Quorum Sensing Inhibitory and Quenching Activity of Bacillus cereus RC1 Extracts on Soft Rot-Causing Bacteria Lelliottia amnigena

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ABSTRACT: The quorum sensing (QS) system of bacteria helps them to communicate with each other in a density-dependent manner and regulates pathogenicity. The concentrations of autoinducers, peptides, and signaling factors are required for determining the expression of virulence factors in many pathogens. The QS signals of the pathogen are regulated by the signal transduction pathway. The binding of signal molecules to its cognate receptor brings changes in the structure of the receptor, makes it more accessible to the DNA, and thus regulates diverse expression patterns, including virulence factors. Degrading the autoinducer molecules or disturbing the quorum sensing network could be exploited to control the virulence of the pathogen while avoiding multidrug-resistant phenotypes. The rhizosphere is a tremendous source of beneficial microbes that has not yet been explored properly for its anti-quorum sensing potential. Lelliottia amnigena causes soft rot diseases in onion, potato, and other species. The present investigation was carried out with the aim of isolating the anti-quorum sensing metabolites and elucidating their role in controlling the virulence factors of the pathogen by performing a maceration assay. The ethyl acetate extracts of various bacteria are promising for violacein inhibition assay using Chromobacterium violaceum MTCC2656 and pyocyanin inhibition of Pseudomonas aeruginosa MTCC2297. Therefore, the extract was used to deduce its role in attenuation of soft rot in potato, carrot, and cucumber. The maximum reduction of macerated tissue in carrot, potato, and cucumber was given by Bacillus cereus RC1 at 91.22, 97.59, and 88.78%, respectively. The concentration-dependent inhibition of virulence traits was observed during the entire experiment. The quorum quenching potential of the bacterial extract was used to understand the regulatory metabolites. The results of the diffusible zone and gas chromatography-mass spectrometry (GC−MS) analysis showed that diketopiperazines, viz. Cyclo(D-phenylalanyl-L-prolyl), Cyclo(Phe-Val, Cyclo(Pro-Ala), Cyclo(β-prolyl-L-valine), Cyclo (Leu-Leu), and Cyclo(Leu-Pro), are prominent metabolites that could modulate the pathogenicity in L. amnigena RCE. The interaction of bacterial extracts regulates various metabolites of the pathogens during their growth in liquid culture compared to their control counterparts. This study might help in exploiting the metabolites from bacteria to control the pathogens, with concurrent reduction in the pathogenicity of the pathogens without developing antibiotic resistance.

INTRODUCTION

Bacterial soft rot is caused by diverse bacteria such as Dickeya dadantii, Pectobacterium carotovorum, and the recently identified bacterium Lelliottia amnigena. The pathogen produces quorum sensing mediated by a wide range of pectin cell wall-degrading enzymes, which leads to maceration of the storage tissue of potato, onion, and other horticultural crops.1,2 The bacteria cause severe damage to the crops during the growing season, storage, and transport. There are no remedies available if infection occurs in the storage tissue.

For pathogenesis, bacteria needs a higher cell density, which results in the expression of pathogenic traits, and this entire process is regulated by the well-operational quorum sensing system.3 Once sufficient cell density is achieved, they produce various quorum sensing-mediated virulence traits such as pigment production, secretion of hydrolytic enzymes, biofilm formation, and toxin and antibiotics production.4 Pathogens secrete N-acyl homoserine lactone (AHL) or autoinducing peptide (AIP), which binds to its cognate receptors and regulates several pathogenic traits exhibited by the bacteria.5 The entire process is based on detection of signal molecules and activation of the signal cascade that will lead to transcriptional regulation of the pathogen.6 To control pathogens, several antibiotic compounds have been used, but there is always the possibility of development of resistance among pathogens. Thus,
there is the need for development of new antimicrobial metabolites or exploitation of quorum quenching (QQ) systems to regulate the pathogenesis of microbes without affecting the growth of microbes. Quorum quenching (QQ) can be considered as any process involving metabolites in the degradation of signaling lactones, competitive inhibition, inhibition of signal molecule synthesis, or suppression of transduction pathways. The lactonase, acylase, and oxidoreductase can cleave or modify the lactone ring present in the signal molecule, while quorum sensing inhibitor metabolites influence the quorum sensing by other mechanisms.

Several quorum sensing inhibitory compounds have been isolated from various microorganisms. Quorum sensing is the major factor leading to the virulence of the soft rot-causing pathogens. Therefore, quorum quenching strategies could be useful for controlling L. amnigena infection. The information pertaining to the pathogenicity of L. amnigena and its control measures is limited in the literature. As per our knowledge, this might be the first report indicating that potato soft rot is caused by L. amnigena in India. The present investigation aimed to identify quorum sensing inhibitor microbes and exploit their metabolites to control pathogens. However, identification and characterization of metabolites produced by the quorum quenching strain through gas chromatography–mass spectrometry (GC–MS) during co-culture has still not been performed. This study provides evidence that bacterial extracts could have the potential to disturb the quorum sensing system.

## METHODS

### Microorganisms Used in the Experiment

The rhizospheric soil samples were collected from the waste-decomposed sites around Navsari Agricultural University, Navsari, Gujarat, India. The samples were serially diluted to obtain an isolated colony. The pure organisms were stored as glycerol stock at −80 °C until further use. The rotten potatoes were collected from the local market and washed under aseptic condition to remove dirt and other impurities. The soft jelly-like rotten part was cut using a sterile scalpel and kept in 5 mL of distilled water for 5 min to diffuse the pathogen (Leiitottia amnigena) out. Hundred microliters of the suspension was spread on the Luria-Bertini (LB) agar plates and incubated at 37 °C for 24 h. Two monitor strains (Biosensor) were used in the experiments, viz. Chromobacterium violaceum MTCC2666 (C. violaceum) and P. aeruginosa MTCC2297 (P. aeruginosa), which were purchased from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The vial was cut open to transfer a little amount of lyophilized culture to LB broth and incubated for 24 h at 37 °C and 180 rpm. Half of the overnight-grown culture was stored as glycerol stock, while the rest half was streaked on the plates to observe purple and green pigment production by C. violaceum and P. aeruginosa, respectively.

### Plate Incubation Assay for Screening of Anti-Quorum Sensing Bacterial Isolates

LB agar was spread with 100 μL of a 24 h-old culture of C. violaceum MTCC2666 (OD_{000} 0.2) and allowed to dry under aseptic condition. Bacterial isolates were spot inoculated on the same plates containing a lawn of biosensor strain and incubated for 24 h at 37 °C. The ring of purple colorless and viable organisms with a colorless bacterial colony indicated the anti-quorum sensing activity of the isolated bacteria.

### Extraction of Anti-Quorum Sensing Metabolites

The organisms showing the anti-quorum sensing activity were grown in 30 mL of LB broth in a 150 mL flask for 72 h at 37 °C. The culture medium was centrifuged at 8000 rpm for 15 min at 4 °C to separate the cells. The supernatant was mixed with an equal volume of ethyl acetate and kept for 30 min with intermittent vortexing. The separating funnel was used to obtain the solvent phase, and the aqueous phase was again mixed with an equal volume of ethyl acetate. The extraction and phase separation process was repeated thrice, followed by combining all of the organic phase. The combined organic phase was evaporated in a rotary evaporator to dryness in a vacuum oven at 50 °C. The dry extract was dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C for further assay.

Analysis of the bacterial extract was done to evaluate its quorum sensing inhibition activity.

### Well Diffusion Assay

Agar well diffusion assay was used with little modification. The overnight-grown culture of the monitor strain C. violaceum (OD_{600} 0.200) was spread on the LB agar plates followed by air drying in a biosafety cabinet for a brief period of time. The well was created with an 8 mm sterile borer. Hundred microliters of the extract was poured into the well and incubated for 24 h at 37 °C, while 100 μL of DMSO was used as the negative control. Quorum sensing inhibitory activity was determined by measuring the halo zone around the wells.

### Violaecin Inhibition Assay

The actively dividing monitor strain C. violaceum (OD_{600} 0.400) was transferred to the 10 mL LB broth and supplemented with 50, 100, and 200 μL of purified culture extract separately. The test tubes were incubated in an incubator shaker at 37 °C and 180 rpm for 24 h for quantification of violacein. The 1 mL culture was centrifuged at 10,000 rpm for 10 min. Cells were collected and 1 mL of DMSO was added to the pellet. The DMSO and cells were mixed vigorously to dissolve pigments and subsequently centrifuged to remove the cells. The DMSO with the pellet of untreated cells was used as control during the experiment and absorbance was monitored at 585 nm. The experiment was repeated thrice to eliminate errors, and the inhibition percentage was calculated as follows:

\[
\text{violacein inhibition %} = \frac{\text{OD}_{585} \text{of control} - \text{OD}_{585} \text{of treated}}{\text{OD}_{585} \text{of control}} \times 100
\]

where OD_{585} control and OD_{585} test are the absorbance of the sample treated with DMSO and the one treated with the bacterial crude extract, respectively.

### Pyocyanin Inhibition Assay

The pyocyanin inhibition ability of the bacterial crude extract was tested using P. aeruginosa MTCC2297. The monitor strain was cultured overnight and the pigment was extracted by the method described in ref 16 with little modifications. Briefly, 100 μL (OD_{600} 0.400) of the monitor strain was inoculated in 10 mL of LB broth with 50, 100, and 200 μL of crude extract in a 50 mL test tube and incubated 37 °C for 24 h and 180 rpm. DMSO of the respective volume was used as control to measure the reduction in the pigment production. The cultures were centrifuged at 8000 rpm for 10 min, and the supernatant was treated with 6.0 mL of chloroform followed by vigorous shaking. The chloroform layer (blue layer) was acidified by adding 2 mL of 0.2 N HCl and mixed gently. The OD of the HCl layer (pink layer) was measured at 520 nm against the suitable blank using a UV–VIS spectrophotometer (Shimadzu Europe—UV-2600).
motility inhibition %
\[= \frac{\text{OD520 of control} - \text{OD520 of treated}}{\text{OD520 of control}} \times 100\]
where OD520 control and OD520 are the absorbance of the sample treated with DMSO and the one treated with the bacterial crude extract culture, respectively.

Swimming and Swarming Motility Inhibition Assays. Hundred microliters of the bacterial crude extract was added to the LB agar media during preparation of plates in a semisolid manner (0.5% w/v agar). The monitor strain P. aeruginosa MTCC2297 was spot inoculated using a toothpick at the center of the plate and incubated at 37 °C for 24 h. The swimming motility within the semisolid agar was evaluated and the mean areas of the swimming motility zones were measured. The control plates were supplemented with 100 μL of DMSO during assays. The movement of the colony through the interface between the medium and Petri dish was observed. For swarming motility assay, the plates were prepared in a similar manner, but the monitor strain was kept at the surface of the plate and solid agar plates were used to carry out the experiment.

\[\text{motility inhibition %} = \frac{\text{motility area of control} - \text{motility area of treated}}{\text{motility area of control}} \times 100\]

Quantitative Assay of Biofilm Inhibition of L. amnigena. The entire experiment was divided into two separate experiments, viz. inhibition and destructive activity. For inhibitory activity assay, the overnight-grown culture of pathogen was supplemented with 100 μL of crude extract and incubated further, while DMSO was used as control for the entire experiment. After incubation, the samples were decanted and the tubes were rinsed with phosphate buffer saline (pH 7.3) two times, followed by deionized water. The tubes were allowed to air dry for sometime and 200 μL of crystal violet (0.4%) was added to the tubes. The stain was discarded and the tubes were rinsed with deionized water three times. The bound dye was solubilized by adding 1 mL of 95% ethanol. The absorbance was recorded at 595 nm using a spectrophotometer (Shimadzu Europe—UV-1600). Conversely, growth inhibition assays were performed by monitoring the optical density (OD600) of the culture media with and without bacterial extracts. The DMSO amended media was considered as control. The observations were recorded at 24, 48, 72, 96, and 120 h.

\[\text{biofilm inhibition %} = \frac{\text{OD595 of control} - \text{OD595 of treated}}{\text{OD595 of control}} \times 100\]
where OD595 control and OD595 are the absorbance of the sample treated with DMSO and the one treated with the bacterial crude extract culture, respectively.

Identification of Selected Isolates at Molecular Level. The quorum sensing inhibiting bacteria as well as pathogenic bacteria were identified by the 16S rRNA gene sequencing method using universal primers 27F 5′AGAGTTTGATCCTGGCTCAG-3′ and 1492R 5′GTTACCTTGTATCGACTT3′. The genomic DNA was isolated using the LSP buffer method.21 Fragments of the amplified product from all of the potential isolates were analyzed and sequenced at SLS Research Ltd., Surat, India, and submitted to the NCBI using Banklt to receive accession numbers (https://www.ncbi.nlm.nih.gov/WebSub/Banklt). The neighbor-joining method was used to develop a phylogeny tree using the Molecular Evolutionary Genetics Analysis software (MEGA7) tool.22

In Vitro Soft Rot Attenuation Assay on Potato, Carrot, and Cucumber. Potato tubers, carrot, and cucumber were purchased from the local market and washed with sterile deionized water. Surface sterilization was done in an aseptic inoculation chamber by immersing in 70% ethanol followed by rinsing with deionized sterile water. The mentioned plant samples were cut into 5–7 mm thick slices and placed in a sterile Petri dish. Each slice was weighed under sterile conditions (using a sterile container) before inoculation. The overnight-grown culture of L. amnigena was inoculated evenly on the slices and incubated. Furthermore, 100 and 200 μL of crude extract were applied on the slices along with L. amnigena and incubated at 37 °C for 24 h. The maceration area (in mm²) was calculated using the diameter of the macerated region measured by a foot ruler. The macerated tissue weight was also recorded by scooping out the macerated region.23 Maceration (%) was calculated using the following formula

\[\text{maceration (%)} = \left(\frac{\text{macerated tissue wt of control}}{\text{macerated tissue wt of treated}}\right) \times 100\]

Characterization of the QSI Compound Using GC−MS. Extraction of Metabolites Using Well Diffusion Assay. The LB agar plate was spread evenly with 100 μL of the overnight-grown culture of L. amnigena. The wells were formed using a sterile cup borer, and 200 μL of the ethyl acetate extract of Bacillus cereus RC1 was loaded to the wells. The plates were incubated at 37 °C for 24 h. The clear agar zone around the well was cut deliberately using a sterile scalpel and dipped into 5 mL of GC-grade acetonitrile (Sigma-Aldrich). DMSO was used as control in the experiment and the LB agar was also dipped into the solvent to nullify the effect of agar. The metabolites present in the bacterial extract were prepared in acetonitrile and analyzed through GC−MS.

Extraction of Metabolites Using Liquid Assay. To extract the bacterial metabolites, the bacterial extract (200 μL) was added to 10 mL of the overnight-grown culture (LB broth) of pathogenic bacteria. The samples were incubated at 37 °C for different time periods at 180 rpm in a shaker incubator. The DMSO alone was considered as control in the entire experiment. At time intervals of 24 h, each sample was centrifuged at 8000 rpm for 10 min. to settle down the cells and the supernatant was mixed with ethyl acetate for the extraction of metabolites. The dried extracts of different time points were mixed with GC-grade acetonitrile (Sigma-Aldrich) and analyzed for the change in the metabolites produced by the pathogens during the interaction with the anti-quorum sensing metabolites produced by the B. cereus RC1.

Gas Chromatography−Mass Spectrometry (GC−MS) Analysis. The extract was filtered through a 0.22 μm size filter to remove any cell debris and contaminants. GC−MS analysis of the samples was performed by a Thermo Scientific TSQ 9000 triple quadrupole GC−MS /MS system coupled with a TRACE 1300 GC using a TOL-5 (30.0 m × 0.25 mm i.d., 0.25 μm film thickness, composed of 100% dimethylpolysiloxane). Helium gas (99.999%) was used as the carrier and the samples were injected in splitless mode. The injector temperature was kept at 280 °C. The oven temperature programme was as follows: 90 °C
for 5 min, increased at the rate of 25 °C/min. till 180 °C, increased from 180 to 280 °C at the rate of 5 °C/min and then 10 °C/min. until it reached 300 °C, followed by holding for 1.4 min. The ion source temperature was set at 280 °C, while the interface temperature was set at 310 °C and the mass measurements were done using electron impact ionization (70 eV) in the full scan mode (m/z 35–550) to detect the metabolites. Identification of metabolites among the different samples was carried out by comparing the ions in the National Institute for Standard and Technology (NIST 20).

Statistical Data Analysis. The descriptive statistics were used wherever applicable to represent the standard error of the mean and the standard deviation. MetaboAnalyst 5.0 was used to analyze the GC−MS raw data. To deduce the changes during the interaction of the extract with pathogenic bacteria, the principal component analysis (PCA), partial least-square discriminate analysis (PLS-DA), heat map, and other analyses were conducted. A Venn diagram was generated by comparing lists of different chemicals present in groups of samples using Venny 2.1 (Oliveros, J. C.) (2007–2015) (https://bioinfogp.cnb.csic.es/tools/venny/index.html).

## RESULTS

### Isolation of Quorum Sensing Inhibitor Bacteria.

Initially, 178 organisms were isolated and screened for their ability to inhibit the purple color pigmentation of the monitor strain *C. violaceum* MTCC2656. The purple pigmentation is a result of the N-acyl homoserine lactone (AHL)-based quorum sensing system working in these Gram-negative bacteria, and disruption of such communication would lead to discoloration in the vicinity of the organisms (Figure 1). By considering this strategy, a total of four organisms (F, AD28, AD38, and BN19) were isolated based on colorless haloes around the organisms and the supportive results are depicted in Figure 1.

### Plate Assay for Anti-Quorum Sensing Bacterial Isolates.

Based on previous reports, ethyl acetate was considered as the solvent for the extraction of quorum sensing inhibitors from the four organisms. The organisms were incubated for 48 h and metabolites were extracted in DMSO. The DMSO-containing extracts of F, AD28, AD38, and BN19 were loaded in the wells and a zone of purple discoloration of *C. violaceum* MTCC2656 was noticed. Organism F exhibited a
higher discoloration zone of 11 mm diameter around the wells (Figure 2). The concentrations and chemical nature of the compounds produced by the microorganisms may be different, which could be a reason for the difference in the zones of discoloration.

**Violacein Inhibition Assay.** To determine the concentration-dependent inhibition of the quorum sensing system in the monitor strain *C. violaceum* MTCC2656, different concentrations were used to see the reduction of the violacein pigment of the reporter strain. The results of the inhibition percentage of violacein production are summarized in a bar graph along with their mean of three replications and SEM (Figure 3). In quantitative determination, a higher concentration of F bacterial crude extract inhibited the violacein pigments by 62.26%, followed by AD38 (57.92%), BN19 (52.14%), and AD28 (42.97%). There was a remarkably higher purple pigmentation at lower concentrations of the extracts, indicating a dose-dependent inhibition of the purple color pigmentation in *C. violaceum* MTCC2656.

**Inhibition of Virulence Determinants Using the Model Organism** *P. aeruginosa MTCC2297.*

**Pyocyanin Inhibition Assay.** *P. aeruginosa* MTCC2297 has been widely used to establish the quorum sensing inhibition properties of several bacterial extracts. Disruption in the quorum sensing system plays a role in regulating pyocyanin production. Similar to the violacein inhibition result, concentration-dependent inhibition was shown by all isolates (Figure 4). From the findings, inhibition of pyocyanin was reported to be 68.64% (F), 59.03% (AD38), 53.18% (AD28), and 51.70% (BN19).

**Swimming and Swarming Motility Assay.** Swimming and swarming motility is generally considered as a mode of movement of cells driven by rotating flagella on or under the surface of semisolid media. For pathogenic bacteria such as *L. amnigena*, the strain RCE (MZ712952) moves by using...
peritrichous flagella and its inhibition can be studied indirectly using the monitor strain P. aeruginosa MTCC2296. The bacterial extract inhibits the swarming and swimming motility of P. aeruginosa MTCC2296 strains in vitro in plate assay (Figure 5). There was reduction in the swimming and swarming motility by 67.90 and 64.36%, respectively, by the F extract (Figures 6 and 7). All other tested organisms showed very little inhibition in swimming and swarming motility. This indicates that F could be the potential candidate for further experimental procedures.

Biofilm Inhibition and Effect on the Growth Curve of L. amnigena in the Presence of Crude Extract. All bacterial crude extracts were capable of showing antibiofilm activity against the pathogen L. amnigena. The biofilm formation is the major virulence factor of L. amnigena and is governed through the quorum sensing system. Addition of bacterial extracts to the pathogen reduced the biofilm formation in L. amnigena by 70.00−80.00% from 24 to 96 h of incubation. AD28, AD38, and BN19 also showed more than 40% reduction in biofilm formation by the pathogen (Figure 8). The growth curve of L. amnigena in the Luria broth amended with crude extracts of various bacteria was similar to the growth curve of the control supplemented with DMSO (Figure 9). This indicated that the extracts did not show antibacterial effects; rather they disturb the quorum sensing-dependent growth and morpho-physiology-related traits.

In Vitro Soft Rot Attenuation on Different Host Plants. Pectate cell wall-degrading enzymes released by the pathogen lead to the rotting of plant surfaces. The secretions of cell wall-degrading enzymes are the outcome of quorum sensing-related signal molecules released by the pathogen. To assess the impact of quorum quenching or quorum disturbing on the maceration capacity of L. amnigena, assay was carried out on potato, carrot, and cucumber slices inoculated with L. amnigena alone or co-inoculated with different bacterial extracts. When potato, carrot, and cucumber slices were co-inoculated with the bacterial crude extract, the soft rot symptom of maceration was significantly attenuated compared to control (Figure 10A−C). The bacterial crude extract of F isolates showed greater reduction in the macerated tissue of carrot, potato, and cucumber by 91.22, 97.59, and 88.78%, respectively, in a dose-dependent manner. In similar cases, the bacterial extracts of AD28, AD38, and BN19 reduced the maceration percentages by more than 65, 75, and 60%, respectively. At lower concentrations, all bacterial crude extracts showed 30% reduction in the maceration inhibition percentages compared to their higher-concentration counterparts, which confirms the concentration-dependent quorum sensing inhibition (Figures 11 and 12). DMSO and the
Figure 10. Effect of bacterial crude extract on maceration inhibition in potato, carrot, and cucumber. (A) Quantification of carrot tissue maceration inhibition. (B) Quantification of potato tissue maceration inhibition. (C) Quantification of cucumber tissue maceration inhibition. Values represent the mean of three replications. Bars indicate the standard error of mean.

Figure 11. In vitro soft rot attenuation assay on potato, carrot, and cucumber, respectively, by treatment with 100 μL of bacterial crude extract. (A) L. amnigena alone, (B) L. amnigena + DMSO (Control), (C), F bacterial crude extract + L. amnigena, (D) AD28 bacterial crude extract + L. amnigena, (E) AD38 bacterial crude extract + L. amnigena, and (F) BN19 bacterial crude extract+L. amnigena.
pathogen were used as negative and positive controls; as a result, rotting appeared on all slices.

Molecular Identification and Phylogenetic Analysis. The 16s rRNA gene sequence of the potential isolate was amplified and sequenced. The sequencing result was aligned to the NCBI database using BLASTN tool. The F isolate showed 99.05% identity with \textit{B. cereus} strain NA-28 (MN882654.1). The 16s rRNA gene sequence was submitted to the genebank database under accession number MZ068218. The isolate had a higher sequence similarity with the respective reference strain in the database; hence, the F isolate was tentatively named as \textit{B. cereus} RC1 (Figure 13).

Evaluation of QSI Compounds Present in the Inhibition Zone through GC–MS. From the results of all of the above
studies, *B. cereus* RC1 was found to be most prominent quorum sensing inhibitor strain due to its inhibitor metabolites. The extracts of the mentioned bacteria were shown to inhibit violacein production, biofilm formation, and also the swarm−swimming motility when supplemented with agar. Therefore, the experiment was carried out to identify the quorum sensing inhibitor metabolites moved from the wells to the pathogen, as well as towards *C. violaceum* MTCC2656 (Figure 14). *B. cereus* RC1 extracts and metabolites diffused from the wells in the surrounding agar media were analyzed through GC−MS (Figure 15). The data thus obtained were matched with the NIST library, and names with their probabilities were obtained. The metabolites present in the *B. cereus* RC1 (F) extract, and the zone of diffused metabolites as well as agar were used to identify the similarity and differences present within different samples. This diagram showed that one metabolite, viz. *n*-hexadecanoic acid, was found exclusively in the agar only; 51 metabolites were found to be diffused from the extract through the agar towards the pathogen, while 46 metabolites were present in the extract. There were 15 common metabolites found in the diffusible zone as well as extract, indicating their role in disturbance of the pathogen quorum sensing system and henceforth suppression of the growth pattern of the pathogens. Circles that overlap had common metabolites, while circles that do not overlap showed unique metabolites (Figure 16). The higher number of metabolites in the zone of diffusion could be attributed to the metabolites released by the pathogen and metabolites diffused from the well. The majority of them belong to the diketopiperazine group.

The raw data obtained from the chromatogram were very complex and needed to be processed as well as normalized to be used for metabolomics analysis. PLS-DA analysis of the data

![Figure 14](image1.png)

*Figure 14.* Plate assay for the extraction of metabolites from the clear holo zone around the wells; wells having crude extract and *L. amnigena* were spread on the plate. The control has the same concentration as DMSO.

![Figure 15](image2.png)

*Figure 15.* GC−MS spectra of the ethyl acetate extract of F (*B. cereus* RC1).

![Figure 16](image3.png)

*Figure 16.* Venn diagram of metabolites present in the F (*B. cereus* RC1) extract, zone of diffused metabolites, and agar.
gave important features to draw valuable conclusions, with a VIP score > 1.5 being considered (Figure 17). The metabolites were found to be palmitoyl chloride, 1,6-dioxacyclododecane-7,12-dione, Gancidin W, Cyclo-Phe-Pro, and Cyclo-L-prolyl-L-valine. These metabolites might have a significant role in quorum sensing inhibition. During the analysis of the zone of diffused metabolites, the metabolites were found to be at a lower concentration compared to the extracts, suggesting that the mentioned metabolites travel to the pathogens and disturb their QS mechanism. The heat map also reflects similar results and metabolite concentrations, which were found to be less but significant due to the migration of metabolites; moreover, some metabolites, viz. benzamide, Tris_2_4-di-tert-butylphenyl___-phosphite, benzoic acid, 4-[(trimethylsilyl)oxy]_, phenyl ester, 1-octylsilatran, N-glycylproline, 3-benzylidene-hexahydro-pyrrolo_1_2-a_pyrazin-1_4-dione, n-hexadecanoic acid, cis-11-eicosenamide, and pent-4-enoylamine__2-methyl-N-2-butyl-N-pentyl, might be the product of pathogens during their interaction with crude extracts and might have been diffused in the zone (Figure 18).

Differential Evaluation of QSI Metabolites during Their Interaction with the Pathogen at Different Time Intervals. To further analyze the impact of metabolites on the quorum sensing inhibition of the pathogen, the extract was mixed with the pathogen and effects were monitored at 24 h intervals. The metabolites listed in the Supporting Information (Table S1) are regulated during the interaction, and the heat map as well as VIP plot explains the results (Figures 19 and 20). There could be downregulation of some metabolites during the interaction with the quorum quencher present in the extract and also with those produced during the active growth phase once sufficient density of the pathogens is attained. The metabolites might disturb the QS of the pathogens in the present experiment and reduce the biofilm formation and other pathogenic factors, while other metabolites are common to pathogens and B. cereus RC1 extract. The PLS-DA analysis of the normalized data gave important features to draw valuable conclusions from the complex data sets by considering a VIP score > 1.5 for the analysis (Figure 19). 2-Quinolinyl methanol, palmitoyl chloride, 4-tetradecylmorpholine, hexahydro-3-(1-methylypropyl) pyrrolo [1,2-a]pyrazine-
1,4-dione, 3-benzylidene-hexahydro-pyrrolo_1_2-a_pyrazin-1_4-dione, 7-ethyl-4_6-pentadecandione, and 4-deoxypyridoxine were considered to draw some conclusions out of the results. These metabolites were also shown to be regulated during the interaction, as indicated from the heat map.

**Figure 18.** Clustering result shown as a heat map of the metabolites extracted from the clear zone around the well and analyzed along with metabolites found in the extract (distance was measured using Euclidean and clustering algorithms using ward D).

**DISCUSSION**

The colonization of bacteria is an outcome of their quorum sensing system and it depends on the synthesis, secretion, and binding of signal molecules to receptors. These signaling molecules accumulate in the vicinity of the organisms; once the sufficient threshold is attained, they bring changes in the phenotype of organisms, which leads to coordinated changes in their behavior within the population. Many Gram-negative pathogens, viz. *P. aeruginosa*, *Erwinia carotovora*, *Ralstonia solanacerum*, *Pantonea stewartii*, *D. dadantii*, and *L. amnigena*, produce signaling molecules to regulate their pathogenic phenotypes such as formation of the biofilm, production of pigments, secretion of cell wall-degrading enzymes, antibiotics, adaptation to diverse environments, and secretion of toxins. The repeated use of antimicrobial agents leads to the development of resistant bacterial strains, which supports the concept of survival of the fittest. It is of prime importance to develop novel strategies by employing the use of quorum quenching molecules to disturb and suppress the pathogenesis-related traits of the pathogens while not affecting the growth of the pathogen. The present work aimed to explore such
bioactive molecules from the B. cereus RC1 to hamper the pathogenicity of the soft rot-causing pathogen L. amnigena RCE. To hamper the quorum sensing system, microbes utilize any of the following four mechanisms: enzymatic hydrolysis of signal molecules, mimicking the signal molecules, impeding the signal transduction, and inhibiting the synthesis of signal molecules.27

In the present work, initially there were four bacterial isolates showing promising results, while B. cereus RC1 was identified to be a potent quorum sensing inhibitor strain due to its higher purple discoloration of C. violaceum MTCC2656. C. violaceum MTCC2656 has been used widely as a model strain for detecting quorum quenching metabolites due to its simple detection and visualization.28 Quorum sensing-dependent pathogenesis-related traits such as pyocyanin, violacein, and biofilm have been expressed by P. aeruginosa MTCC2297.29 Therefore, any alteration in any of these traits is believed to be the outcome of the quorum sensing inhibitory effect of metabolites.12 These pigments, EPS, and biofilm are not related to their growth; hence, their inhibition does not lead to bactericidal impact. In this study, the bacterial extract was subjected to organic extraction using ethyl acetate and mixed with DMSO. The violacein production of C. violaceum MTCC2656 has been used widely as a model strain for detecting quorum quenching metabolites due to its simple detection and visualization. Moreover, the extracts inhibit biofilm formation without inhibiting the growth of the organisms. The ethyl acetate extracts used in this study inhibit pyocyanin production by 68% and violacein production by 62%. The control and treated flask showed similar growth patterns, indicating that no bacteriostatic metabolites were present in the extract.30 Similarly, the ethyl acetate extracts of Bacillus pumilus S8-07,31 Staphylococcus hominis D11,32 Halobacillus salinus C42,33 and Brevibacterium casei strain Alu 134 have been exploited against P. aeruginosa for their inhibitory effects on pyocyanin production and biofilm formation. The experimental results indicated that potent anti-quorum sensing metabolites are non-enzymatic in nature.

In the present study, L. amnigena was found to belong to the rotten potato tuber, and no previous reports, specifically in India, have supported that the soft rot of potato is caused by L. amnigena. Only few previous reports have indicated that the soft rot in potato and onion bulb was caused by L. amnigena.33,34 The pathogen bears peritrichouse flagella, which is responsible for its motility. The swarming and swimming motility is responsible for the virulence of the pathogens, and controlling such motility has been shown to be a major antipathogenic strategy.35 In addition to that, the metabolites that block swarming represent an alternative tool for controlling the pathogen movement, surface attachment, and population dynamics.36 In the present experiment, supplementation of the extract to the media inhibited the motility of P. aeruginosa MTCC2297. The quorum sensing signal surfactin produced by Bacillus subtilis, which reduces the surface tension, might play a role in disturbing the biofilm.37 For instance, Lactobacillus acidophilus and Lactobacillus plantarum supernatants inhibit the swarming motility in a dose-dependent...
manner in *S. marcescens*.\(^{38}\) *E. carotovora, D. dadantii,* and *L. amnigena* are reported to be soft rot-causing pathogens.\(^{39,40}\) The ethyl acetate extract of the *B. cereus* RC1 efficiently inhibits the biofilm formation of the pathogen *L. amnigena* RCE by 70%. The pathogen secretes diverse plant cell wall-degrading enzymes, which are considered to be pathogenic factors, and their synthesis is regulated by the quorum sensing system operational in them. Due to the release of pectinase, cellulase, protease, and polygalacturonase, there was visible tissue maceration in the control samples and rotting, leading to tissue damage and formation of a watery body. In the present experiment, the application of a crude purified bacterial extract led to reduction in the macerated tissue of carrot, potato, and cucumber by 91.22, 97.59, and 88.78%, respectively, at higher concentrations. Therefore, it can be inferred from the experimental results that metabolites present in the extract have a quorum quenching effect on the soft rot-causing pathogen *L. amnigena* RCE.

The impact of the extract on the metabolome of the pathogen during growth in liquid medium was analyzed by gas chromatography coupled to a mass spectrometer (GC–MS) to elucidate the metabolic modulation during quorum sensing inhibition. From the PLS-DA, very important features were identified among the variables and they were compared with the heat map, which showed distinct metabolite patterns. The present results indicate that mono-culture extracts after different time periods show diverse abundances of metabolites and that the pattern changed in the medium supplemented with the bacterial extract (Figure 20). Moreover, the extracts from the zone of diffused metabolites of agar showed metabolites belonging to the extract of bacteria as well as pathogen-releasing molecules. The heat map clearly showed that several metabolites were regulated when the extract was added to the pathogens \(\text{Supporting Information; Table S1C}\). The Venn diagram gave very interesting results as they were the raw qualitative data and were not analyzed statistically. There were 15 features common in the extracts and zone of diffused metabolites. This result was further confirmed by the addition of the extract to the liquid culture of pathogen and evaluation of its metabolic regulation (Figure 20).

Figure 20. Heat map for different metabolites regulated during the interaction with extracts at different time intervals (distance was measured using Euclidean and clustering algorithms using ward D).
Table 1. Metabolites Present in the Ethyl Acetate Extract of B. cereus RC1

| metabolites present in the ethyl acetate extract of B. cereus RC1 | area | % (total area) | RT (min) |
|---------------------------------------------------------------|------|----------------|---------|
| benzamide                                                     | 915,493 | 0.20 | 8.63 |
| d-mannose                                                     | 424,766 | 0.09 | 8.80 |
| tyrosol                                                       | 457,055 | 0.10 | 9.30 |
| 2,3,4,5-tetrahydro-1,4-benzoazepin                           | 405,677 | 0.09 | 9.42 |
| 5-formyluracil                                                | 632,486 | 0.14 | 10.22 |
| 1,6-dioxacyclodecane-7,12-dione                               | 222,080 | 0.05 | 10.39 |
| n-tetradecyl methyl imine                                     | 617,930 | 0.13 | 10.61 |
| 2-quinolinyl methanol                                         | 279,171 | 0.06 | 11.49 |
| cyclo-Ala-Pro-diketopiperazine                                 | 3,706,846 | 0.80 | 11.83 |
| cyclo(Leu-Ala)                                                | 3,711,629 | 0.81 | 11.96 |
| tetradecanoic acid                                            | 910,130 | 0.20 | 12.04 |
| N-glycylproline                                               | 3,135,786 | 0.68 | 12.22 |
| tryptophol                                                    | 834,550 | 0.18 | 12.30 |
| benzoic acid, 4-[(trimethylsilyl)oxy], phenyl ester            | 501,608 | 0.11 | 12.41 |
| octadecanol                                                   | 1,096,872 | 0.24 | 12.68 |
| cyclo(1-prolyl-1-valine)                                      | 41,149,556 | 8.93 | 12.96 |
| benzoic acid, 4-[(trimethylsilyl)oxy], phenyl ester            | 2,053,394 | 0.45 | 13.03 |
| 12-hydroxy-14-methyl-oxa-cyclotetradec-6-en-2-one              | 1,489,512 | 0.32 | 13.11 |
| 4-tetradecylmorpholine                                        | 3,130,766 | 0.68 | 14.23 |
| hexahydro-3-(1-methylpropyl)pyrrolo[1,2-a]pyrazine-1,4-dione or cyclo(1-prolyl-1-valine) | 54,687,576 | 11.87 | 14.31 |
| 7,9-di-tert-butyl-1-ozaazepino[4,5]deca-6,9-diene-2,8-dione    | 6,357,596 | 1.38 | 14.36 |
| pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-or cyclo(Pro-Leu) | 137,935,133 | 29.93 | 14.46 |
| n-hexadecanoic acid                                           | 7,521,463 | 1.63 | 14.65 |
| 2-(2-oxo-2,3-dihydro-1H-imidazol-4-yl)malonic acid, diethyl ester | 2,199,287 | 0.48 | 15.12 |
| 1-octylsilatrane                                              | 794,888 | 0.17 | 17.20 |
| palmitoyl chloride                                            | 2,305,364 | 0.50 | 17.41 |
| 2,5-piperazinedione, 3-(phenylmethyl)-pent-4-enoylamide, 2-methyl-N-(2-butyl)-N-pentyl- | 995,752 | 0.22 | 18.48 |
| 2,5-piperazinedione, 3-benzyl-6-isopropyl-                    | 1,336,142 | 0.29 | 19.09 |
| 1-cyclohexylmethylhexahydro-3,5-dimethylbenzene               | 9,308,123 | 2.02 | 20.37 |
| pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-  | 137,475,058 | 29.83 | 20.89 |
| or cyclo(n-phenylalanyl-1-prolyl)                              | 6,373,339 | 1.38 | 21.05 |
| 6-benzyl-3-methyl-6,7-dihydro-4H-isoxazolo[5,4-c]pyrind-5-one  | 2,392,501 | 0.52 | 22.52 |
| cyclo(prolyl-tirosyl)                                         | 959,753 | 0.22 | 23.95 |
| 1,3-benzenedicarboxylic acid, bis(2-ethylhexyl) ester          | 2,705,333 | 0.59 | 26.10 |
| cis-11-icosanamide                                            | 681,649 | 0.15 | 26.52 |
| squalene                                                      | 485,347 | 0.11 | 27.22 |
| phenol, 2,4-bis(1,1-dimethylethyl)-, phosphate (3:1)           | 2,328,118 | 0.51 | 28.28 |
| cyclo(1-3-trp-1-Pro)                                          | 3,242,282 | 0.70 | 30.85 |

In the ethyl acetate extract of B. cereus RC1 (Table 1), 86.93% of the compounds were found to be diketopiperazines (DKPs), which inhibit the quorum sensing mechanisms of the reporter strain and biofilm inhibition of pathogen during the assay. The DKPs, viz. Cyclo(d-phenylalanyl-1-prolyl), Cyclo Phe-Val, Cyclo(Pro-Ala), Cyclo(1-prolyl-1-valine), Cyclo (Leu-Leu), and Cyclo(Leu-Pro), were found to be exclusively in the extracts as well as in the zones, while few DKPs were regulated in the liquid culture supplemented with extracts. This indicated that they were moved through the gel and reached the pathogen to modulate its QS system (Table 2). On the other hand, bacterial extracts amended in the liquid culture of pathogens showed a varied pattern of metabolites. DKPs such as Cyclo (d-phenylalanyl-1-prolyl), Cyclo(1-prolyl-1-valine), and Cyclo(Leu-Pro) were regulated (Supporting Information; Table S1C) at different time periods after the interaction. Moreover, cyclo(1-Ala-1-Val), cyclo(1-Phe-1-Pro), and cyclo(1-Pro-1-Tyr) were found to be present in the culture supernatant of Pseudomonas spp. This indicated that DKPs acted antagonistically and suppressed the quorum-mediated bioluminescence of E. coli (pSB401) and swarming motility in S. liquefaciens. There might be competition for the binding sites of LuxR receptors with homoserine lactone by mimicking signal molecules. As per the authors, this would be the first report indicating the role of B. cereus RC1 extracts containing DKPs as biofilm inhibitory agents for the soft rot-causing pathogen L. amnigena RCE. It is speculated from the results that DKPs were predominantly found in the extracts as well as in the zones, while few DKPs were regulated in the liquid culture supplemented with extracts. Cyclo(1-prolyl-1-valine), cyclo(1-isoleucine-1-valine), and cyclo(1-alanine-1-proline) were secreted by the pathogen during different time periods in the control, indicating that there was modulation of LuxR-dependent quorum sensing systems of the pathogens and there could be more LuxR homologues present in the cells with respect to different DKPs. During the course of this work, it can be inferred that the pathogenesis of Gram-negative bacteria can be suppressed by the DKPs. Similarly, several scientists had previously isolated and identified quorum quenching metabolites belonging to the diketopiperazine class from Streptomyces spp. These molecules exhibited quorum...
sensing inhibition activity against *P. aeruginosa* PAO1 with concomitant reduction in virulence factors. The heterocyclic compound LH-pyrrrole-2-carboxylic acid from *Streptomyces* spp. disturbs the QS signal transduction process of las, rhl, and pqs. Thus, it inhibits biofilm formation and pyocyanin production in *P. aeruginosa* PAO1. Likewise, the ethyl acetate extract of *B. pumilus* S8-07 was shown to have biofilm inhibition activity against *P. aeruginosa* PAO1. It was reported that the metabolites present in the extracts could be competing with lactones for the available sites of the receptors. In another work, chemically synthesized pyrrolo derivatives were shown to inhibit biofilm formation of *P. aeruginosa* MH602. GC–MS analysis of the ethyl acetate extract of *Brevibacterium cesei* showed that DKPs along with the unsaturated fatty acids (UFAs), viz. hexadecanoic acid and butanoic acid, inhibit biofilm formation as well as synthesis of virulence factors in *P. aeruginosa*. Heterocyclic DKPs bear nitrogen atoms, which makes them physiologically more stable compared to their counterpart lactones. Unsaturated fatty acids (UFAs) such as cis-9-hexadecenoic acid and cis-9-tetradecenoic acids were reported to inhibit the quorum sensing system and subsequently reduce biofilm formation and suppress motility in *V. cholerae* and *Acinetobacter baumannii* ATCC 17978. The fatty acid prevents the interaction of the transcription factors with DNA and thus regulates the expression of virulence factors. In this study, we demonstrated that hydroxyl fatty acid ((E)-4-hydroxy-4-[4-hydroxy-2-[(E)-6-hydroxyhept-1-enyl]cyclopentyl] but-2-enoic acid), octadecanoic acid, n-hexadecanoic acid, and tetradecanoic acid were found to be higher in the bacterial extract as well as in the zone of diffused metabolites. Therefore, we speculated that unsaturated fatty acids present in the extract might inhibit biofilm formation of *L. amnigena* RCE. Moreover, fatty acids and their derivatives reduced the virulence characteristics of *Chromobacterium violaceum* and Vibrio spp.

Benzamide interferes with the quorum sensing regulator MvR (PqsR) of *P. aeruginosa*, which leads to the interference in the biofilm formation. In silico and in vitro analyses of unsaturated fatty acids, with virulence factor production from *A. baumannii*, suggested that interaction with AHL synthase reduces AHL production. *L. amnigena* RCE is resistant to 1,2-dihydro-2,2,4-trimethyl- with its role as a quorum sensing inhibitor. Phenol, 2,4-bis(1,1-dimethylethyl), or DTBP from the sea weed-associated marine bacterium inhibits biofilm formation. 

Table 2. Metabolites Present in the Clear Zone around the Wella

| metabolites present in the ethyl acetate extract of *B. cerasus* RC1 | area | % (total area) | RT (min) |
|---------------------------------------------------------------|------|---------------|---------|
| 1-propanamine, 3-(diethoxymethylsilyl)- | 290,792 | 1.15 | 7.66 |
| 2-oxo-3-phenylpropanal | 118,331 | 0.47 | 8.17 |
| benzamide | 695,521 | 2.75 | 8.64 |
| dodecanal | 383,623 | 1.52 | 9.18 |
| 2-hydroxy-3-methoxybenzaldehyde | 304,321 | 1.21 | 9.65 |
| ethyl 4-ethoxybenzoate | 99,260 | 0.39 | 9.98 |
| 5-formyluracil | 198,622 | 0.79 | 10.12 |
| 1,6-dioxacyclocdecane-7,12-dione | 2,033,636 | 8.05 | 10.40 |
| n-tetradecyl methyl imine | 148,745 | 0.59 | 10.55 |
| 4-(4-tert-butylphenyl)-1,3-thiazol-2-amine | 128,654 | 0.51 | 11.06 |
| 2,4-dimethylbenzo[k]quinoline | 154,155 | 0.61 | 11.74 |
| cyclo-Ala-Pro-diketopiperazine | 686,224 | 2.72 | 11.83 |
| tetradeconoic acid | 150,479 | 0.60 | 12.20 |
| N-glycylproline | 3,139,841 | 12.44 | 12.28 |
| tryptophol | 290,096 | 1.15 | 12.46 |
| benzoic acid, 4-[(trimethylsilyl)oxy]-, phenyl ester | 293,381 | 1.16 | 12.69 |
| octadecanal | 366,188 | 1.45 | 12.96 |
| cyclo-[1-prolyl-3-valine] | 553,032 | 2.19 | 13.02 |
| benzoic acid, 4-[(trimethylsilyl)oxy]-, phenyl ester | 639,282 | 2.53 | 13.12 |
| 4-tetradecylmorpholine | 287,722 | 1.14 | 13.76 |
| 7,9-di-tetra-butyl-1-oxaspiro[4,5]deca-6,9-diene-2,8-dione | 361,952 | 1.43 | 14.31 |
| pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- or cyclo(Pro-Leu) | 519,908 | 2.06 | 14.41 |
| n-hexadecanoic acid | 3,886,469 | 15.39 | 14.65 |
| 1-octylsilatran | 577,694 | 2.29 | 17.21 |
| palmitoyl chloride | 2,367,653 | 9.38 | 17.42 |
| 2,5-piperazinedione, 3-(phenylmethyl)- | 480,608 | 1.90 | 17.67 |
| pent-4-enylamide, 2-methyl-N-(2-butyl)-N-pentyl- | 741,174 | 2.94 | 18.48 |
| 1-cyclohexylmethylsulfoxo-3,5-dimethyldieiene | 1,572,717 | 6.23 | 20.43 |
| pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)- or cyclo-(n-phenylalanine-1-prolyl) | 512,287 | 2.03 | 20.86 |
| 1,3-benzenedicarboxylic acid, bis(2-ethylhexyl) ester | 1,590,285 | 6.30 | 26.09 |
| cis-11-eicosanamide | 484,046 | 1.92 | 26.52 |
| phenol, 2,4-bis(1,1-dimethylethyl) -, phosphate (3:1) | 961,168 | 3.81 | 28.29 |

*a*Zone of diffused metabolites.
formation in S. marcescens.\textsuperscript{36} \textit{Exiguobacterium indicum}, a Gram-positive bacteria, produces the quorum sensing inhibitor 3-benzyl-hexahydro-pyrrolo[1,2-a]pyrazine-1,4-dione. This molecule inhibits biofilm formation in 	extit{Pseudomonas aeruginosa} PAO1 and \textit{P. aeruginosa} PAH, while it did not show any growth retardation in them.\textsuperscript{37} This indicates that the bacterial extract changed the conformation of the biofilm, which resulted in the prevention of bacterial adherence. This molecule has thus shown the potential to inhibit the virulence factors of \textit{P. aeruginosa} by transcriptional regulation of pathogens.

**CONCLUSIONS**

\textit{B. cereus} RC1 release diverse metabolites, which act as quorum quenching molecules and have the potential to inhibit biofilm formation in the soft rot-causing pathogen \textit{L. amnigena} RCE, as well as pyocyanin production in the monitor strain \textit{P. aeruginosa} MTCC2297. GC–MS analysis of the \textit{B. cereus} extract and the zone of diffused metabolites showed diketopiperazine as the predominant metabolite. There might be the possibility that metabolites moved through the agar from the well and modulated the growth pattern of the pathogen. Even in the liquid culture of pathogen, the extract exhibited downregulation of various metabolites. One can speculate that the metabolites released by certain bacteria can act as a possible alternative strategy to control the pathogenesis of bacteria as well as help to avoid drug-resistant phenotype development.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c02202.

(A) Metabolites found common in extract of \textit{B. cereus} RC1 and zone of diffusible metabolites as per Venn diagram (B) metabolites found in high concentration in \textit{B. cereus} RC1 extract as per the heat map (C) metabolites concentration regulated during interaction with crude extract (Table S1) (PDF)

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**Notes**

The authors declare no competing financial interest.

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