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Research Article

QUORUM QUENCHING POTENTIALS OF PROBIOTIC *ENTEROCOCCUS DURANS* LAB38 AGAINST METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Objective: The focus of this study was to explore the nuance strategy to combat the virulence factors of the pathogens by probiotic *Enterococcus durans* LAB38.

Methods: Probiotic attributes was determined by bile salt tolerance (0.5%) and *Artemia* gnotobiotic assay. Quorum sensing (QS) inhibitory activity of the supernatant and ethyl acetate (EA) extract of LAB38 was evaluated using the indicator strains, includes *Chromobacterium violaceum* CV026 (mini-Tn5 mutant of ATCC 31532), methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* (PA). Reporter strains, *Vibrio harveyi* BB170 (*luxN* mutant), BB886 (*luxP* mutant) and *Escherichia coli* pSB401 (pACYC184-derived) were used for bioluminescence-based target specificity analysis. Gas chromatography-mass spectrometry (GC-MS) analysis of EA extract was performed using standard protocol.

Results: LAB38 has shown bile salt tolerance and positive probiotic effect toward *Artemia salina*. In addition, 100 µg/ml EA extract has significantly reduced the violacein production (37±1.4%) in CV026, biofilm formation in MRSA (94±0.9 %) and PA (22±0.08%). Further, 200 µg/ml of EA extract has shown inhibition against both autoinducer-1 (AI-1) and AI-2 mediated QS system. Bioluminescence inhibition is directly proportional to the time of exposure. GC-MS result revealed that bromine, sulfur containing molecule and azulene derivative were found in the EA extract.

Conclusion: This is the first report on probiotic *E. durans* for quorum quenching activity. Hence, the bacterium could be used for future therapeutics application.

Keywords: Autoinducer, Biofilm, Methicillin resistant *Staphylococcus aureus*, Quorum quenching, Probiotics, CV026.

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INTRODUCTION

Biofilm is a matrix of extra polymeric substances often exerted by a bacterial community to protect themselves from external factors such as antibiotics and host defenses, consequently turned as a barrier against killing factors [1]. Notably, 80% of the bacterial infections are facilitated by biofilm [2]. Biofilm formation is relied on quorum sensing (QS) process, where gene expression is mediated by extracellular signaling molecules called autoinducers (AIs) upon population density [3]. QS process also plays a key role in the expression of virulence factors [4] and antibiotic production [5].

Human gastrointestinal tract is a suitable place for horizontal gene transfer from a diverse group of pathogens, making non-pathogens as virulent ones [6]. Methicillin resistant *Staphylococcus aureus* (MRSA) emerged as a commonly reported pathogen in the gastrointestinal tract [7]. Astonishingly, 96% of *S. aureus* causes biofilm-associated infections [8]. However, biofilm-assisted chronic infections of *S. aureus* are difficult to treat due to its fractious nature [9]. Similarly, it has been repeatedly observed that *Pseudomonas aeruginosa* (PA) colonize in the intestinal tract of the hospitalized and immunosuppressed patients [10]. Besides, PA causes chronic respiratory disease and prolonged chronic rhinosinusitis [11]. Obviously, virulence factors of PA, regulated by QS network are involved in the pathogenesis [12].

Hence, the inhibition of QS became an important target to attenuate pathogenesis, since resistance to antibiotics among pathogens is increasing rapidly and posing a major global threat. Although the pathogens are killed by antimicrobials in a host, their determinants of virulence may persist to harm [13]. Thus, the inhibition of QS network would be a productive alternate way, as it impedes the virulence gene expression without disturbing its growth [14]. Furthermore, it is very interesting to note that QS inhibition enhances the impairment of bacterial biofilm, thereby ease the antibiotic treatment [15].

Ample of reports are found to demonstrate the probiotics potentials of *Enterococcus* spp. For instance, intestinal tract of mammals is frequently colonized with *Enterococcus* sp., for the beneficial effects [16]. Probiotics with quorum quenching potential would be a unique dual strategy to control antibiotic-resistant pathogens and to support the host in a positive manner. At the best of our knowledge, there is no report on quorum quenching potential of the probiotic *Enterococcus durans*. Hence, the aim of this work is (i) to study the probiotic attributes and quorum quenching potential of *E. durans* LAB38 and (ii) to determine the spectrum of quorum quenching activity.

METHODS

Bacterial strains and culture condition

Unpasteurized cow's milk sample was serially diluted in 0.9% saline and plated on de Man, Rogosa and Sharpe (MRS) agar for 48 hrs at 30°C. For experimental purposes, the isolate LAB38 was cultured in Luria-Bertani (LB) medium with 2% glucose (w/v). Indicator organisms such as PA (ATCC 27853) and clinical isolate MRSA were cultured in LB medium. *Chromobacterium violaceum* CV026 and *Escherichia coli* pSB401 were maintained in LB medium with respective antibiotics. *Vibrio harveyi* BB170 and BB886 mutant strains were routinely subcultured in Luria Marine agar, and AI bioassay medium was used for the experimental purpose [17]. Identification of the bacterium was done based on the biochemical and molecular characteristics. Universal

primers 27F and 1492R were used for 16S rRNA gene amplification. Purified amplicon product was sequenced and analyzed using NCBI nucleotide database, and phylogenetic tree was constructed with highly resembled sequences using MEGA 5.03.

Probiotic attributes

Bile salt tolerance

To confirm the bile salt tolerance, the method described earlier was adopted [18]. Briefly, bacterial pellet was adjusted to 0.1 optical density (OD) using phosphate-buffered saline and it was incubated with or without 0.5% bile salts for 2 hrs. Survival of the bacterium was checked by plating 100 µl of inoculum from the respective tubes on MRS agar plate. After 48 hrs of incubation, viable colonies were enumerated.

Artemia gnotobiotic assay

Artemia salina cysts were cultivated routinely in 3.2% filtered and autoclaved saline water (FASW). Decapsulation of cysts was performed as described earlier [19]. Decapsulated axenic *Artemia* was transferred to the tube containing 10 ml of aerated FASW, and then probiotic inoculum (LAB38) was added to reach the final concentration of 0.1 OD. *Nannochloropsis* sp. was given as a feed at 25×10^3 cells per nauplii. The tubes containing *Artemia* without bacterial inoculum were used as control. All the tubes were kept under shaking condition for 24 hrs with constant incandescent light. All the experiments were done in axenic conditions and were performed in triplicates.

Quorum quenching assay

Cell-free supernatant of LAB38 was extracted with half volume of hexane and subsequently with half volume of ethyl acetate (EA) [20]. Extracts were concentrated using Buchi Rotavapor R-210, which were then stored in pre-weighed tubes. Hexane and EA extracts were dissolved in dimethylsulfoxide (DMSO) to make 100 mg/ml stock solution. Sterile DMSO was used as a control in all experiments to check the solvent effect on QS inhibition.

CV026 plate assay

Preliminary screening of QS inhibition was determined using *C. violaceum* CV026 (mini Tn5 mutant of *C. violaceum* ATCC 31532, Km^r) [21]. LB soft agar containing 0.1 OD of CV026 inoculum and 1 µg/ml of C6-HSL (Sigma-Aldrich, USA), was poured over the molten LB hard agar. After solidification, 10 µl of filter-sterilized supernatant of the LAB38 was placed and incubated for 48 hrs at 30°C. LB medium and cinnamaldehyde (Himedia, India) were used as the negative and positive control, respectively.

Pigment quantification assay

Violacein pigment quantification was performed as described previously [22] with some modification. A volume of 500 µl inoculum consist of CV026 and C-6-HSL (1 µg/ml) was added to each well in a 24-well microtitre plate. Hexane and EA extract were added at a concentration of 100 µg/ml. Then, the extract containing inoculum was serially diluted with fresh inoculum by half-dilution technique. After 24 hrs, pigment extraction was done from bacterial pellet using 1 ml of DMSO. After a brief vortex, cell-free pigment extract was measured at 585 nm. CV026 without compound was acts as a control. To confirm the exclusive quorum quenching inhibition (independent from cell growth) of the extract, cell density of CV026 was measured at 600 nm using ultraviolet visible spectrophotometer (Elico SL-159). The following formula was used to calculate the percentage of inhibition:

$$\left[\frac{(\text{DMSO control OD} - \text{Compounded treated OD})}{\text{DMSO control OD}}\right] \times 100$$

Pyocyanin of *Pseudomonas* spp. plays a key role in pathogenesis. It was quantified as described elsewhere [23]. Briefly, 0.1 OD adjusted inoculum of PA was incubated for 48 hrs with or without 50 µg/ml of EA extract. Then, the supernatant was extracted with 3 ml of chloroform followed by vortex for few seconds. Chloroform layer was separated and transferred to a new tube along with 1 ml of 0.2 M HCl. After a gentle mixing, all tubes were centrifuged and pink color layer was measured at 520 nm.

Biofilm inhibition assay

Biofilm inhibition was done by static ring tube assay with filter-sterilized supernatant of LAB38. Biofilm inhibition was quantitatively determined (microdilution method) using polystyrene 96-well U bottom plate [24]. Briefly, indicator organisms such as MRSA and PA were adjusted to 0.1 OD, respectively, and 200 µl of inoculum was added to each well. EA extract of LAB38 was added into the well at a concentration of 100 µg/ml. Then, it was subsequently serial diluted for four times to the next well to make half the concentration of the extract. After 36 hrs of static incubation, planktonic cells and non-adhered cells were removed by washing the well with 0.9% saline thrice. In each well, 0.1% w/v of crystal violet was added and left for 20 minutes. Then, crystal violet was discarded, and the well was washed with de-ionized water to remove excess crystal violet. After 2 minutes of air drying, 33% acetic acid was used to dissolve crystal violet, and the absorbance was measured at 570 nm using enzyme-linked immunosorbent assay reader (Bio-Rad Model 680). To confirm the quorum quenching effect (independent of cell density) of the EA extract against MRSA and PA, protocol was followed as mentioned in pigment quantification assay, where incubation was kept in shaking condition. Percentage of growth inhibition was calculated as described for violacein inhibition.

Bioluminescence-based target analysis

Target specificity analysis was performed according to the previously described protocol [25] with little modification. Briefly, *V. harveyi* reporter strains, BB170and BB886 were grown for 18 hrs and diluted in fresh AI bioassay medium in the ratio of 5:100, respectively. Then, 200 µg/ml of EA extract was added and incubated at 28°C. As far as *E. coli* pSB401 is concerned, 0.1 OD of inoculum supplemented with 3-oxo-C-6-HSL (50 µg/ml) was used. Luminescence reading was measured at 30 minutes, 1 hr and 3 hrs. All the luminescence readings were recorded using Modulus multimode reader (Turner Biosystem) and expressed in relative light units.

Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis was performed on Thermo GC-Trace Ultra Version 5.0, equipped with DB 5-MS capillary standard non-polar column (30 Mts × 0.25 mm ID × 0.25 µm film). Helium was used as the carrier gas with a flow rate of 1 ml/minute. GC oven temperature was initiated at 70°C and increased at a rate of 6°C/minute to 260°C. 1 µl of the EA extract was injected and run for 37.51 minutes in 1:10 split ratio. GC-MS peaks were analyzed by the relative abundance of the compounds from each retention time. Peaks were matched with the database of the National Institute of Standard and Technology (http://www.sisweb.com/software/ms/nist.htm).

RESULTS AND DISCUSSION

This study is an initiative to explore pharmaceutical and commercial importance bacterium, which has exhibited probiotics' attributes and quorum quenching activity. Biochemical and molecular characteristics revealed that the isolate LAB38 belongs to the genera *Enterococcus* sp. The sequence was submitted to GenBank database and accession no. (KJ720654) was obtained. Phylogenetic analysis revealed that the sequence of LAB38 is highly matched with the sequence of existing probiotic strains. *E. durans* is a potential probiotic, which additionally produces antimicrobials to defend against pathogens [26]. Although probiotic *E. durans* has been reported to produce the antimicrobial peptide, this is the first report for quorum quenching activity, isolated from unpasteurized milk. However, many reports are endorsed the concept of quorum quenching activity in probiotics [27,28].

Probiotic attributes

Tolerance to bile salts is an inevitable credential for probiotics, as bile salts reduce bacterial survival in the intestine by impairing its cell membrane [29]. Viable colony of LAB38 was found about 4.5×10^3 CFU/ml in treated and 5.5×10^3 CFU/ml in the control media. Although bile salt delayed the growth of LAB38, the bacterium has survived successfully in the bile environment. Evidently, many reports are found to describe the bile salt tolerance of *Enterococcus* spp. [30]. *Artemia* is an excellent model organism to study the impact of probiotic bacteria on its survival and development [31]. *Artemia* has shown 100% survival in both control and LAB38-treated medium. It clearly indicated that the tested LAB38 has not exhibited any pathogenic effect on *Artemia* nauplii. In the presence of LAB38, 5.7±0.5% µm of length has increased in *Artemia* than that of control. Moreover, the growth of the *Artemia* was promoted, in which the development of thoracic segments is clearly observed in the presence of live LAB38 (Fig. 1). Apparently, it delineated that *Artemia* nauplii acquired probiotic effect from LAB38.

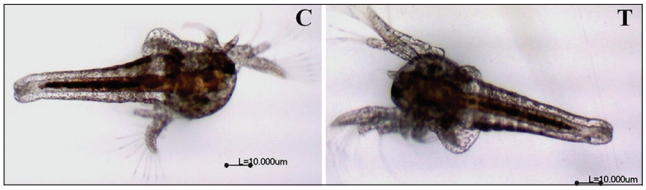

Fig. 1: *Artemia* gnotobiotic assay. After 24 hrs of incubation, LAB38 treated *Artemia* (T) has promoted to the next stage of growth than that of control (C)

Quorum quenching assay

Inhibition of QS-regulated pigment production

Violacein is a purple pigment, which is regulated by QS network. Zone of nonpigmented CV026 was observed as a consequence of the addition of supernatant. Moreover, hexane extract of LAB38 has shown neither antimicrobial nor quorum quenching activity, whereas EA extract has shown inhibitory activity on pigment production without disturbing the growth of CV026. In addition, EA extract (100 µg/ml) has shown a maximum of 37.1±1.4% inhibition against violacein pigment production. It was reported that *Cuminum cyminum* methanol extract (500 µg/ml) has exhibited nearly 30% inhibition of violacein production [32]. Remarkably, 100 µg/ml of EA extract of LAB38 was sufficient to show 37% inhibition of violacein production. Moreover, inhibition of pigment production was found even in the one-fourth diluted EA extract containing medium (Fig. 2a). There was a significant negative correlation (r^2=0.8738) between inhibition of violacein production and the concentration of the EA extract. This described that the violacein production is affected in a dose-dependent manner.

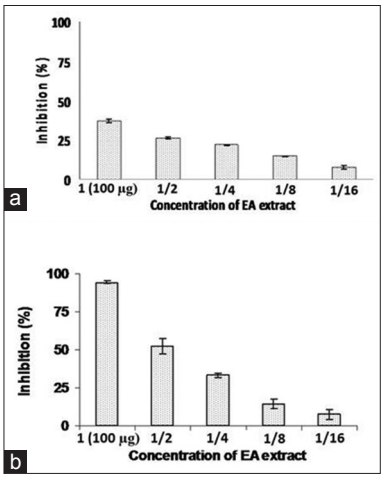

Fig. 2: (a) Inhibition of violacein pigment production by ethyl acetate (EA) extract of LAB38 (n=3). Pigment was estimated spectrophotometrically at 585 nm. Bar 1 represents the inhibition of violacein production by full strength (100 µg/mL) of EA extract. Subsequent bars represent the inhibition of pigment production by half strength of the proceeding concentration. (b) Inhibition of methicillin resistant *Staphylococcus aureus* (MRSA) biofilm by EA extract of LAB38 (n=3). Bar 1 indicates the inhibition of MRSA biofilm by full strength (100 µg/ml) of LAB38 EA extract. Adjacent bars represent the inhibition of MRSA biofilm by half strength of the proceeding concentration of EA extract

Pyocyanin is a blue phenazine redox active secondary metabolite of PA causes adverse invasive pulmonary infection by inhibiting the ciliary function, epidermal cell growth and prostacyclin release [33]. In the presence of 50 µg/ml of EA extract, 19.3±1.2% of pyocyanin production has inhibited. Thus, the EA extract of the potential probiotic strain has additional value of bearing quorum quenching activity.

Biofilm inhibition

Biofilm is a prominent virulence factor that eases the colonization and protects the cells from host immune system [34]. Supernatant of LAB38 has shown inhibition against biofilm formation of MRSA and PA. Furthermore, 100 µg/ml of EA extract has exhibited 94±0.9% and 22±0.08% of inhibition against biofilm formation of MRSA and PA, respectively. Even one-fourth concentration of 100 µg/ml of EA extract has shown inhibition against biofilm of MRSA (Fig. 2b). Significant negative correlation (r^2=0.9158) between biofilm inhibition and concentration of the EA extract was observed. As the biofilm of PA received moderate inhibition, further dilutions were not made to check the biofilm inhibitory concentration. The ratio of biofilm formation and plantonic cells (OD570/600) clearly indicates that biofilm density of MRSA was significantly reduced ($p<0.0001$) in EA extract treated sample (0.318±0.007), compared to DMSO control (5.379±0.149) . Moreover, cell density was unaffected by the extract, whereas adherence was significantly reduced. Many studies reported that intestinally colonized MRSA spread to the skin surfaces to facilitate the transmission vigorously [35]. Colonization of *S. aureus* in the intestinal tract can be eliminated by probiotic lactic acid bacteria, which eventually kill by its antimicrobial substances [36]. Thus, it is proved that probiotic LAB38 has devastated the biofilm formation of MRSA and PA.

Bioluminescence-based target analysis

Bioluminescence of *V. harveyi* is regulated by AI-1 and AI-2 type of signaling molecules [25]. *V. harveyi* BB170 can respond only to AI-2 type of signaling molecule, as it is sensor 1^- sensor 2^+, whereas BB886 is sensor 1^+ sensor 2^-, so it can respond to AI-1 type of signaling molecules [37]. In this study, 200 µg/ml of EA extract has started to show 14.8±4.8% of bioluminescence inhibition in BB170 after 1 hr incubation. Increased trend of bioluminescence inhibition (53.7±0.3%) has exhibited after 3 hrs of incubation (Fig. 3a). Similarly, the same trend of inhibition was found in BB886. After 3 hrs of incubation, 60±1.8% of bioluminescence inhibition was observed in *V. harveyi* BB886 in the presence of 200 µg/ml of EA extract (Fig. 3b). There was no reduction in bioluminescence at 30 minutes in both BB170 and BB886. There was a significant positive correlation (r^2=0.8676) between bioluminescence inhibition and increasing incubation time in BB170. Similarly, a significant correlation (r^2=0.7902) was found in the reporter BB886 as well. Based on the results obtained from these two reporters in response to time of incubation, it is revealed that the inhibition of both QS systems (AI-1 and AI-2) is directly proportional to the time of exposure. In support of this results, it was reported that AI-2 is involved in the biofilm formation and virulent gene expression of Staphylococci [38].

E. coli pSB401 (pACYC184-derived, Tet^r) is an AHL based biosensor, which can produce bioluminescence in the presence of 3-oxo-

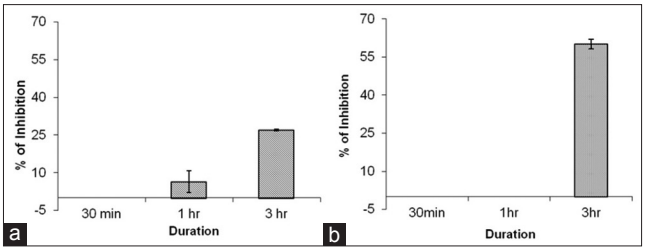

Fig. 3: (a and b) Represent the effect of ethyl acetate (EA) extract on inhibition of bioluminescence against *Vibrio harveyi* BB170 and *V. harveyi* BB886, respectively. Bioluminescence-based target analysis was evaluated at different incubation time with 200 µg/ml of EA extract of LAB38 (n=3)

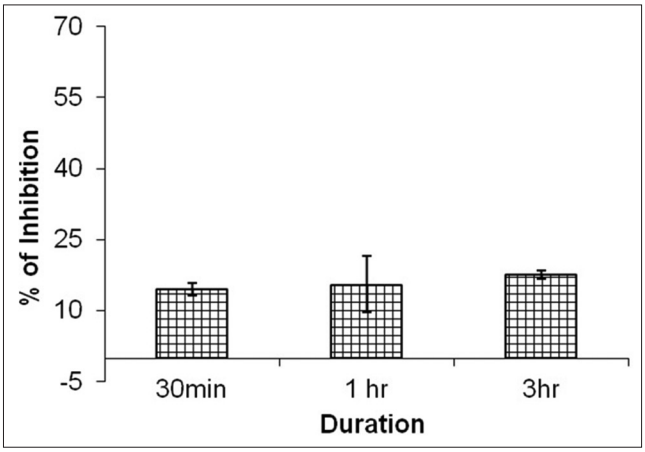

Fig. 4: Bioluminescence inhibition by ethyl acetate extract (n=3) against *luxR* based reporter strain pSB401

C6-HSL [39]. There was no significant bioluminescence inhibition found in the reporter strain pSB401 (only around 10% of inhibition was found at all incubation time intervals). Furthermore, there was no significant (r^2=0.1692) correlation between inhibition of bioluminescence and increasing time intervals (Fig. 4). It implied that the extract has not interfered in *luxR* sensor-based bioluminescence. Obviously, the result delineated that EA extract is unable to quench 3-oxo-C6-HSL signaling molecule or interferes with the receptors of this molecule. In conclusion, from the present work, the EA extract of LAB38 has not exhibited antimicrobial activity against any of the tested strains, which apparently expounded that the inhibition merely was on QS network. Perhaps, EA extract was involved in either the process of inactivating signaling molecules/receptor proteins or competing for the receptor proteins. However, further studies are required to confirm the speculation.

GC-MS analysis

GC-MS chromatogram revealed about 30 types of different compounds in EA extract. Indeed, five compounds has existed with high percentage of probability, in which four compounds occupied almost 50% of the total extract. Furthermore, those four peaks at the retention time 11.87, 14.13, 19.69, and 12.86 were not matched with the commercial database, revealing the novelty of the compounds (Table 1). Based on the relative abundance, it was speculated that 7,9-di-*tert*-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione is possibly involved in QS inhibition activity. This compound was an azulene derivative. Moreover, it has not matched with the existing compound databases, which designated the compound as novel. However, some of the metabolites of the extract have been previously reported for biofilm inhibition. Bromine-containing furanones are potential inhibitors of biofilm of several pathogens [40]. Sulfur atom plays a role in biofilm inhibition [41]. Though many QS inhibitors were ascertained from various biological resources [42-44] this study highlighted the significance of the bacterial source especially *E. durans* and its possible quorum quenching mechanisms.

Table 1: GC MS analysis of ethyl acetate extract

Retention time	Compound name	p	Relative abundance
7.2	Acetic acid, 2-propenyl ester (CAS)	21.99	0.54
7.83	1-butanol (CAS)	12.67	0.75
10.16	Isobutyric acid-2-d1	41.00	6.62
12.08	l-alanine, N-propoxycarbonyl-, dodecyl ester	54.26	0.34
17.65	E-15-heptadecenal	9.69	0.48
18.7	Tetradecanoic acid (CAS)	84.65	2.97
19.32	(E)-2-(2H (1)-4-methoxyphenylethene	13.03	0.45
19.72	(E)-2-(2H (1)-4-methoxyphenylethene	18.01	1.32
21.76	1-octadecene (CAS)	8.54	2.75
22.93	Hexadecanoic acid	78.74	5.82
23.09	2-(dimethylhydrazono) butanal	63.86	2.27
23.84	7,9-di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione	92.34	0.97
24.98	2,6-dibromo-3-pyridinyl 3-methyl-2-butenyl ether	16.64	11.87
25.55	1-docosanol, methyl ether	5.58	3.42
26.85	(erythro)-5-methyl-2-phenylsulphonylhexan-3-ol	36.65	14.13
27.18	1à,5à,9,12,12-pentamethyl-4,8-dioxo-15á-cetyloxy-11à,13à-tetracyclo[8.5.0.0 (3,7).0 (11,13)]pentace-9-ene	29.58	19.69
27.40	1-methyl-3-(methylamino)-4-pyrazolecarboxamide	20.85	0.92
28.34	2-(n-butylidene)6-n-butylcyclohexanone	28.98	1.76
28.94	5-(N-methylanilino)-4-(N-methyl-N-phenylcarbamoyl)-2,3-dioxo-2,3-dihydrofuran	62.29	1.90
30.26	Cyclohexane, 1,3,5-trimethyl-2-octadecyl (CAS)	53.09	0.89
31.38	n-tetracosanol-1	7.92	1.38
32.46	2-benzyl-3,6-dioxo-5-isopropylpiperazine	94.02	0.44
32.71	2-nonadecanone	9.07	0.31
33.14	Di-(2-ethylhexyl) phthalate	21.28	1.31
33.79	Cyclo-(l-leucyl-l-phenylalanyl)	82.27	0.37
34.42	Octacosyl trifluoroacetate	3.92	0.78
34.75	4-(3-hydroxypropynyl)-3-(2-nitroethyl)indole	69.08	1.83
35.52	1-acetyl-4-methyl-1,3-dihydropyrrole-2,2-dicarboxylic acid, diethyl ester	7.13	12.86
36.23	Diethyl 2-methylphenyl(methylthio)methanephosphonate	46.11	0.29
39.11	Hexatriacontyl pentafluoropropionate	3.14	0.58
Total			100.1

GC-MS: Gas chromatography-mass spectrometry, P: Probability

CONCLUSION

In this comprehensive study, the probiotic *E. durans* showed immense potential to curb the pathogenesis in terms of quorum quenching. Indeed, it has the potential to inhibit the biofilm of MRSA and PA. The results implied that the extract has the inhibitory activity against both AI-1 and AI-2 mediated QS systems. Furthermore, a broad range of QS inhibition was explored with clear evidence using reporter strains such as *C. violaceum* CV026, *E. coli* pSB401, *V. harveyi* BB170 and BB886. It was speculated that the key azulene derivative might be involved in the quorum quenching mechanism, as the molecule was relatively abundant in the extract, but confirmation and its mechanisms of activity remain to be elucidated. Our results suggested that LAB38 has commercial value, as it has both probiotic and quorum quenching attributes.

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