

RESEARCH ARTICLE

Impact of non-nutritive sweeteners on bacterial quorum sensing and phenotypic expression: implications for gut microbiome dynamics

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Abstract

Non-nutritive sweeteners (NNSs) are popular sugar substitutes, valued for their potential to reduce caloric intake and associated health risks. However, their long-term effects on the human gut microbiome remain debatable. This study investigates the impact of tagatose, allulose, Rebaudioside-A (Reb-A), and saccharin on quorum-sensing (QS)-regulated phenotypes and gene expression in QS biosensor model bacteria. It sheds light on their potential influence on the gut microbiome. Our study revealed diverse effects among the NNSs. Tagatose and allulose demonstrated QS phenotypic inhibition in *Chromobacterium violaceum* (~50%) and *Pseudomonas aeruginosa* (20–50%) in a concentration-dependent manner. Additionally, tagatose and allulose decreased the *P. aeruginosa lasI* gene expression. Reb-A and saccharin presented a significant, however less prominent, phenotypic inhibition on *C. violaceum* (25–30%) and *P. aeruginosa* swarming motility (~20%). Both NNSs decreased the expression of the *lasI* gene of *P. aeruginosa*. Molecular docking of QS regulatory proteins showed that saccharin and Reb-A have significantly higher binding affinity compared to allulose and tagatose, relative to native inducers. These results suggest the complex interactions mediated by NNSs in QS regulatory pathways. These findings provide valuable insights into the varied, species and dose-dependent effects of NNS on microbial communication, suggesting potential implications for the gut microbiome.

Keywords: allulose; tagatose; Rebaudioside-A; saccharin; *Chromobacterium violaceum*; *Pseudomonas aeruginosa*

Introduction

Non-nutritive sweeteners (NNSs) have become increasingly popular as sugar substitutes in the food industry, primarily due to their capacity to lower caloric intake and mitigate health risks associated with high sugar consumption (Castro-Muñoz et al., 2022; Zare et al., 2024). As consumers gravitate towards natural ingredients for their perceived health benefits and clean-label appeal, these NNS, particularly the naturally sourced ones, present a compelling alternative to refined sugars. Despite their widespread use, the long-term health effects of NNS remain debated (Rathaus et al., 2024), with their effect on the microbiome gaining significant attention (Suez et al., 2022).

The human microbiome, composed of complex and diverse microbial communities, is increasingly recognized for its crucial role in human health (Joos *et al.*, 2025). The composition of the gut microbiome is well known to be affected by the diet (Wu *et al.*, 2011; David *et al.*, 2014; Chai *et al.*, 2023). Different diet components, with particular attention to refined carbohydrates, can alter the microbial landscape in the gut by supporting or disrupting the balance of the microbial community (Sarda and Giuntini, 2023).

Most NNSs are not metabolized by the gut microbiome (Richardson and Frese, 2022). Additionally, the acceptable daily intake (ADI) for these NNSs has been established as safe for human consumption by regulatory agencies such as the European Food Safety Authority (EFSA) and the US Food and Drug Administration (FDA) (Meenakshi and Mohan, 2024). However, it remains uncertain how NNSs might influence the composition of the gut microbiome (Hosseini *et al.*, 2023). An overview of studies investigating the effects of natural origin and artificial NNSs on gut microbiomes is provided in Supplementary Table S1. This compilation includes research conducted using *in vitro* culture methods, animal models, and human studies. This information provides valuable insights into the potential impacts of natural NNS on the complex ecosystem of the gut microbiome based on the kind of NNS, the model, and study conditions applied.

Quorum sensing (QS) is a highly evolved communication system that bacteria utilize to synchronize group activities in response to changes in population density (Santos *et al.*, 2021). In addition to modulating group behaviours within the species, this type of communication plays a crucial role in the gut host–microbiome interaction (Zhang *et al.*, 2024). This intricate cell-to-cell signalling process relies on synthesizing, releasing, and detecting specific chemical signalling molecules named autoinducers (Fuqua *et al.*, 1994; Bassler, 2002). As the number of bacterial cells increases, the autoinducers build up in the surrounding environment, enabling bacteria to perceive when a critical concentration or threshold has been achieved. Upon reaching this threshold, they bind to specific receptors on bacterial cells, triggering a signalling cascade that leads to changes in gene expression across the bacterial community (Bassler and Losick, 2006). This coordinated response facilitates the execution of collective behaviours, such as biofilm formation, virulence factor production, or bioluminescence, that are advantageous to the population to adapt efficiently to environmental changes, optimizing their survival and competitiveness (Bassler, 2002; Defoirdt *et al.*, 2013; Lima *et al.*, 2023).

QS systems are widespread among both Gram-positive and Gram-negative bacteria. Typically, Gram-negative bacteria (Pseudomonadota) use acylated homoserine lactones (AHLs) as autoinducers. AHLs are small molecules that diffuse freely within bacterial cells and into the surrounding environment (Lade *et al.*, 2014). While Gram-positive bacteria (Bacillota) utilize autoinducer peptides, typically secreted by ABC-type carrier proteins (Fuqua and Greenberg, 2002; Lima *et al.*, 2023).

Food components, such as phenolic compounds, have been tested, and their influence on QS is well-known (Paczkowski *et al.*, 2017; Santos *et al.*, 2021). Compounds such as curcumin, resveratrol, gallic acid, and capsaicin have shown potential as QS inhibitors, interfering with bacterial signalling pathways and blocking autoinducer molecules (Lima *et al.*, 2023). The disruption of these communication pathways may possibly lead to an imbalance in the gut microbial community (dysbiosis), leading to associated health issues (Zhang *et al.*, 2024).

The delicate equilibrium of the gut microbiome is crucial for maintaining gut health and overall well-being (Joos *et al.*, 2025). However, disruptions in the microbiome balance lead to dysbiosis associated with a range of health issues, including inflammatory bowel disease, obesity, and neurological disorders (Singh *et al.*, 2021; Mitrea *et al.*, 2022; Markus *et al.*, 2023). Extensive literature connects dietary components with the gut microbiome imbalances (Wu *et al.*, 2011). Therefore, understanding the impact of NNSs on the QS and, consequently, on gut microbiome dynamics is a promising avenue for developing strategies to prevent and treat these health conditions.

QS systems in bacteria orchestrate complex regulatory networks that control crucial phenotypes, as exemplified in *Chromobacterium violaceum* ATCC12472 and *Pseudomonas aeruginosa* PAO1. *C. violaceum* employs an acyl-homoserine lactone (AHL)-based QS system, where CviI synthesizes the autoinducer homoserine lactone (HSL), which binds to the transcriptional regulator CviR (Stauff and Bassler, 2011). This CviR-HSL complex directly activates the *vioABCDE* operon by binding to the *vioA*

promoter, regulating violacein production, with additional modulation by the repressor *vioS* (Devescovi et al., 2017).

In contrast, *P. aeruginosa* PAO1 utilizes a more intricate QS network comprising two AHL-based systems (*las* and *rhl*) and an alkylquinolone-dependent system, *Pseudomonas* quinolone signal (PQS), which collectively regulate several behaviours, including motility (Birmes et al., 2019). Swarming motility is positively influenced by *lasI*, *lasR*, and *rhlI* expression, and is regulated by *rhl*-controlled rhamnolipid biosynthesis, while PQS independently represses swarming (Shrout et al., 2006). Swimming motility is similarly affected by *lasI*, *lasR*, and *rhlI*, and is further modulated by the GacAS-RsmA pathway (Heurlier et al., 2004). Both motility types are influenced by QS-controlled flagella and pili production, as well as cyclic-di-GMP level (Birmes et al., 2019). These sophisticated QS pathways in *C. violaceum* and *P. aeruginosa* demonstrate the intricate regulatory mechanisms governing essential bacterial behaviours, highlighting the importance of cell-to-cell communication in microbial physiology and adaptation.

This study aims to investigate the influence of tagatose, allulose, Rebaudioside-A (Reb-A) (E960), and saccharin (E954) on QS-regulated phenotypes by using two well-known biosensor model bacteria. The study assessed violacein production by *C. violaceum* ATCC12472 and swarming and swimming motility by *P. aeruginosa* PAO1 as phenotypic measures of QS activity and their modulation by NNSs. Gene expression studies were carried out for the genes *lasI*, *lasR*, *cvrI*, and *cvrR* to assess their involvement in the phenotypes observed. We hypothesized that artificial and naturally sourced NNS would have different effects on QS biosensor organisms. By examining these specific bacterial models, this study aims to gain insights into how NNS might affect microbial communication and, by extension, the overall balance of the gut microbiome.

Materials and methodology

Bacterial strains and culture conditions

The microorganisms used in this study were the QS biosensor strains *C. violaceum* ATCC 12472 and *P. aeruginosa* PAO1 DSM 19880. The strains were grown in Luria-Bertani (LB) broth at 30 and 37 °C, respectively.

Calculation of sugar concentrations

Test concentrations were established, considering each NNS's daily maximum allowable dosage (ADI) and the average human gut volumes (Schiller et al., 2005; Han et al., 2018; Fitch et al., 2021). Additionally, concentrations were established using the minimum and maximum gut volumes and the amount of NNS consumed in one dose (Figure 1 and Supplementary Tables S1(a) and S1(b)).

For the *in vitro* tests, solutions with different concentrations of NNS were prepared using sterile distilled water. To enhance the robustness of the experimental design, saccharin was included as a reference control in the study. This well-established NNS was selected due to its widespread use and extensive research history (Markus et al., 2021). The inclusion of saccharin provided a reference point for comparing the effects of the other selected natural NNSs under examination.

Violacein production

The quantification of violacein production was carried out according to a published protocol with modifications (Santos et al., 2021). The assay was conducted in a 96-well plate containing 90 µL of the NNS in LB broth at selected concentrations as shown in Figure 1. The plate was inoculated with 10 µL of a suspension containing 10⁶ CFU/mL of *C. violaceum* and incubated for ~36 h at 30 °C and 150 rpm. After the incubation period, the plates were kept at 50 °C until completely dried. Pure dimethylsulfoxide (100 µL) was added to each well and incubated for 24 h at room temperature. The optical density (OD) at

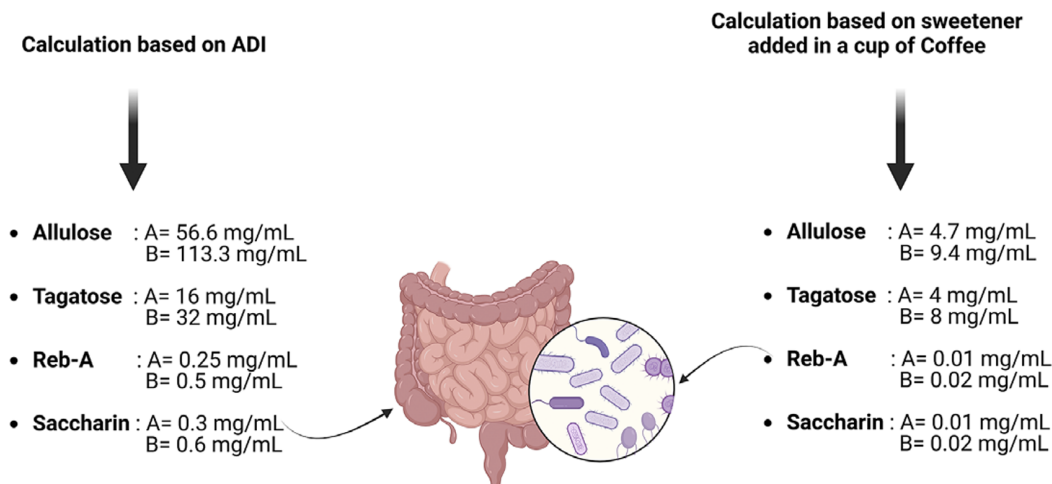


Figure 1. Sweetener concentration calculations. The left side illustrates the calculated concentrations (mg/mL) of allulose, tagatose, Reb-A, and saccharin in the intestinal environment, assuming full acceptable daily intake (ADI) consumption at once. The right side shows the calculated concentrations of the same NNS in the intestinal environment resulting from their addition to a cup of coffee. Values A and B refer to the NNS concentration at maximum gut volume (1,112 mL) and minimum gut volume (556 mL) (Schiller et al., 2005), respectively. Detailed calculations are provided in Supplementary Tables S2(a) and S2(b). Created in BioRender. Hoffmann Sarda, F. (2025) <https://BioRender.com/p88m840>.

595 nm was then measured using a spectrophotometer (Biotek, Eon, USA). LB broth without the NNSs was used as the untreated control. Results were expressed as percentages, comparing OD measurements obtained for NNSs and the untreated control (without NNS), which was considered 100% of violacein production.

Motility assays

The experiment was conducted following the methodology of Santos et al. (2021) with some alterations. For the swarming assay, aliquots of the NNSs giving the required final concentrations were mixed with 10 mL of the molten agar LB 0.5% (w/v) in a 90 mm-diameter sterile petri plate. Then, once the agar had solidified, 2 μ L of the overnight culture of *P. aeruginosa* PAO1 was inoculated at the centre of the plate. Once the inoculum dried, the plates were closed and incubated at 37 °C for 24 h. Inhibition of swarming motility was considered when a visual reduction of the swarm was observed in the presence of the NNS. The untreated sample was considered a control.

For the swimming motility assay, 2 μ L of an overnight culture of *P. aeruginosa* PAO1 was inoculated at the centre of semi-solid LB agar 0.3% (w/v) with different concentrations of the NNSs. The plates were then incubated for 24 h at 37 °C, and the motility diameters were measured and compared with untreated control plates.

Growth curves for *C. violaceum* ATCC 12472 and *P. aeruginosa* PAO1

To study the growth curve of *C. violaceum* and *P. aeruginosa* PAO1 in the presence of various NNS, the methodology described by Wiegand, Hilpert, and Hancock was followed with modification (Santos et al., 2021; Wiegand et al., 2008). Initially, the bacterial strains were cultured in LB broth to obtain a standardized 1×10^6 CFU/mL inoculum. The inoculum was then introduced into microtiter plates containing different concentrations of NNS, each dissolved in the medium to achieve the desired test concentrations. The growth of *C. violaceum* and *P. aeruginosa* was monitored by measuring the OD at 600 nm at regular intervals using a spectrophotometer, allowing for the construction of growth curves.

Molecular docking

The molecular docking study was focused on the main proteins involved in QS in *P. aeruginosa* and *C. violaceum* (De Kievit, 2009; Dimitrova et al., 2023; Vadakkan et al., 2024). The receptors and inducers analysed were CviR, LasR, LasI, and RhlR, with their structures obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) (Burley et al., 2023). The structure for CviI was obtained using DeepMind's artificial intelligence model, AlphaFold (AF) (Scardino et al., 2023). By analysing receptors, such as CviR, LasR, and RhlR, which are essential for signal reception and the response of autoinducers, we aimed to identify potential binding sites for QS inhibitors. Additionally, including LasI and CviI in the analysis allowed us to explore the possibility of interfering with signal production.

The ligands involved were C6-HSL (10058590), 3-oxo-C12-HSL (3246941), N-butyryl HSL (C4-HSL) (10130163), furanone (CID:140765), tagatose (CID:439312), saccharin (CID:5143), allulose (CID:441036), and Reb-A (CID: 6918840). The three-dimensional (3D) structures were obtained from the PubChem database (Kim, 2021), except for Reb-A, which was drawn using the MarvinSketch (version 24.1.2) due to the unavailability of the 3D structure in the PubChem database. All ligands were minimized and converted to mol2 files using OpenBabel software (O'Boyle et al., 2011). Ligands were prepared by minimizing their structures and adding charges, while receptors were prepared using University of California San Francisco (UCSF) ChimeraX by removing non-standard residues and adding necessary charges. Docking was performed using AutoDock Vina (Trott and Olson, 2010), and the best docking scores were recorded after a minimum of three runs. UCSF ChimeraX (Meng et al., 2023) was used for the visualization and rendering of the docking results.

The PDB files of the docked ligands were then combined with their respective receptor proteins into a single PDB file using PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC, save function. These combined structures were analysed using the Protein–Ligand Interaction Profiler (PLIP) web tool to identify and characterize the interactions between the ligands and receptors (Adasme et al., 2021).

We conducted tests on furanone (Supplementary Table S5), a known inhibitor of *C. violaceum* (Santos et al., 2021), and found that it had a lower binding score than the natural ligand, C6-HSL. Furanone derivatives like Meldrum's acid are known QS inhibitors due to their ability to mimic natural ligands while disrupting signalling pathways (Sadik et al., 2024). Furanone consistently showed a lower binding affinity compared to the natural ligands 3-oxo-C12-HSL and C4-HSL.

The accuracy of the docking protocol was validated by comparing the predicted binding poses of native ligands C6-HSL with CviR (PDB ID: 3QP6) and 3-oxo-C12-HSL with LasR (PDB ID: 3IX3) against their experimentally determined structures using root mean square deviation (RMSD) values (Nwabueze et al., 2022). This validation step ensured that the computational predictions closely matched the known binding modes, thereby confirming the reliability of the docking protocol for further analyses.

RNA extraction, cDNA synthesis, and qPCR testing

The assay was conducted in tubes containing 900 μ L of the NNSs in LB broth at selected concentrations. The tubes were inoculated with 100 μ L of a suspension containing 10^6 CFU/mL of *C. violaceum* and incubated for ~36 h at 30 °C and 150 rpm. Another set of tubes was inoculated with 100 μ L of a suspension containing 10^6 CFU/mL of *P. aeruginosa* and incubated for ~24 h at 37 °C. RNA extractions of the samples were performed using the TRIzol method (Pahlevan Kakhki, 2014). Reverse transcriptions and complementary DNA (cDNA) syntheses were performed according to the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative polymerase chain reaction (qPCR) experiments were performed using PowerUp SYBR Green Master mix (Applied Biosystems, Foster City, CA, USA) using StepOnePlus™ Real-Time PCR System, and data analyses were performed with the accompanying StepOne™ Real-Time PCR Software v2.3.

The selection of NNS concentration for the gene expression study was done based on the concentration with the most prominent phenotypic inhibition, and where the growth curve was not affected.

The objective was to test at least a pair of receptors and inducer enzymes for *C. violaceum* (*cviR* and *cviI*) and for *P. aeruginosa* PAO1 (*lasI* and *lasR*). The selected genes and their primer pairs are listed in Supplementary Table S3.

Statistical analyses

Statistical analysis was performed using IBM Statistical Package for the Social Sciences version 28.0.1.1 (14) (IBM Corp., Armonk, NY, USA). Before statistical analysis, data were analysed for normality using the Shapiro–Wilk test and homogeneity of variances using Levene’s test, where applicable (Wang *et al.*, 2017).

For statistical analysis of parametric data, we used one-way analysis of variance (ANOVA) followed by the least significant difference Fisher test for mean comparisons. Homogeneity of variances was tested using Levene’s test where applicable. Where homoscedasticity was not detected, Walsh ANOVA was performed.

For non-parametric data, we used the Kruskal–Wallis test. Data are shown as mean $\pm$ standard error of mean. Statistical significance was set at $p < 0.05$. Graphical representations were created using GraphPad Prism software version 8.0.c (GraphPad Software, Inc., San Diego, CA, USA).

Results

Violacein production

The analysis of violacein production by *C. violaceum* ATCC12472 in the presence of various NNSs reveals significant effects ($p < 0.05$) on this QS-regulated phenotype. The study tested allulose, tagatose, Reb-A, and saccharin at different concentrations, with results that were compared to the untreated control group, set at 100% violacein production. For allulose, a statistically significant decrease in violacein production was observed at 56 and 113 mg/mL concentrations (Figure 2). The substantial reduction in violacein production at the highest concentration of allulose tested (113 mg/mL) is associated with the antimicrobial activity observed in the growth curve analysis presented in Supplementary Figure S2. This decrease in violacein production can be attributed to growth inhibition rather than inhibition of QS. Tagatose also showed a statistically significant decrease in all tested concentrations (4, 8, 16, and 32 mg/mL) (Figure 2). In contrast, Reb-A and saccharin did not consistently reduce violacein production in a clear, dose-dependent manner across all tested concentrations.

Motility assays

Swarming motility

Figure 3 shows the comparison of swarming percentages for allulose, tagatose, Reb-A, and saccharin at different concentrations. Allulose and Reb-A exhibit the most significant reduction in swarming motility as their concentrations increase, indicating a stronger inhibitory effect in a dose-dependent manner. The substantial reduction in swarming motility at the highest concentration of allulose tested (113 mg/mL) is associated with the antimicrobial activity observed in the growth curve analysis presented in Supplementary Figure S2. This decrease in motility can be attributed to growth inhibition rather than inhibition of QS. Tagatose and saccharin also reduce swarming to a smaller degree of swarming percentage reduction compared to the more pronounced reductions caused by allulose and Reb-A. The untreated control group maintains a 100% swarming percentage, serving as a baseline. This indicates that higher concentrations of these NNSs can potentially inhibit bacterial swarming.

Swimming motility

Swimming motility assays demonstrated that increasing concentrations of allulose significantly reduced swimming motility, with the highest concentrations (56.6 and 113 mg/mL) showing the most

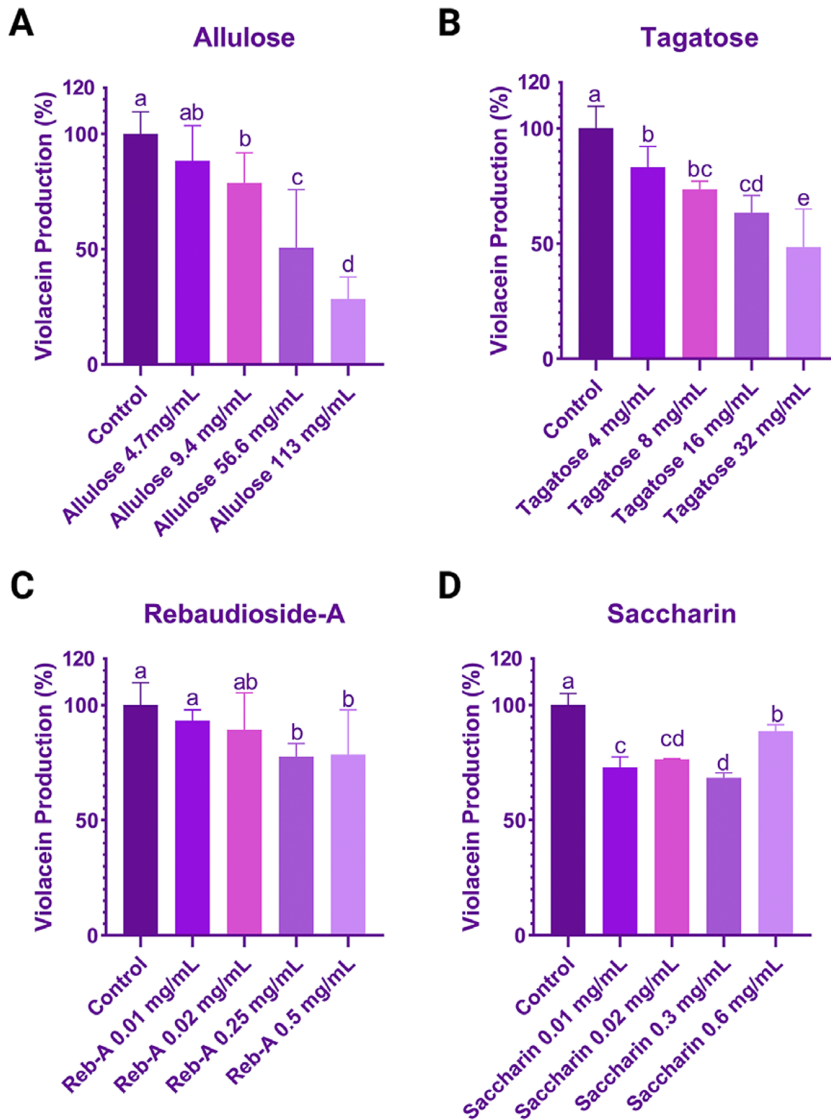


Figure 2. Effect of NNSs on violacein production by *C. violaceum* ATCC 12472 expressed as percentage of production, in comparison with the untreated control (100%). (A) Allulose; (B) tagatose; (C) Rebaudioside-A; (D) saccharin; Control = violacein production in LB broth. Bars represent mean values $\pm$ standard deviation. Different letters comparing various treatments indicate statistically significant differences ($p < 0.05$). Created in BioRender. Hoffmann Sarda, F. (2025) <https://BioRender.com/p89d153>.

pronounced inhibitory effect ($p < 0.05$). However, the highest concentration of allulose (113 mg/mL) exhibited antimicrobial activity, as observed in the growth curve analysis performed in this study (Supplementary Figure S2).

Tagatose exhibited a mild reduction ($\approx 20\%$) in swimming motility only at the higher concentrations (32 mg/mL, $p < 0.05$), though the effect was less prominent compared to allulose ($\approx 50\%$, 56.6 mg/mL, $p < 0.05$). Reb-A and saccharin had no notable impact on swimming motility across all tested concentrations, as there were no statistically significant differences compared to the untreated control ($p > 0.05$).

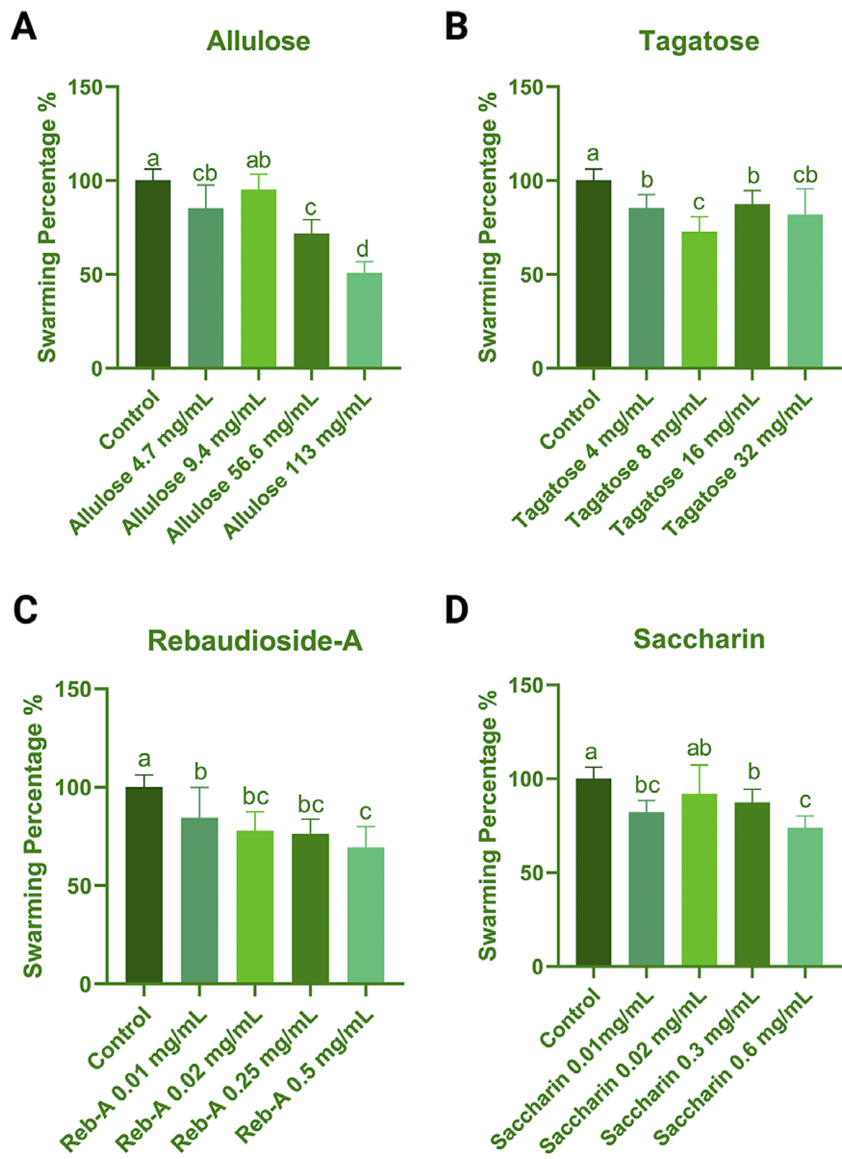


Figure 3. Effect of NNSs on swarming motility in *P. aeruginosa* PAO1 expressed as swarming percentage, in comparison with the untreated control (100%). (A) Allulose; (B) tagatose; (C) Rebaudioside-A; (D) saccharin; Control = Swarming in swarming agar. Bars represent mean values $\pm$ standard deviation. Different letters comparing various treatments indicate statistically significant differences ($p < 0.05$). Created in BioRender. Hoffmann Sarda, F. (2025) <https://BioRender.com/v54c250>.

Growth curves for *C. violaceum* and *P. aeruginosa* PAO1

The data indicate that allulose (tested concentrations of 4.7, 9.4, 56.6, and 113 mg/mL) at higher concentrations of 113 mg/mL influences the growth of *C. violaceum* and *P. aeruginosa* PAO1. This suggests that higher concentrations of allulose may have antimicrobial effects on the two tested bacteria. Growth curve results can be found in the Supplementary [Figure S2](#).

Tagatose (tested concentrations of 4, 8, 16, and 32 mg/mL) did not affect growth in either of the QS biosensor organisms at the tested concentrations.

Reb-A (0.01, 0.02, 0.25, and 0.50 mg/mL) and saccharin (0.01, 0.02, 0.3, and 0.6 mg/mL) were also tested. Similar to tagatose, there were no significant effects on growth, suggesting that tagatose, Reb-A, and saccharin may not substantially impact the growth of *C. violaceum* and *P. aeruginosa* PAO1 at the tested concentrations.

Molecular docking

Based on the obtained docking scores (Supplementary Table S5–S12), saccharin consistently exhibits strong binding affinity across multiple receptors, including CviR, CviI, LasR, LasI, and RhIR, with notably negative docking scores indicating a strong affinity. For instance, saccharin scored -7.3 with CviR (3QP6) and -7.2 with LasR (3IX3), suggesting it could effectively interact with these bacterial proteins. Similarly, Reb-A shows strong binding, particularly with LasR (3IX3, score -7.6) and RhIR (7R3E, score -6.8).

In Figure 4(X), we illustrate molecular docking studies of the CviI protein from *C. violaceum*, modelled using AF, with six compounds, including C6-HSL, furanone, saccharin, Reb-A, allulose, and tagatose. Panels A–F show the molecular surface representation of the protein with each compound bound in its active site, while panels G–L provide close-up ribbon diagrams highlighting specific ligand–protein interactions. C6-HSL (A, G), the native QS ligand, fits well into the binding pocket.

Figure 4(Y) illustrates molecular docking of the CviR protein (PDB ID: 3QP6) from *C. violaceum* with several compounds, including C6-HSL, furanone, saccharin, Reb-A, allulose, and tagatose. The CviR protein, with PDB ID 3QP6, is a luxR-type transcription factor from *C. violaceum*. It plays a crucial role in the control of QS, serving as its master transcriptional regulator in *C. violaceum*. The left column (A–F) shows the surface representation of the protein in purple, highlighting the binding sites for each compound in different colours. The right column (G–L) focuses on hydrogen bonding interactions between the protein's amino acid residues and the compounds, with blue arrows indicating these bonds.

A more detailed image of the protein–ligand interactions is provided in the Supplementary Figure S4.

In Figure 5(X), the molecular docking analyses of LasI (1RO5) highlight the binding interactions of various ligands, including 3-oxo-C12-HSL, furanone, saccharin, Reb-A, allulose, and tagatose, within the enzyme's active site. The surface representations (A–F) and detailed binding interactions (G–L) illustrate how these molecules occupy the active site and interact with key residues. The native ligand, 3-oxo-C12-HSL (A, G), forms critical hydrogen bonds with residues such as Arg30 and Thr145, essential for enzymatic activity (Liu et al., 2024).

The molecular docking analyses of the RhIR (7R3E) and LasR (3IX3) transcriptional regulators from *P. aeruginosa* with various ligands provide insights into their binding interactions. Data regarding RhIR (7R3E) is provided in Supplementary Figure S3. For RhIR, ligands such as C4-HSL, furanone, saccharin, Reb-A, allulose, and tagatose were analysed. The surface and backbone representations show how these ligands interact with the protein, highlighting hydrogen bonding sites. Similarly, the LasR analysis with 3-oxo-C12-HSL, furanone, saccharin, Reb-A, allulose, and tagatose reveals binding energies and the number of hydrogen bonds formed. The visualizations demonstrate the binding sites and interactions, with blue arrows indicating hydrogen bonds.

The results obtained from the PLIP web tool are presented in Supplementary Image S4, providing insights into the nature and strength of these interactions.

We used RMSD as a protein comparison method, as it is the most commonly used quantitative measure of the similarity between two superimposed atomic coordinates. The most accepted range for RMSD values is <3.0 Å, with lower RMSD values signifying enhanced stability within the system (Kufareva and Abagyan, 2012; Nwabueze et al., 2022). The protocol was performed by comparing the docked poses with experimentally determined structures, yielding RMSD values of 1.871 Å for LasR with 3-oxo-C12-HSL. The RMSD for CviR with C6-HSL was 1.358 Å, indicating accurate reproduction of the

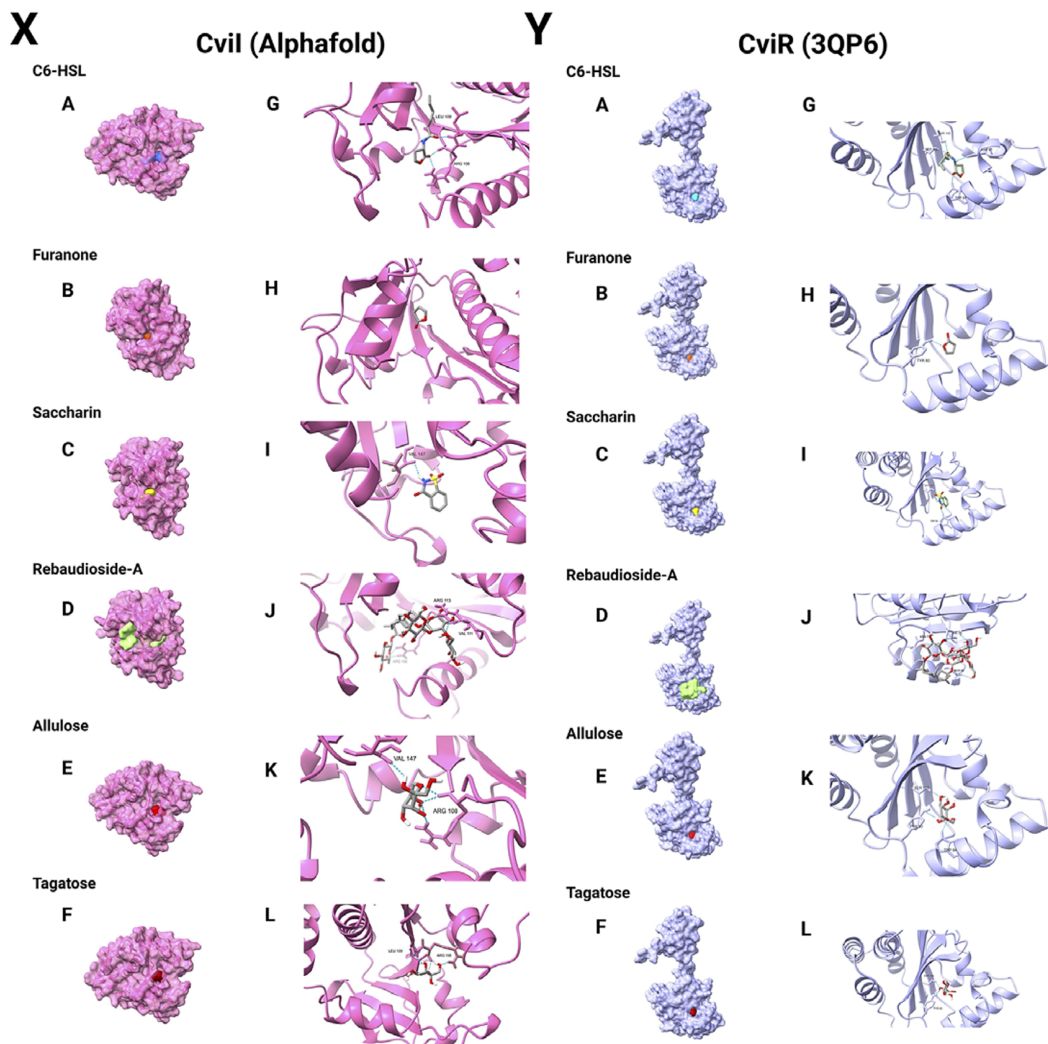


Figure 4. Molecular docking results for X column: *C. violaceum* CviI (AlphaFold 3D structure) tested for C6-HSL, furanone, saccharin, Reb-A, allulose, and tagatose. Y column: *C. violaceum* CviR (PDB: 3QP6) was tested for C6-HSL, furanone, saccharin, Reb-A, allulose, and tagatose. Panels A–F show the molecular surface representation of the protein with each compound bound in its active site, while panels G–L provide close-up ribbon diagrams highlighting specific ligand–protein interactions. Created in BioRender. Hoffmann Sarda, F. (2025) <https://BioRender.com/4bsmydg>.

experimental binding mode. These results support the use of docking to predict interactions and validate the protocol's accuracy for studying QS pathways (Supplementary Table S13).

Gene expression of target QS-related genes

For *lasI* expression, there was a significant difference for all NNS treatments ($p < 0.05$). For *lasR*, there are no statistically significant differences between any of the treatment groups, suggesting that this gene is less affected by the tested NNSs (Figure 6). Similarly, *cviI* and *cviR* expression also did not have any statistically significant difference between the treatment groups (Figure 6). Overall, treatments with the NNSs at the tested concentrations appear to influence *las* QS genes more prominently than *cvi* genes, indicating potential selective effects on specific signalling pathways.

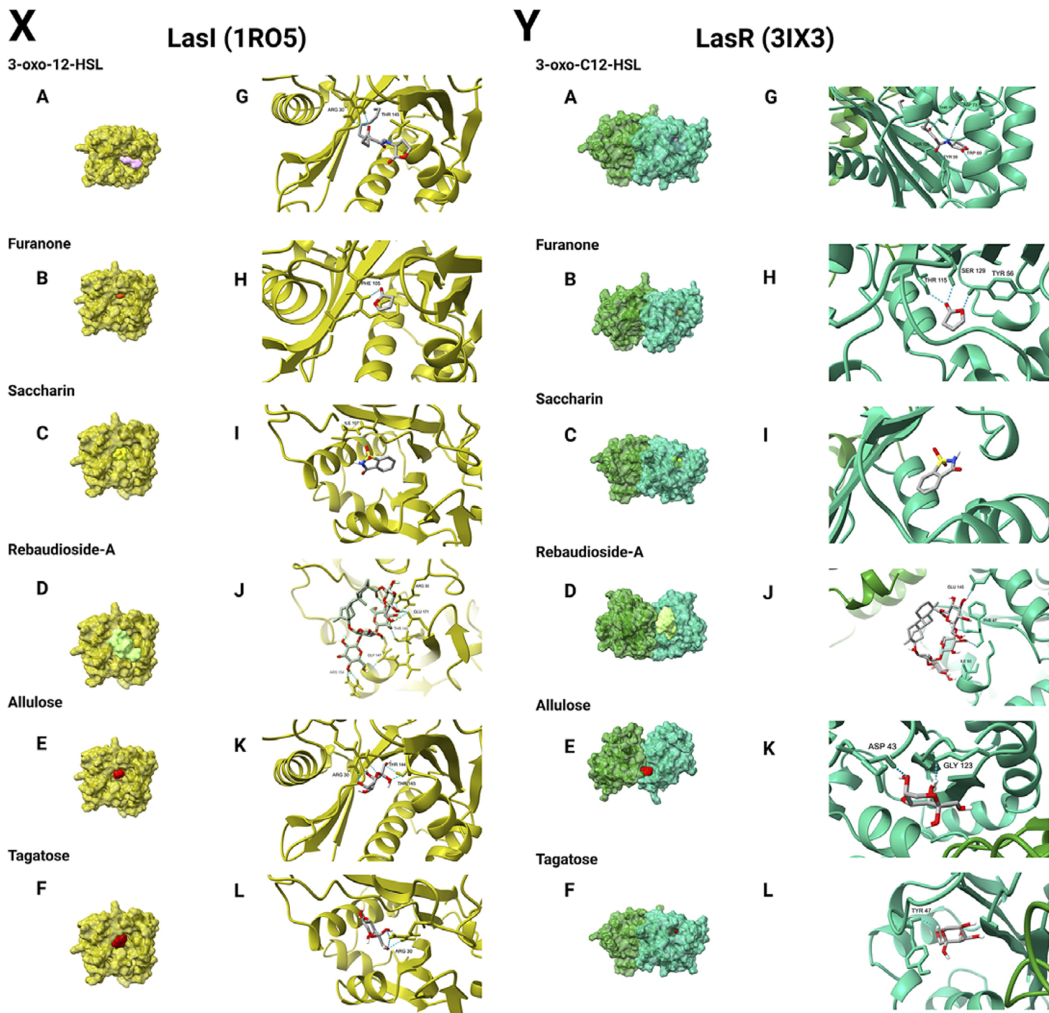


Figure 5. Molecular docking results for X column: *P. aeruginosa* LasI (PDB:1R05) tested for 3-oxo-C12-HSL, furanone, saccharin, Reb-A, allulose, and tagatose. Y column: *P. aeruginosa* LasR (PDB:3IX3) tested for 3-oxo-C12-HSL, furanone, saccharin, Reb-A, allulose, and tagatose. Panels A–F show the molecular surface representation of the protein with each compound bound in its active site, while panels G–L provide close-up ribbon diagrams highlighting specific ligand–protein interactions. Created in BioRender. Hoffmann Sarda, F. (2025) <https://BioRender.com/tn92e5u>.

Discussion

The findings of this study underscore the complex interactions between various NNSs and bacterial QS systems. The observed variability in responses emphasizes the critical need to consider the specific type and concentration of NNS when evaluating their effects on microbial communities.

In recent years, several studies suggest that the microbiome is critically important for normal host functions, while impaired host–microbiome interactions contribute to the pathogenesis of non-communicable diseases (Suez et al., 2015; Bello et al., 2018). The diet has been acknowledged as able to shape the microbiome (David et al., 2014; Wu et al., 2011), and NNSs have been implicated with contradictory results on aberrant microbiome modifications in animal models and humans (Serrano et al., 2021; Suez et al., 2014).

Contradictory *in vitro* and animal studies on stevia effects have shown its capacity to increase (Kunová et al., 2014) and decrease (Deniņa et al., 2014) *Lactobacillus* strains count. However, only a

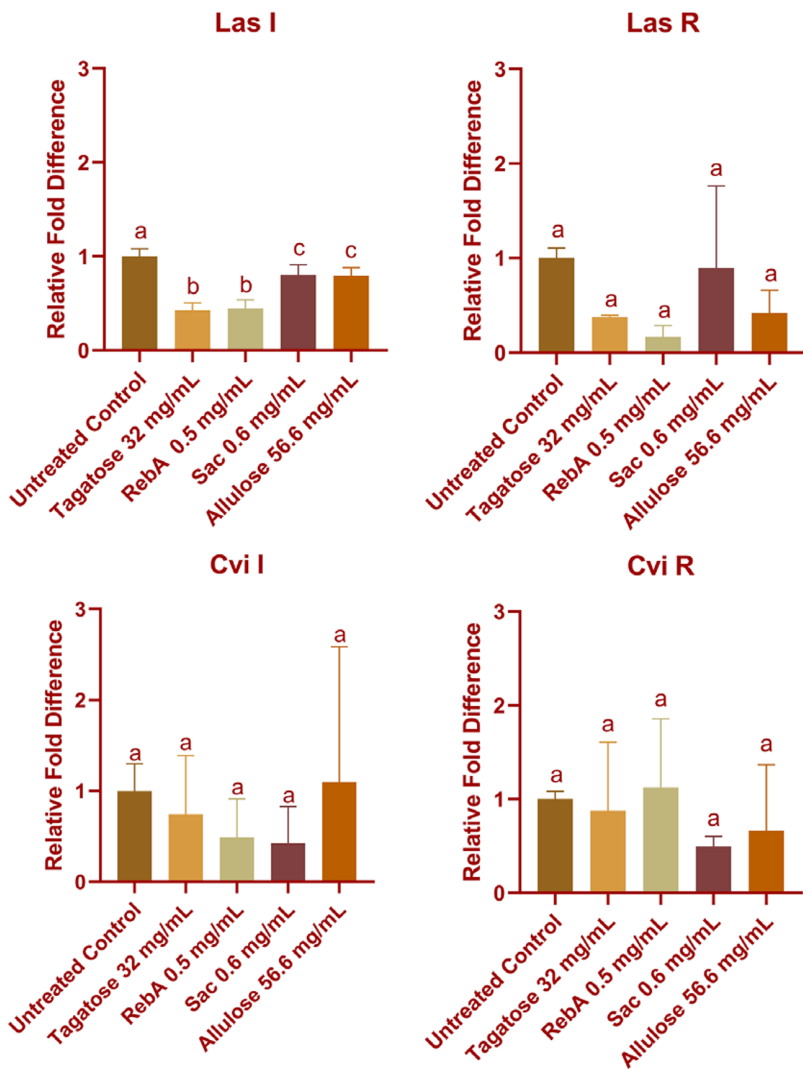


Figure 6. Relative fold difference ($\Delta\Delta CT$) in the expression of QS genes (*lasI*, *lasR*, *cviI*, and *cviR*) in response to selected treatments (the highest concentrations with inhibitory effect). The treatments include untreated control, tagatose (32 mg/mL), Rebaudioside A (0.5 mg/mL), saccharin (0.6 mg/mL), and allulose (56.6 mg/mL). Bars represent mean values $\pm$ standard deviation. Different letters comparing various treatments indicate statistically significant differences between groups ($p < 0.05$) as determined by post hoc analysis. Groups sharing the same letter are not significantly different. Created in BioRender. Hoffmann Sarda, F. (2025) <https://BioRender.com/ptycxsk>.

single 2-week clinical trial evaluating stevia’s effects on the human gut microbiome has been published to date (Suez et al., 2022). In this study, participants received saccharin, sucralose, aspartame, or stevia in doses below the daily limit for two weeks, compared to those receiving glucose or no supplement. Each NNS changed gut and oral bacteria and blood metabolites differently (Suez et al., 2022). This highlights the need for further research to unravel the underlying mechanisms and assess potential health implications, particularly in relation to the gut microbiome.

NNSs are regulated substances that undergo stringent safety evaluations before market authorization. However, due to the new techniques available and the controversial published studies, the EFSA is calling

for technical data, and there is a scheduled re-evaluation currently underway (EFSA Scientific Committee et al., 2023).

In this study, we simulated a real-life condition as we calculated the concentrations that would, hypothetically, reach the commensal or pathogenic bacteria harboured in the gut. The ingested dose would represent the ADI for each NNS (Wierzbicka, 2021) or the recommendation in the case of allulose and tagatose (Schiller et al., 2005; Han et al., 2018; Fitch et al., 2021).

Regarding the QS assays, violacein is a purple pigment synthesized by *C. violaceum* by CviI/CviR when this QS system is active. Therefore, this test has been widely used as a model for QS research (Dimitrova et al., 2023). In the present study, the reduction of violacein production observed in the presence of NNS indicates QS inhibition in a concentration/type-dependent manner.

Swarming motility is also regulated by QS. It is an organized movement of bacteria across surfaces, dependent on extensive flagellation and cell–cell interactions, which collectively contribute to biofilm formation and infection (Rütschlin and Böttcher, 2020; Santos et al., 2021). A reduction in swarming motility caused by NNSs is noteworthy from the perspective of QS inhibition, which aims to reduce bacterial virulence.

The QS systems in bacteria, such as the CviIR system in *C. violaceum*, share structural and functional similarities with the LuxIR systems found in other Gram-negative bacteria, including those present in the gut microbiota (Stauff and Bassler, 2011). The QS system of *C. violaceum* is characterized by two key components, the *cviI* and *cviR* genes, which are homologues of the *luxI/luxR* family (Venkatramanan et al., 2020). For instance, LuxIR-like systems in gut bacteria, such as *Escherichia coli* and *Hafnia alvei*, use AHLs to synchronize gene expression across populations (Li et al., 2019). The parallels between CviIR and gut-associated QS systems suggest that studying these genes and their systems can provide insights into how dietary compounds, such as NNSs, modulate bacterial communication.

Our *in-silico* assessment focused on evaluating a possible competitive or non-competitive effect of the tested NNS with the native autoinducers and their receptors for *C. violaceum* and *P. aeruginosa*. While CviI is the AHL synthase of the signalling molecule C6-HSL, the CviR protein is a transcriptional regulator that governs gene expression following binding to C6-HSL. This system specifically regulates violacein production and other phenotypes in this bacterium (Santos et al., 2021; Venkatramanan and Nalini, 2024).

P. aeruginosa has two LuxIR QS systems. LasR is a critical component in the QS system of *P. aeruginosa*. LasR is a transcriptional activator that regulates the expression of numerous virulence genes in response to cell density. This regulation is mediated by the interaction of LasR with a signalling molecule, *N*-3-oxo-dodecanoyl-L-HSL, synthesized by LasI (Kiritisin et al., 2002). LasR binds its native ligand 3-oxo-C12-HSL through interactions with residues such as Trp-60 and Ser-129.

The *rhlR* gene in *P. aeruginosa* encodes a transcriptional regulator that plays a crucial role in the QS system, particularly in regulating virulence factors and biofilm formation. RhlR works in conjunction with its autoinducer, C4-HSL synthesized by RhlI, to control gene expression based on cell density (Lee and Zhang, 2015; Lima et al., 2023).

Details of homologous system to the LasIR or CviIR systems in other gut-related bacteria can be found in Supplementary Table S14.

For each of the NNSs, we were able to uncover distinct effects and potential mechanisms by exploring molecular docking and gene expression of the main QS pathways for the two QS biosensor models evaluated.

Rebaudioside-A

In the current study of *C. violaceum* ATCC 12472, Reb-A demonstrated a statistically significant inhibition of violacein production at higher concentrations. Reb-A also displayed high binding affinity in molecular docking studies with CviI/CviR. However, despite this strong affinity, Reb-A did not exhibit a clear, significant effect on the expression of *cviI* or *cviR* genes. This discrepancy suggests that the

concentration of Reb A used in the experiments may not have been sufficient to impact gene expression. Another possibility is that the time point at which we analysed gene expression may not represent the right time at which the effect may have occurred. Future studies should also consider exploring the other genes in the pathway. Additionally, the *in silico* docking assay suggests binding of Reb-A to the proteins CviI or CviR, which may not directly implicate in changes in gene expression.

In *P. aeruginosa*, our phenotypic tests revealed a statistically significant inhibition of swarming motility, whereas no significant inhibition was observed for swimming motility. At the genetic level, a statistically significant decrease in the *lasI* gene expression was noted. Molecular docking studies showed high-affinity scores for Reb-A with both LasI and LasR proteins.

In a previous study, Reb A was tested on the recombinant bioluminescent *E. coli* K802NR-pSB1075 reporter strain at a concentration range of 10–300 mg/mL, and it did not show QS inhibitory effect in that biosensor strain (Markus *et al.*, 2020). However, when the mutant of *P. aeruginosa* PAO-JP2 (pKD-*rhlA*) strain was exposed to Reb A, it demonstrated significant inhibitory activity across all concentrations tested (10–300 mg/mL), even though no growth curve was performed in the study (Markus *et al.*, 2020); a growth inhibitory effect may explain the difference in gene expression. This contrasts with the K802NR strain results, highlighting variability in Reb A's effectiveness depending on the strain and concentrations used.

Saccharin

Saccharin demonstrated a statistically significant inhibition of violacein production in *C. violaceum* at higher concentrations. Molecular docking studies revealed that saccharin exhibited high binding affinity with CviI/CviR, similar to that of Reb A. Saccharin did not show a significant effect on *cviI* or *cviR* gene expression.

Additionally, saccharin and Reb-A exhibited higher docking scores, similar to C6-HSL (Supplementary Table S5); however, their molecular agreement and position in the receptor were not consistent with those of the natural ligand (Figure 4 and Supplementary Figure S4). The natural ligand C6-HSL interacts with amino acid residues in the ligand-binding domain of CviR, forming hydrogen bonds and hydrophobic contacts that stabilize the complex. This highlights the importance of cautiously interpreting these predictions, particularly given that *in vitro* experiments have not demonstrated inhibition at the tested concentrations.

In *P. aeruginosa*, phenotypic tests showed that saccharin exhibited a statistically significant inhibition of swarming motility, whereas swimming motility remained unaffected. At the genetic level, a statistically significant decrease in the *lasI* gene expression was noted, whereas no statistically significant differences were observed in the *lasR* gene expression compared to the untreated control. However, molecular docking studies indicated high-affinity scores for saccharin with both LasI and LasR proteins.

Saccharin evaluated at other concentrations (2.7 mM) showed around 50% inhibition in the motility test for *P. aeruginosa* (Markus *et al.*, 2021), demonstrating that higher concentrations than the physiological ones we tested can elicit stronger phenotypes. This same study investigated the binding of artificial sweeteners, such as saccharin, sucralose, and aspartame, to QS proteins, including LasR, a key receptor in the *N*-acyl homoserine lactone (AHL)-based communication system of Gram-negative bacteria. Molecular docking demonstrated that these sweeteners interact with the ligand-binding pocket of LasR, forming hydrogen bonds with residues, such as Tyr56, Thr75, Thr115, and Ser129 (Markus *et al.*, 2021). These interactions likely disrupt the proper housing of native ligands, impeding protein folding and QS signalling without bactericidal effects (Markus *et al.*, 2021).

Allulose

Allulose exhibited a statistically significant inhibition of violacein production in *C. violaceum* at the two highest tested concentrations. However, it is clear from the growth curve analysis that the highest concentration tested, 113 mg/mL, showed significant growth inhibition, suggesting that the observed reduction in violacein production at 113 mg/mL was secondary to the impact on bacterial growth.

Therefore, it is important to note that QS assays should be performed in concentrations that do not interfere with bacterial growth (Defoirdt et al., 2013). Furthermore, gene expression analysis of *cviI* and *cviR*, which are involved in violacein production, did not reveal any significant decrease in the presence of allulose. Additionally, molecular docking studies indicated that allulose had a lower binding affinity (docking scores) compared to the native ligand for both CviR and CviI proteins. This indicates that allulose is less likely to interfere with the normal functioning of CviR and CviI, as it does not bind as strongly as the natural ligand. Overall, these findings suggest that allulose does not significantly impact the violacein production pathway through these proteins, suggesting a future opportunity to test the other genes in the pathway, as a possibility to explain the phenotype.

In *P. aeruginosa*, allulose significantly reduced both swarming and swimming motility at higher concentrations. Similar to *C. violaceum*, the highest concentration of 113 mg/mL caused growth inhibition in *P. aeruginosa*. Gene expression analysis of *lasI* and *lasR*, key components of the QS system, showed no inhibition of *lasR* by allulose. However, it did show a statistically significant inhibition for *lasI*. Consistent with the findings in *C. violaceum*, allulose also had lower docking scores compared to the native ligands of LasR and LasI proteins.

The disagreement between the phenotypic expression of certain genes and gene expression in the presence of allulose and its lower binding affinity for these proteins could be due to several factors. Gene expression does not always correlate with protein activity, and allulose may indirectly influence downstream effects or affect other genes in the pathway. Additionally, molecular docking predictions may not fully capture biological complexities (Ghasemi et al., 2017).

Tagatose

Tagatose exhibited a statistically significant inhibition of violacein production in *C. violaceum*, although it did not significantly reduce the gene expression of *cviI* and *cviR*. In terms of protein interaction, tagatose shows lower docking scores compared to the native ligand for both CviR and CviI proteins.

In *P. aeruginosa*, higher concentrations of tagatose led to a significant decrease in both swarming and swimming motility. Gene expression analysis revealed that *lasI* was statistically decreased in the presence of tagatose, while *lasR* remained unaffected. Additionally, tagatose had lower docking scores compared to the native ligand for both LasR and LasI proteins.

The discovery that NNSs exert antimicrobial effects presents a complex scenario when considered alongside their possible role in promoting antibiotic resistance (Yu and Guo, 2022). While NNSs can inhibit bacterial growth and even kill antibiotic-resistant pathogens, they also have the potential to accelerate the exchange of resistance genes and stimulate bacterial evolution toward antibiotic tolerance (Yu and Guo, 2022). This effect raises concerns about the unintended consequences of NNS consumption, including disruption of gut microbiota and the potential selection of resistant bacterial strains. One study has shown that common NNSs, such as saccharin, sucralose, aspartame, and acesulfame potassium, can enhance conjugative gene transfer, which is a key mechanism for spreading antibiotic resistance genes (Yu et al., 2021). Consequently, the widespread use of NNSs may inadvertently exacerbate the growing issue of antibiotic resistance, underscoring the need for comprehensive research into their long-term effects on bacterial ecosystems (Yu and Guo, 2022).

A recent review highlighted how enteropathogens can use their QS system to intensify their invasion abilities to the intestinal mucus layer in an unbalanced gut microbiome (Su and Ding, 2023). The study also showed how QS modulators could be used as a therapeutic strategy to mitigate microbial infections, for example, using QS signalling molecules or degradation enzymes.

The potential application of this study's findings to gut bacteria, despite the use of non-native model organisms, can be justified through several key points. First, the conservation of QS mechanisms across bacterial species suggests that the effects observed in *C. violaceum* and *P. aeruginosa* could be indicative of broader impacts on bacterial communication in various species, including those found in the gut (Giannakara and Koumandou, 2022). Second, this study serves as a proof of concept, demonstrating that NNSs can interfere with bacterial QS, which opens new avenues for research using more relevant gut

bacterial species. Third, although *C. violaceum* and *P. aeruginosa* are not typical gut residents, alterations in their behaviour could potentially impact the gut environment if introduced through food or other means, as transient bacteria can influence the gut ecosystem. Lastly, the molecular docking results provide a mechanistic basis for the interactions between NNS and QS receptors, which could potentially apply to other bacterial species with similar receptors, given that many bacteria share homologous QS receptors. These findings collectively suggest that the observed molecular interactions might extend to gut-relevant species, warranting further investigation into the effects of NNS on gut bacterial communication and behaviour.

In conclusion, this study underscores the potential of NNS to modulate bacterial QS and associated phenotypes within concentration-dependent ADI limits. The molecular docking evaluation of the NNS interactions with the QS auto-inducer receptor provides a possible mechanism of action for the observed phenotypes. Further research is necessary to fully comprehend these mechanisms and to explore the practical applications of these findings in clinical and environmental settings.

Future directions

The exploration of NNSs and their effects on QS and the gut microbiome presents several promising avenues for future research. Comprehensive studies are essential for evaluating the long-term effects of NNSs on the gut microbiome and QS pathways, providing insights into potential chronic health implications. Further, gaining mechanistic insights into how NNS influence QS at the molecular level and gene expression could clarify the specific interactions between NNS and bacterial signalling molecules or receptors.

Another area to explore is the interaction among NNS and their potential combined or synergistic effects on the gut microbiome. Currently, the ADIs are established only for individual NNS. Regulatory agencies, such as the FDA and EFSA, do not mandate the labelling of NNS quantities used, only their listing. However, in December 2024, initiatives in South American countries were noted, where soft drinks labels display the amounts of NNSs used, often in combinations of two or three different NNSs.

Expanding research to include a wider range of microbial species beyond the commonly studied *C. violaceum* and *P. aeruginosa* will provide a more comprehensive understanding of how different NNS affect various bacterial communities and their ecological roles.

Human clinical trials are crucial to assess the real-world effects of NNS on gut microbiome composition and QS-related health outcomes, considering factors such as genetic variability and lifestyle.

Abbreviations

ADI	Acceptable Daily Intake
EFSA	European Food Safety Authority
FDA	U.S Food and Drug Administration
NCD	Non-communicable diseases
NNS	Non-nutritive sweeteners
QS	Quorum Sensing
Reb-A	Rebaudioside-A

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- **F.A.H.S.:** Lead the conceptualization of the study, developed the methodology, participated in writing the original draft, reviewed and edited the manuscript, supervised the research, and acquired funding.

- **M.I.W.:** Assisted in developing the methodology, performed formal analysis, curated data, wrote the original draft, and contributed to reviewing and editing the manuscript.
- **U.M.P.:** Contributed to the conceptualization of the study, participated in developing the methodology, reviewed and edited the manuscript, supervised the research, and acquired funding.
- **E.M.F.L.:** Participated in developing the methodology, conducted formal analysis, curated data, and contributed to reviewing and editing the manuscript.
- **M.S.:** Contributed to developing the methodology, performed formal analysis, and curated data.
- **B.X.V.Q.:** Contributed to the development of the methodology.
- **D.G.:** Participated in developing the statistical methodology, reviewed and edited the manuscript, supervised the research, and acquired funding.
- **C.H.:** Assisted in developing the methodology, contributed to reviewing and editing the manuscript, and acquired funding.
- **A.S.:** Reviewed and edited the manuscript, supervised the research, and acquired funding.

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