations of 0.059 mM, 0.59 mM, and 5.9 mM. The observed decline in the bacterial population may represent a survival response mechanism to xenobiotics [41], indicating the potential existence of distinct response pathways under varying glyphosate concentrations.

The findings presented herein may hold considerable significance for research pertaining to the degradation of glyphosate, particularly given that the strain exhibits tolerance to elevated concentrations of this herbicide. Prior investigations have examined both the degradation of glyphosate and its potential utilization as an energy source. Notably, the

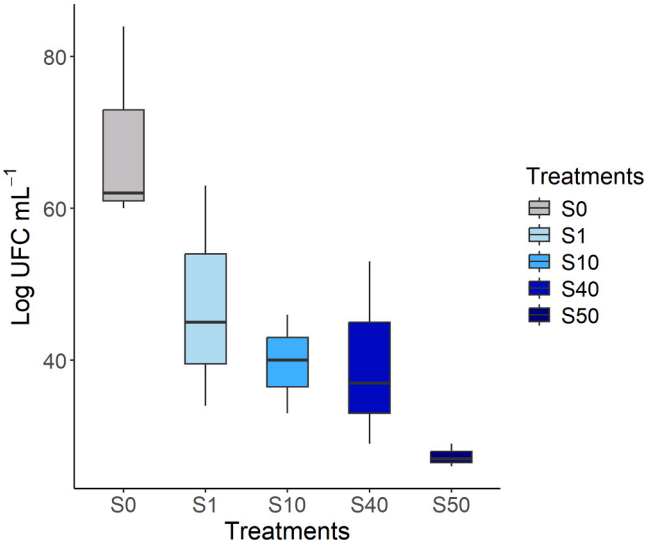

Fig. 5. The cell viability of *Enterobacter* sp. CMA55A was evaluated after 15 h of incubation in LB medium supplemented with variable concentrations of the herbicide saflufenacil (S0, S1, S10, S40 and S50).

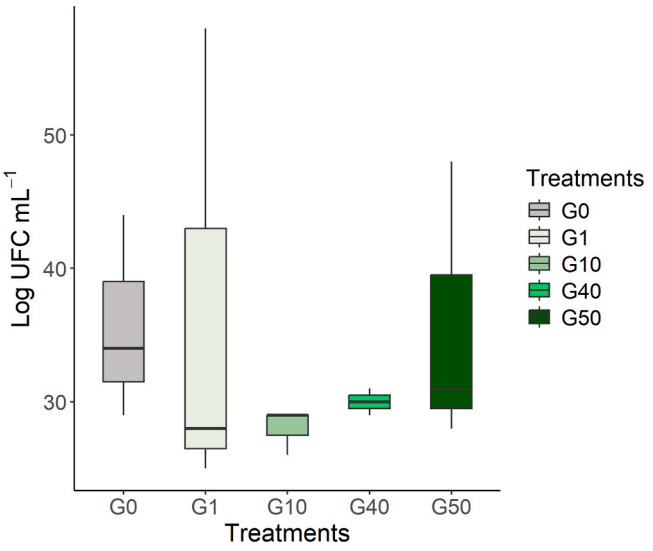

Fig. 6. The cell viability of *Enterobacter* sp. CMA55A was evaluated after 15 h of incubation in LB medium supplemented with variable concentrations of herbicide glyphosate (G0, G1, G10, G40 and G50).

genera *Pantoea* and *Enterobacter* have been identified as capable of tolerating glyphosate and utilizing it as a phosphorus source [12]. Specifically, *Enterobacter cloacae* demonstrated the ability to reduce glyphosate levels by 50 % within a five-day timeframe by converting harmful byproducts into sarcosine and glycine [14].

Although glyphosate treatment is recognized as detrimental to human health and the environment [11], with substantial environmental accumulation documented [37,47], it was noted that treatments containing saflufenacil demonstrated increased toxicity to the *Enterobacter* sp. strain.

Herbicides have been shown to induce oxidative stress in bacterial populations [8], which can result in diminished bacterial growth and potentially reduced bacterial diversity [19]. Therefore, it was hypothesized that increasing concentrations of herbicides would lead to a decline in bacterial populations. Consequently, it is essential to conduct comparative studies on enzymatic systems designed to alleviate oxidative stress caused by the herbicides saflufenacil and glyphosate.

3.4. Biofilm formation by Enterobacter sp. strain CMA55A

Biofilm formation can serve as an indicator of phenotypic plasticity in response to various stimuli, extending beyond mere reactions to chemically induced stress [46]. EPS are matrices produced collectively by bacterial communities, consisting of organic molecules such as proteins, lipids, and polysaccharides [33]. These substances facilitate the adhesion of bacterial populations to both biotic and abiotic surfaces [18]. EPS plays a crucial role in biofilm formation, wherein bacterial populations are encapsulated within an EPS layer, which provides protection against environmental fluctuations [4], offers structural support to biofilm communities both physically and chemically, and shields them from antimicrobial agents, thereby enhancing the pathogenicity of microorganisms [33].

Figs. 7 and 8 depict the biofilm formation of the *Enterobacter* sp. CMA55A strain following treatment with the herbicides saflufenacil and glyphosate after 15 h of growth. Despite the visual representation of variation among the medians, no statistically significant differences were observed across any of the treatments (see Supplementary Material 1). The variability among replicates may suggest differing biological responses; however, no differences were detected between the means. Additionally, biofilm formation remained unchanged across the various treatments and concentrations.

Gram-negative bacteria, including *Enterobacter* sp., employ

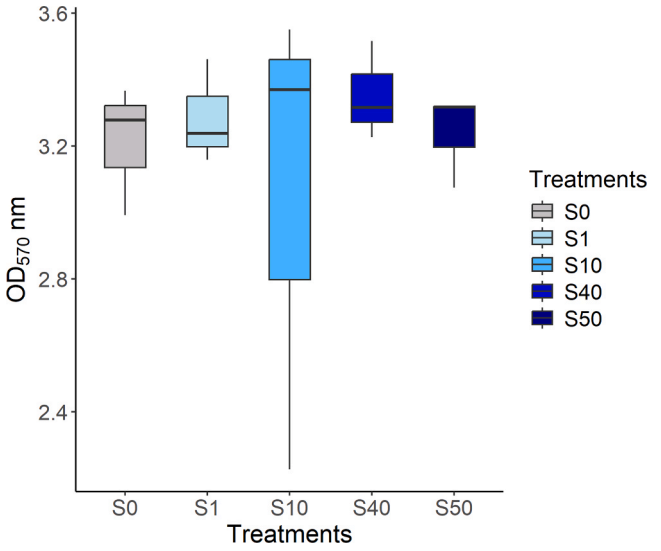

Fig. 7. Quantification of biofilm formation of *Enterobacter* sp. CMA55A after 15 h of incubation in LB S0, S1, S10, S40 and S50 medium of the herbicide saflufenacil.

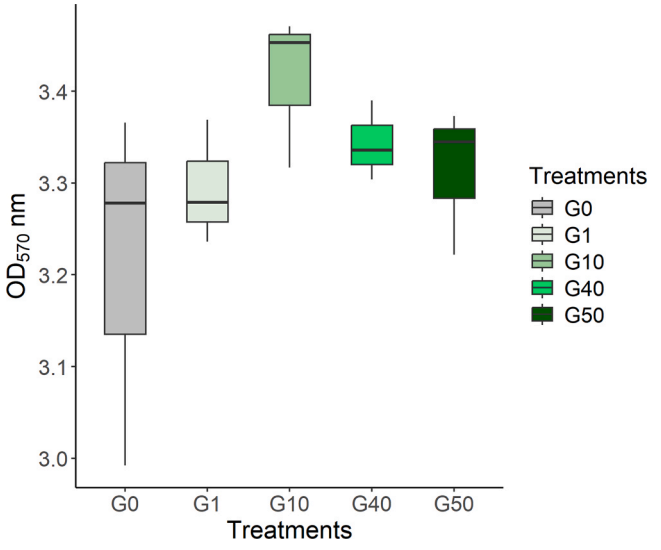

Fig. 8. Quantification of biofilm formation by *Enterobacter* sp. CMA55A after a 15 h incubation in LB medium supplemented with varying concentrations of herbicide glyphosate (G0, G1, G10, G40 and G50).

autoinducer molecules known as AHLs to elicit responses and adapt to various environmental conditions. The primary AHLs implicated in biofilm formation are N-butanoyl-homoserine lactone (C4-HSL) and N-hexanoyl-l-homoserine lactone (C6-HSL), which are crucial for biofilm maturation and the production of virulence factors. In contrast, N-(3-oxododecanoyl)-l-homoserine lactone (3OC12-HSL) is involved in the initiation of biofilm formation [1]. Additionally, C8-HSL not only mitigates oxidative stress but also plays a significant role in the production of EPS [22].

Fig. 9 illustrates that biofilm formation is associated with increased concentrations in both herbicide treatments; however, it is distinctly categorized from the production of QS signaling molecules. The production of all three QS signaling molecules demonstrated a negative correlation with varying concentrations of glyphosate, indicating that the bacterium utilizes distinct response mechanisms to this herbicide that are not observed in its isolated environment. This phenomenon was particularly pronounced for S50, which, despite being a molecule

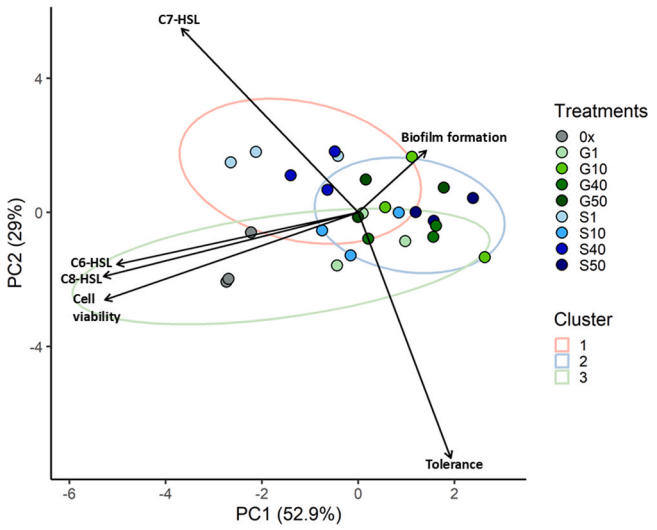

Fig. 9. PCA analysis on the profile of QS signaling molecules in *Enterobacter* sp. strain CMA55A. The experiment included quadrupled treatments denoted by the symbols (0X, S1, S10, S40, S50, G1, G10, G40 and G50). The variances explained for PC1 and PC2 were 52.9 % and 29 %, respectively.

previously identified in this bacterium, was detected at a significantly elevated concentration.

3.5. Quorum sensing of signaling molecules in the presence of the herbicides saflufenacil and glyphosate

Water contaminated with 30 different pesticides created unfavorable conditions for the survival of microorganisms. Among the genera identified in this environment, *Enterobacter* possesses the potential to harbor genes that encode traits associated with phenotypic plasticity, including xenobiotic degradation and biofilm formation and structure, potentially mediated through quorum sensing [19].

In the context of *Enterobacter*, QS may involve signaling pathways that utilize autoinducers, specifically AI-2 and AI-3, which are predominantly found in *Enterobacter aerogenes* and *Enterobacter cloacae* [34]. However, these pathways were not the focus of the current study. Instead, this research concentrated on the signaling mechanisms mediated by autoinducer molecules known as acyl-homoserine lactones (AHLs), which are prevalent in Gram-negative bacteria, including *Enterobacter*.

In the study conducted by Lau et al. [15], it was demonstrated that a strain of *Enterobacter asburiae* primarily produced C4-HSL as the main signaling molecule, with C6-HSL identified as a secondary signaling molecule. These signaling molecules interact with their respective receptor proteins, thereby initiating the transcription of genes associated with QS and its phenotypic manifestations related to biofilm formation [34]. Additionally, Martins et al. [23] investigated two strains of *Enterobacter*; one strain exhibited no detectable AHL in its extracts, while the other strain was found to produce a variety of signaling molecules, including C4-HSL, 3-hydroxy-C4-HSL, C5-HSL, 3-oxo-C6-HSL, C6-HSL, 3-oxo-C8-HSL, C8-HSL, C10-HSL, 3-hydroxy-C12-HSL, and 3-oxo-C12-HSL [23].

The biosynthesis of C8-HSL by *Enterobacter* sp. remains underexplored; however, evidence suggests its production by *Citrobacter* and *Klebsiella*, both of which are members of the Enterobacteriaceae family [16]. Similarly, C7-HSL has received limited attention in the literature, although there are documented instances of its synthesis by *Serratia*, which is also classified within the Enterobacteriaceae family [45].

Consequently, it has become pertinent to investigate the relatively underexplored acyl-homoserine lactones (AHLs) within the *Enterobacter* genus, which exhibit intriguing adaptive responses in phylogenetically

related bacterial species. In this study, the primary AHLs examined were N-hexanoyl-L-homoserine lactone (C6-HSL), N-heptanoyl-L-homoserine lactone (C7-HSL), and N-octanoyl-L-homoserine lactone (C8-HSL) (Supplementary Material 2). These molecules were detected and identified based on their retention times utilizing a semiquantitative LC-MS/MS approach.

The existing literature indicates that the C6-HSL molecule plays a significant role in conferring resistance to nutrient deprivation and facilitating biofilm formation [10,26]. Additionally, it has been shown to modulate resistance to temperature fluctuations and enhance the levels of adenosine triphosphate (ATP), superoxide dismutase (SOD), peroxidase (POD), and glutathione peroxidase (GSH), while simultaneously reducing the concentration of reactive oxygen species (ROS) [42]. Although there are limited studies on the C7-HSL molecule, its involvement in colonization [26] and its role in providing resistance to both biotic and abiotic stresses have been documented [45]. Furthermore, the C8-HSL molecule has been reported to contribute to resistance against nutrient deprivation [10] and biofilm formation [2], while also significantly enhancing the production of acetyl-CoA, a critical component for successful bacterial metabolism [17]. In the study conducted by Wang et al. [44], the combined effects of C6-HSL and C8-HSL were examined, revealing an increase in EPS secretion, which subsequently led to enhanced biofilm formation.

The late logarithmic phase of bacterial growth is characterized by an increased population density, indicating that this phase is associated with the highest levels of QS signaling [20]. Consequently, the 15 h time point was chosen for the extraction of QS signaling molecules in *Enterobacter* sp. CMA55A, as it is anticipated to be the period during which xenobiotic-induced QS-activated responses are most pronounced [8].

The relationships between the generation of QS signaling molecules and treatments with the herbicides saflufenacil and glyphosate, across various concentrations, as well a biofilm formation, tolerance levels, and viability, were analyzed using PCA, as illustrated in Fig. 9. The results indicated that both the control and S1 treatment conditions resulted in elevated production of the C6-HSL and C8-HSL molecules, which are primarily associated with biofilm maturation and protection against oxidative stress, respectively [1]. This observation likely indicates a non-stress condition, as the S1 treatment may represent an environment conducive to *Enterobacter* sp. CMA55A, as encountered during its isolation. Conversely, at concentrations of S10, S40, and S50, there was an increase in the production of C7-HSL molecules, which may contribute to resistance against biotic and abiotic stresses, as well as to biofilm formation [45]. It is plausible that the production profile of the C7-HSL molecule is modified at higher doses of saflufenacil, the herbicide to which *Enterobacter* sp. CMA55A has adapted; however, this adaptation may not occur at these elevated concentrations, resulting in the expression of this QS signal.

The findings of our study indicate that the bacterial strain *Enterobacter* sp. CMA55A demonstrated survival in the presence of herbicides within its growth medium, a phenomenon that may be attributed to biofilm formation facilitated by QS molecule signaling. This observation aligns with the research conducted by Freitas et al. [8], which reported that the bacterial strain *Pseudomonas fluorescens* CMA55 exhibited growth in the presence of the herbicide glyphosate at the G1 concentration. Furthermore, this growth was associated with the signaling molecules C6-HSL and C8-HSL. Notably, *P. fluorescens* was isolated from the same location as *Enterobacter* sp. CMA55A. However, no association was observed with higher concentrations of glyphosate, which were not assessed in the study by Freitas et al. [8]. This discrepancy may be linked to gene regulation and various metabolic pathways influenced by QS molecule signaling, as well as the production of biofilm-promoting molecules, all of which are strongly related to the concentration and presence of the xenobiotic at the isolation site. The diversity of adaptive strategies enhances the resilience of bacteria to herbicide-induced oxidative stress in their growth environment [8].

3.6. System of responses to various herbicide treatments

The accumulation of QS molecules, upon reaching a critical threshold, triggers the production and secretion of EPS. Bacteria utilize QS regulation to enhance the composition of EPS and promote bacterial growth within biofilms, thereby facilitating increased cell adhesion [40].

The integration of tolerance tests, cell viability assessments, biofilm formation, and QS signaling molecules (C6-HSL, C7-HSL, and C8-HSL) of *Enterobacter* sp. CMA55A was elucidated through PCA. This analysis encompassed the control (0X) as well as treatments involving the herbicides saflufenacil (S1, S10, S40, and S50) and glyphosate (G1, G10, G40, and G50). Fig. 9 shows the results of the PCA analysis.

The PCA reveals the presence of three primary clusters among the treatments, each exhibiting distinct response patterns to the stress induced by herbicides. The first principal component (PC1) encompasses treatments characterized by elevated production of AHL, tolerance, and biofilm formation. The second principal component (PC2) reflects cellular variability associated with varying levels of production of QS signaling molecules. The factor loadings suggest that AHL production and biofilm formation are prominent features in treatments subjected to higher concentrations of herbicides, as evidenced by the results for G40 and G50, which imply the activation of QS mechanisms under increased stress conditions. Conversely, treatments such as 0x and S1 are situated in regions indicative of higher cell viability, potentially reflecting reduced metabolic stress. These findings support the hypothesis that the strain employs QS-regulated responses as an adaptive strategy, particularly in more challenging environments.

Furthermore, the formation of biofilms demonstrated a negative correlation with cell viability and the associated treatments, indicating that biofilm formation may represent a generalized response to increased and, consequently, more toxic concentrations of herbicides, which may suggest an adaptive response of *Enterobacter* sp. CMA55A to previously experienced environmental conditions, thereby reducing the necessity for metabolic adjustments in response to stress.

The study documented an increase in the production of C6-HSL and C8-HSL molecules in the control groups and at concentrations up to S40. These molecules are mainly associated with genes related to biofilm and protection against oxidative stress, respectively [1]. A similar pattern was observed for C7-HSL, which may contribute to resistance against both biotic and abiotic stresses, as well as to biofilm formation [45]. Overall, no glyphosate concentration exhibited a positive correlation with these QS signaling molecules, including S50. We hypothesize that *Enterobacter* sp. CMA55A possesses a signaling mechanism that was selected in the tank containing water used for washing agrochemicals, enabling it to adapt to the specific conditions present. At high concentrations of both herbicides, particularly glyphosate—which was absent at the isolation site—alternative response systems, not explored in this study, likely facilitated adaptation and enabled bacterial growth that exceeded those of the control.

Additionally, C6-HSL is implicated in the production and regulation of phenazines, siderophores, and proteases, which contribute to the defense responses in various genera, including *Burkholderia* and *Serratia* [5]. Notably, the production profile of C7-HSL in *Enterobacter* sp. CMA55A is modified at elevated concentrations of saflufenacil, whereas the production profiles of C6-HSL and C8-HSL are exclusively associated with specific concentrations of saflufenacil. It has been observed that the QS molecules exhibit a stronger correlation with control conditions and in S1.

This observation suggests that the strain may produce an increased quantity of these molecules when it undergoes selective adaptation. According to Moroshi et al. [25], C7-HSL is synthesized by a limited number of bacterial species; for instance, in *Serratia marcescens*, its production was only detected during the stationary phase. Although research on this molecule is relatively scarce, it has been documented that both *Serratia* sp. and *Ochrobactrum* sp. produce 3-oxo-C7-HSL,

which plays a role in the synthesis of pyrrolnitrine, a compound that exhibits antibacterial properties, as well as siderophores that facilitate iron acquisition from the soil, thereby inhibiting opportunistic plant pathogens [21]. In the study conducted by Nawaz et al. [26], it was discovered that in two strains of *Aeromonas* sp., 3-oxo-C7-HSL may have contributed to disease control within the rhizosphere of saline soil, an environment considered toxic for bacterial survival. Additionally, in conjunction with the molecules 3-OHC6-HSL, 3-oxo-C10-HSL, and 3-oxo-C12-HSL, it may have enhanced protection against both biotic and abiotic stresses by increasing the activity of SOD, CAT and POD enzymes.

It is well-established that QS molecules play a significant role in the production of extracellular polymeric substances (EPS) and the formation of biofilms. However, further research is necessary to elucidate how various stressors may interfere with the production of these molecules, thereby affecting biofilm formation, particularly in the context of herbicide application. In the study conducted by Sazykin et al. [35], the impact of glyphosate on biofilm formation was examined in a strain of *Escherichia coli* CDC F-50. The findings indicated that at all tested concentrations, glyphosate either completely or partially inhibited the matrix structure and metabolic activity of the bacterial cells [35]. In contrast, there is a paucity of literature regarding the herbicide saflufenacil and its effects on bacterial metabolism. The specific genes regulated by QS molecules to elicit responses remain largely unidentified. Consequently, the present study serves as a preliminary screening for future research aimed at utilizing specific genes for the manipulation of bacterial strains.

Numerous microorganisms capable of biofilm formation demonstrate significant potential for the bioremediation of xenobiotics, with QS playing a crucial role in enhancing this process. QS facilitates bacterial growth within biofilms and influences the composition of EPS [40]. The research conducted on the bacterial strain *Enterobacter* sp. CMA55A provides valuable insights into the QS responses of selected bacteria in contaminated environments, as well as the functionality of their response systems in the presence of novel xenobiotics. These findings may have important implications for exploring potential bioremediation applications, wherein the degradation capabilities of bacteria can be modulated by specific QS molecules, even in cases where the bacterial strain has not previously encountered the xenobiotic targeted for bioremediation.

4. Conclusions

This study demonstrates that the *Enterobacter* sp. CMA55A strain, which was isolated from an environment contaminated with various concentrations of pesticides, employs diverse adaptive strategies to withstand the presence of xenobiotics. The strain exhibited bacterial growth, cell viability, and biofilm formation under both treatment conditions. Biofilm formation serves as a primary mechanism of protection against environmental stressors and is regulated by the quorum sensing (QS) system, which is primarily mediated by signaling molecules known as acyl-homoserine lactones (AHLs). Notably, there is a correlation between the principal QS molecules investigated (C6-HSL, C7-HSL, and C8-HSL) and the herbicide response systems, particularly in relation to saflufenacil, which is present at the strain's isolation site. This observation implies that the QS system, along with the regulatory systems it influences, may have been selected for adaptation to the specific conditions encountered in the isolation environment. Furthermore, the strain demonstrated responses even under conditions that differed from its isolation site, such as varying concentrations of glyphosate and elevated doses of saflufenacil. These responses may also be linked to other QS molecules that were not examined in this study. The findings of this research are significant for understanding bacterial adaptive responses to xenobiotic pressure, emphasizing the role of QS in orchestrating these responses. From an environmental standpoint, this study suggests that strains adapted to contaminated environments could be integrated into bioremediation strategies that are modulated through QS, thereby enhancing their tolerance to such environments. The diversity of response systems illustrates the extensive adaptive potential of bacteria in agricultural settings. Future research should focus on comparative genomic and transcriptomic analyses of this bacterium under varying concentrations of these herbicides. It is essential to explore how these behaviors can be modulated by QS to improve degradation and bioremediation capabilities, particularly in contaminated ecosystems where multiple xenobiotics at varying concentrations are likely to be present.

Environmental implications

The water storage tank used for washing pesticide packaging materials serves as a model environment under selective pressure, allowing for the study of bacterial adaptive traits. The *Enterobacter* sp. strain showed tolerance to both saflufenacil, present at the site, and glyphosate, a widely used herbicide with significant environmental effects. This research suggests that adaptations may occur through distinct quorum sensing signaling molecules, enhancing bacterial adaptability. These findings could also inform bioremediation efforts in ecosystems contaminated with multiple xenobiotics at varying concentrations.

CRediT authorship contribution statement

Caroline Rosa Silva: Writing – original draft, Validation, Software, Formal analysis, Conceptualization. **Eduardo Cesar Meurer:** Methodology. **Luiz Ricardo Olchanheski:** Project administration. **Angélica Priscila Parussolo Tonin:** Methodology. **Sônia Alvim Veiga Pileggi:** Supervision. **Juliane Gabriele Martins:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Vitória Bonfim Iurk:** Visualization, Validation, Resources, Methodology. **Marcos Pileggi:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **de Oliveira Elizangela Paz:** Methodology, Data curation, Conceptualization. **de Oliveira Évelin Lemos:** Validation, Software, Investigation, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2025.139324.

Data availability

No data was used for the research described in the article.

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