http://dergipark.org.tr/trkjnat
Trakya University Journal of Natural Sciences, 24(2): 23-30, 2023
ISSN 2528-9691
DOI: 10.23902/trkjnat.1276562

Research Article

EVALUATION OF QUORUM SENSING SIGNALS OF STRONG BIOFILM PRODUCING BACTERIA VIA LC-MSMS, HPLC AND BIOSENSORS

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Cite this article as:

İpek D. 2023. Evaluation of quorum sensing signals of strong biofilm producing bacteria via LC-MSMS, HPLC and biosensors. *Trakya Univ J Nat Sci*, 24(2): 23-30, DOI: 10.23902/trkjnat.1276562

Received: 04 April 2023, Accepted: 07 July 2023, Online First: 23 July 2023, Published: 15 October 2023

Edited by:
Mustafa Yamaç

Key words:
AHL Signals
AI-2 Signals
Biofilm
Biosensors
HPLC
LC-MSMS

Abstract: This study aimed to show presence of Quorum Sensing (QS) signals of Gram-negative and Gram-positive biofilm producing bacteria isolated from real dairy process lines. Defining the profile and chemical composition of QS-signals is an important factor in control of microbial resistance and biofilm production. We especially focused on unusual behaviour of Gram-positive and Gram-negative isolates. Long-chain acyl-homoserine lactones (AHLs) signals (C14-HSL, C16-HSL and C18-HSL) and DFD (4,5-dihidroksi-2,3-pentanedione)-AI-2 signals of the isolates were studied by High-performance liquid chromatography (HPLC) and Liquid Chromatography with tandem mass spectrometry (LC-MSMS) methods. All Gram-positive isolates were defined as AHL-producers. All Gram-negative isolates, formerly defined as non-AHL producers by both biosensors and HPLC methods, were identified as AHL-producers. DFD signal was only detected from Gram-negative *Klebsiella pneumonia*, *Enterobacter cloacae* and *Klebsiella oxytoca* isolates. The results demonstrated that the QS-system is a complex system and biosensor microorganism may not be the best method for QS-signal identification. The results also provided new insights in defining the profile and chemical composition of QS-signals importance for interrupting the chemical communication completely to reduce biofilm formation and prevent resistance gain of microorganisms.

Özet: Bu çalışma, süt ürünleri proses hatlarından izole edilen hem Gram-negatif hem de biyofilm üreticisi bakterilerin Quorum Sensing (QS) sinyallerini göstermeyi amaçlamıştır. QS sinyallerinin profilinin ve kimyasal bileşiminin tanımlanması, mikrobiyal direnç gelişimi ve biyofilm oluşumunun kontrolü için önemli bir faktördür. Özellikle Gram-pozitif ve Gram-negatif izolatların olağan dışı davranışlarına odaklanılmıştır. İzolatların uzun zincirli açil-homoserin lakton (AHL) sinyalleri (C14-HSL, C16-HSL ve C18-HSL) ve DFD (4,5-dihidroksi-2,3-pentanedion)-AI-2 sinyalleri yüksek performanslı sıvı kromatografisi (HPLC) ve Tandem kütle spektrometresi ile Sıvı Kromatografisi (LC-MSMS) yöntemleri ile incelenmiştir. Sonuç olarak, tüm Gram-pozitif izolatların olağandışı olarak AHL üreticisi olduğu tespit edilmiştir. Biyosensör ve HPLC yöntemleri ile AHL üreticisi olmadığı tespit edilen Gram-negatif bakteriler, LC-MSMS yöntemi ile AHL üreticileri olarak tanımlandı. Gram-pozitif izolatlar tarafından üretilen DFD sinyali, Gram-negatif izolatlardan *Klebsiella pneumonia*, *Enterobacter cloacae* ve *Klebsiella oxytoca*'da tespit edilmiştir. Sonuçlar, QS sisteminin karmaşık bir sistem olduğunu ve biyosensör mikroorganizmaların QS sinyal tanımlaması için en iyi yöntem olmayabileceğini göstermiştir. Bu çalışma, mikroorganizmaların biyofilm oluşumunu azaltmak ve direnç kazanımını önlemek için kimyasal iletişimi tamamen kesmek adına QS sinyallerinin profilini ve kimyasal bileşimini tanımlama konusunda önemli bir bakış açısı sunmaktadır.

Introduction

Microorganisms form biofilm structures to prevent themselves against the harmful effects of various environmental factors, to survive and grow. Biofilm also provide protection in presence of antimicrobials, antiseptics and industrial biocides. A microorganism is 10 to 1000 times more resistant to compared to its planktonic (free-living) form. On the other hand, biofilm may lead to irreversible contaminations, food spoilage and foodborne disease, and may cause corrosion on equipment which may result in energy losses. Quorum Sensing (QS), which is a chemical communication system between microorganisms, is an important factor for biofilm formation. By the increase of QS-signal molecules up to the threshold limit, receptor proteins which are regulated by specific genes recognize

the biofilm formation (Püning *et.al.* 2021, Wang *et.al.* 2022). QS-system defected microorganisms either produce loose structured biofilms or cannot produce any biofilm structure (Shih & Huang 2002, Suntharalingam & Cvitkovitch 2005, Federle & Bassler 2003). Biofilm formation ability of bacteria is positively correlated with the QS-signal production (Duanis-Assaf *et al.* 2016, Mizan *et al.* 2016, Abbondio *et al.* 2019).

QS-signals of microorganisms are usually detected by indicator microorganisms, such as *Chromobacterium violaceum* CV026, *Agrobacterium tumefaciens* A136, *Vibrio harveyi* BB 170 and MM 30. For Gram-negative bacterial isolates, the presence of short-chain acyl-homoserine lactones (hereafter AHLs) (such as C4-HSL, C6-HSL, C8-HSL, and 3-oxo-C4~C8) were screened by using the biosensor strain *Ch. violaceum* CV026, while the medium/long-chain AHLs (such as C8-HSL, 3-oxo-C8HSL, C10-HSL, C12-HSL, 3-oxo-C12-HSL and C14-HSL) by *A. tumefaciens* A136. For both Gram-negative and Gram-positive bacteria, Lux S signals, such as AI-2, can be detected by indicator microorganisms. However, microorganisms can produce more signals than the detectable amount by the indicator microorganisms. In addition to biosensors, Thin Layer Chromatography (TLC) and tandem mass spectrometric methods linked with High-performance liquid chromatography (HPLC), Gas chromatography (GC), Liquid chromatography (LC) and Ultra performance liquid chromatography (UPLC) have been used for detectionof a wide range of QS-signals (Suntharalingam & Cvitkovitch 2005, Tang *et al.* 2013, Biswa & Doble 2013, Lade *et al.* 2014, Patel *et al.* 2016, Zhang *et al.* 2022).

Gram-positive bacteria are not AHL producers in most cases, but recent studies provided evidence on AHL signal production by Gram-positive bacteria. Biswa & Doble (2013) reported that an AHL producing positive bacteria of the genus *Exiguobacterium* was isolated from the surface of seawater around Mamallapuram (Chennai, South India). Naik *et al.* (2017) reported an AHL producer *Listeria monocytogenes* BN3 which was isolated from dairy industry surfaces. It is also stated that the expression of the virulence factor (hemolysin) was reported to be a response to AHL signals of *L. monocytogenes* BN3. Naik *et al.* (2018) has detected the presence of AHL which has 6-carbon long acyl chain, i.e. N-hexanoyl-homoserine lactone molecule (C6-HSL), from *Staphylococcus sciuri* NN14. The authors also indicated that the presence of C6-HSL played a vital role in the biofilm formation by microorganism. The study of İpek (2017) who studied the biofilm formation potentials of Gram-negative bacteria isolated from surfaces water and air of a dairy industry. The findings suggested that the isolated bacteria were strong biofilm producers, but the methods applied failed to identify the production of QS signalling. This drawback underlines the motivation of the current study., with which the production of QS-signals in biofilm forming bacteria isolated from dairy process lines were investigated. The presence of AHL and AI-2 signals in biofilm positive isolates were investigated by High-performance liquid chromatography (HPLC) and Liquid Chromatography with tandem mass spectrometry (LC-MSMS). The LC-MSMS method was chosen for more precise signal detection. It has the capability of high degree separation, chromatographic separation and molecular fragmentation via mass spectroscopy and its success on detection of the same target substances is higher compared to HPLC. The study, which is believed to have a significant role in displaying the relationship between biofilm formation and QS also focused on the production of AHLs by Gram-positive bacteria. A comparison between biosensorial methods and the applied HPLC and LC/MSMS methods was performed.

Materials and Methods

Bacterial strains

To understand QS-signals-biofilm interactions, 6 Gram-negative (*Enterobacter cloacae, Klebsiella pneumonia, K. oxytoca, Mannheimia haemolytica, Ochrobactrum anthropi and Pseudomonas stutzeri*) and 9 Gram-positive isolates (*Aneurinibacillus aneurinilyticus, Bacillus coagulans, B. licheniformis, Listeria innocua, L. seeligeri, L. monocytogenes, Streptococcus bovis, S. thermophilus* and *S. lactis*) were used. All the isolates used were formerly identified, their biofilm and QS-signal formation statues were defined (İpek & Zorba 2016, 2017). *Chromobacterium violeceum* CV026 and *Agrobacterium tumefaciens* A136 were used as biosensor strains for the detection of short and long chain AHLs of Gram-negative bacteria by colour change. AI-2 signals of Gram-positive bacteria were detected by *Vibrio harveyi* BB 170 bioassay. *Vibrio harveyi* BB 170 is a non-AI-2 producer bacterium but becomes luminescent in the presence of exogenous AI-2 signals. Among the Gram-negative isolates, *M. haemolytica*, *O. anthropi* and *P. stutzeri* were defined as non-AHL producers. All Gram-positive bacteria were found to be high AI-2 producers. All isolates were determined as strong-biofilm producers as revealed by the 96-well microtiter plate assay (Fig. 1) (İpek 2017). All isolates were stocked on Tryptone Soy Agar (TSA, Merck 105458, Germany) at -18°C. Overnight cultures were prepared in Tryptone Soy Broth (TSB, Merck 105459, Germany) at 30°C.

To provide more detailed information about QS-signal presence, AHL and AI-2 production status of the isolates were examined more sensitively by HPLC and LC-MS MS methods.

AHL Signal Extraction

100 µl isolate for each strain was inoculated into 100 ml of Luria-Bertani broth (LB, BD-Difco, USA) and incubated at 30°C/24h. The overnight culture was centrifuged at 9000 *g* for 10 min at +4°C (NF800R, Nüve, Türkiye). The supernatant was mixed with ethyl acetate (PubChem CID: 8857) (2:1) and incubated at 30°C, 180 rpm for 2 h (Certomat, Sartorius, Germany). The organic layer of the mixture was separated by separating funnels. After removing ethyl acetate, dry signal molecules were mixed with acetonitrile (PubChem CID: 6342) water solutes on (1:1) and stored at +4°C (Lade *et al.* 2014).

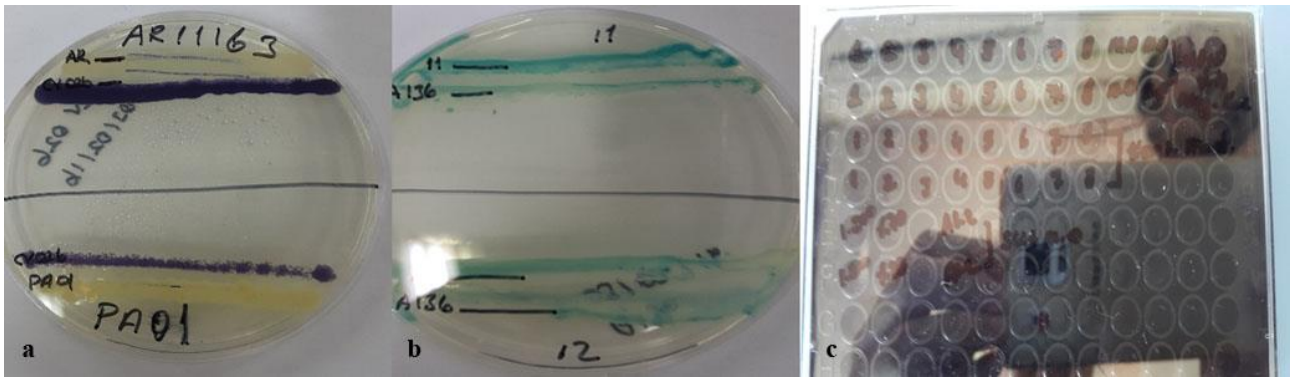

Fig. 1. a. Short Chain AHL Colour Change, **b.** long Chain AHL Colour Change **c.** AI- 2 signals 96-well-Microplate method.

AI-2 signal Extraction

Vibrio harveyi BB 170 was used as the indicator microorganism (reporter strain). They were inoculated onto AB medium and 1:5000 dilution was prepared. 100 µl of 1:5000 dilution was put into 96-well plates (SPL Life Sciences, Pocheon-Si, Korea). The AI-2 producer strain (0.5 ml) was inoculated into 3 ml LB broth (22460, BD-Difco, USA) and 2 ml natural extracts at 30°C/4-6 h. After incubation, overnight cultures and natural extract mixtures were centrifugated at 18405 *g*, 5 min at room temperature for cell-free supernatants (NF800R, Nüve, Türkiye) (Almasoud *et al.* 2016, Zhu *et al.* 2015).

Determination of AHL presence by HPLC

AHL extracts of the Gram-negative bacteria was analysed by the Agilent 1200 HPLC system (Agilent, Santa Clara, CA, USA) equipped with a ZORBAX Eclipse XDB-C18 column (4.6 mm × 150 mm, 5 µm particle size) at 30°C. The injection volume was 15 µl, running time 60 min and post time 15 min. Commercial standards (C4-HSL, C6-HSL, C8-HSL, 3-oxo-C8-HSL, C10-HSL, C12-HSL, 3-oxo-C12-HSL and C14-HSL) (PubChem CID:10130163, 10058590, 6914579, 127293, 10131281, 45266530, 3246941 and 11688418) (Cayman Chemicals, Michigan, USA) were prepared as 1000 µg/ml with acetonitrile (PubChem CID: 6342) (Lade *et al.* 2014).

Determination of AHL and AI-2 presence by LC-MSMS

Both AHL and AI-2 extracts of all bacteria were analysed by the Shimadzu LC-MS MS-8040 (Shimadzu, Kyoto, Japan) with C18 Kinetex column (5 µm, 100 Å, 100 × 2.1 mm). The mobile phase was prepared as 0.1% acetic acid (PubChem CID: 176) 95:5 distilled water: acetonitrile. Commercial standards (C14:HSL, C16:HSL; C18:HSL; 4,5-dihidroksi-2,3-pentanedione-DFD) (PubChem CID: 11688418, 87606261, 69757690, 443434) were prepared as 5 mg/kg with acetonitrile. The injection volume was 10 µl and the flow rate was 200 µl/min. MRM was chosen as 50-500 *m/z*. AHL standards were provided from Cayman Chemicals, Michigan, USA. 4,5-Dihydroxy-2,3-pentanedione was provided from Omm Scientific Inc., Dallas, TX, USA (Zimmer *et al.* 2014, Saurav *et al.* 2016, Xu *et al.* 2017).

Results

AHL Production Status of Gram-Negative Isolates

A previous study investigated the QS presence by using QS indicator microorganisms (İpek 2017). The results were challenged in the present study by applying two different methods (HPLC and LC-MSMS) for the detection of QS presence.

The HPLC analysis was performed for verification of the results obtained by the biosensor microorganisms. According to retention times of the commercial standards, *Ochrobactrum anthropi*, *Mannheimia haemolytica*, and *Pseudomonas stutzeri* were discovered as non-AHL producers. *Klebsiella pneumonia*, *Enterobacter cloacae*, and *Klebsiella oxytoca* were determined as short-chain AHLs producers, similar to C6-HSL, C8-HSL and 3-oxo-C8-HSL. *Klebsiella pneumonia* was identified as C10-HSL and C14-HSL producer. Both *E. cloacae* and *K. oxytoca* were identified as C10-HSL, 3-oxo-C12-HSL and C14-HSL producers (Table 1). HPLC analysis successfully detected the short-chain AHL production of 3 Gram-negative bacteria. It also identified the type of the AHLs (middle/long chain) produced. Although it is expected to see a strong correlation between biofilm formation and QS, the results suggested that there were no AHL signals coming from the strong biofilm producers *Ochrobactrum anthropi*, *Mannheimia haemolytica* and *Pseudomonas sturtzeri*.

AHL and AI-2 Signal Production Status of Gram-Negative and Positive Isolates

AHL and AI-2 signal production status of all isolates were studied by LC-MSMS as a sensitive method for more precise signal detection. Extraction was performed by using 24 h cultures with 50-500 *m/z* MRM to identify QS-signals. Signal standards' *m/z* values were detected as 350.20 for C14-HSL; 360.40 for C16-HSL; 458.50 for C18-HSL and 332.30 for DFD. Mass spectrum graphics and *m/z* values for Gram-negative isolates were given in Fig. 2 and Table 2. All AHL signals (C14-HSL, C15-HSL and C18-HSL) were detected from all Gram-negative isolates, but the DFD signal was only detected from *K. pneumonia*, *E. cloacae* and *K. oxytoca*.

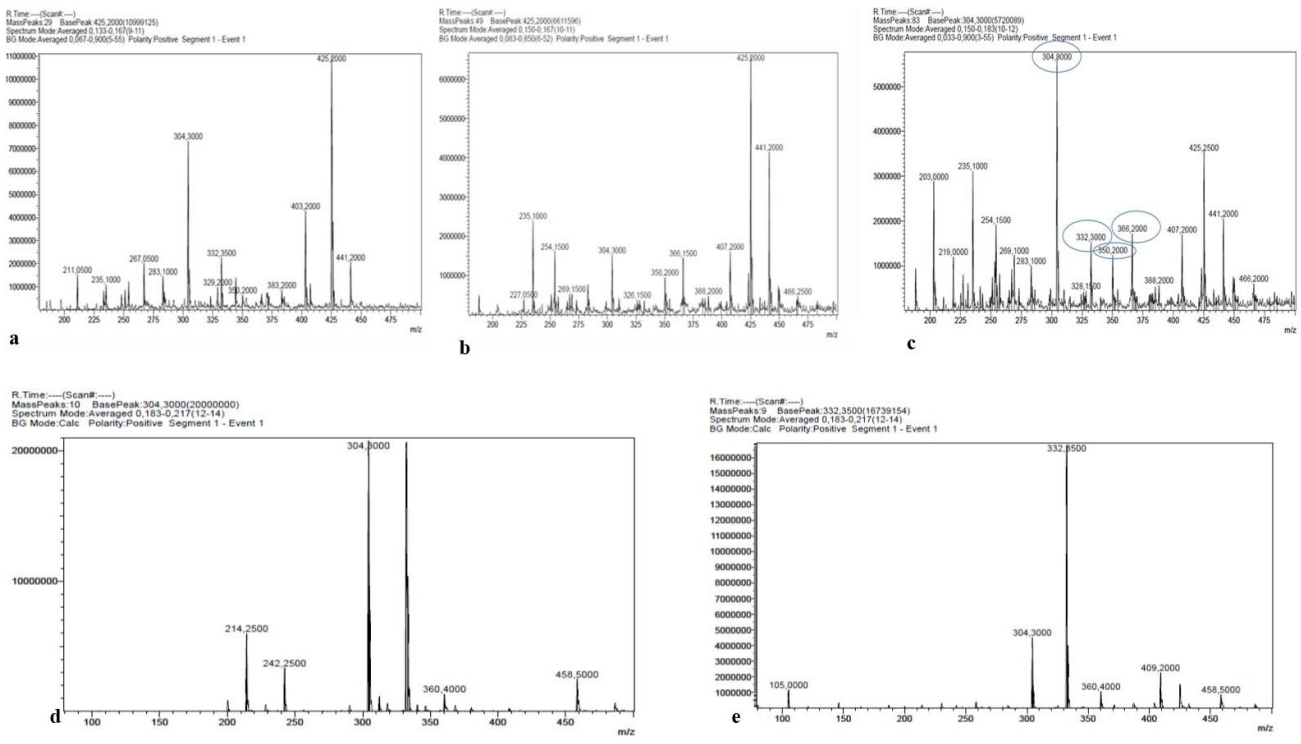

Fig. 2 Mass spectrum graphics of selected Gram-Negative/Positive isolates. **a.** *E. cloacea* AHL and DFD signals, **b.** *P. sturtzeri* AHL signals with no DFD (AI-2) signal (332.30 *m/z*), **c.** *S. bovis* (C14-HSL: 350,20; C16:HSL: 366.20; C18-HSL: 466.20 and DFD: 332.30 *m/z*), **d.** AHL Standards (C14-HSL: 350,80; C16:HSL: 360.40; C18-HSL: 458.50 m/z), **e.** DFD (AI-2) Standard (332.30 *m/z*).

Table 2. *m/z* values of AHL and DFD (AI-2) Signals of Gram-Negative/Positive isolates Bacteria.

Isolates	**Blank** Acetonitrile	**C14:HSL**	**C16: HSL**	**C18:HSL**	**DFD**
Standards	**304.30**	**350.20**	**360.40**	**458.50**	**332.30**
K. pneumonia	304.30	350.60	360.20	456.20	332.30
E. cloacae	304.30	350.20	360.20	441.20	332.30
K. oxytoca	304.30	350.30	360.20	466.20	332.30
O. anthropi	304.30	350.30	360.20	466.20	-
M. haemolytica	304.30	350.30	360.20	466.20	-
P. stutzeri	304.30	350.20	366.15	466.25	-
L. innocua	304.30	350.25	366.25	462.25	332.25
L. seeligeri	304.30	350.25	360.20	466.20	332.30
L. monocytogenes	304.30	350.35	360.20	466.20	332.30
B. coagulans	304.30	350.30	360.20	466.20	332.30
A. aneurinilyticus	304.30	350.30	360.20	466.15	332.30
B. licheniformis	304.30	350.30	360.20	466.20	332.30
S. bovis	304.30	350.20	366.20	466.20	332.30
S. thermophilus	304.30	350.30	360.20	466.20	332.30
S. lactis	304.30	350.30	360.20	466.20	332.30

Table 1. HPLC Retention times of AHL-Signals (min.) of the Gram-Negative *E. cloacae*, *K. pneumonia* and *K. oxytoca*.

Signals	Standards	K. pneumonia	E. cloacae	K. oxytoca
Blank (Acetonitrile)	4.275	4.912	4.894	4.885
C6	13.065	13.129	13.117	13.117
3-oxo-C8-HSL	16.676	16.396	16.352	16.332
C8	23.667	23.785	23.795	23.778
C10	28.500	28.311	28.315	28.304
3-oxo-C12-HSL	32.599	-	32.478	32.479
C12	40.533	-	-	-
C14	41.536	41.701	41.714	41.719

Mass spectrum graphics and *m/z* values for Gram-positive isolates were given in Fig. 2 and Table 2. All Gram-positive isolates were defined as AHL (C14, C16, C18-HSL) and DFD producers.

The results suggested, as expected to see a strong relation between biofilm formation and QS, that all strong biofilm producer Gram-positive and Gram-negative isolates are also QS-signal producers.

Discussion

QS-signals are powerful supporters for biofilm formation. Multicultural biofilm matrices help microorganisms to do horizontal gene transfer, which in turn help them to become more durable to survive for a long time (i.e. have more resistance to antibiotics, disinfectants, etc.). It is crucial to understand and identify the QS-signals of microorganisms to be able to interrupt the resistance gaining. In this study, we aimed to show QS-signal presence of both Gram-negative and Gram-positive biofilm producer bacteria, which were isolated from real dairy process lines. The bacteria were isolated from multicultural biofilm structures after the cleaning process. First focus of this study was to understand the reason for three strong biofilm producer Gram-negative isolates being non-AHL producers via biosensors and HPLC methods. Only three Gram-negative isolates were identified as AHL-producer by HPLC, as similar to the results obtained with biosensors. However, other isolates were strong biofilm-producers. Most HPLC systems might not discriminate co-extracted interfaces and the target effectively. Purification should be done enough to remove interferences for HPLC analysis (Qian *et al.* 2019). Targets can be masked by co-extracted interfaces. Another explanation for this is that unusual long-chain signals can be produced by bacteria. Our isolates might be unusual AHL signal producers. Steindler & Venturi (2007) mentioned detection limits of biosensors. The presence of low concentration AHL signals which are lower than their detectable limit, and the presence of other types of signals which are out of their detectable ranges might stem from false-negative results. The unusual AHL signal (C16, C18 to C20) production was also mentioned in their study. Tang *et al.* (2013) identified non-small chain AHL producer *Pseudomonas* sp. with the biosensor microorganisms *Agrobacterium tumefaciens* 136, *Chromobacterium violaceum* ATCC 31532 and 12472^{T}. In the study using biosensor method, Lade *et al.* (2014) reported non-AHL producer Gram-negative *Pseudomonas* and *Aeromonas* sp. which were isolated from bioreactors. HPLC was used to detect the small-and middle/long-chain AHL producer Gram-negative bacteria. Haslan & Kimiran-Erdem (2013) reported *P. aeruginosa*, which was isolated from cooling water towers, as a non-AHL producer. The authors pointed out that the biosensors may not detect all AHLs. These strains might get inhibited because of AHLs rates of the bacteria. Moreover, AHL levels can be under the detectable limits of biosensor strains and novel AHL signals which are unknown from biosensor strains can be produced by the bacteria. Patel *et al.* (2016) mentioned three major reasons for biosensors' limitations: (i) LuxR-type receptors are specific for particular AHLs, (ii) activation threshold of the biosensors, (iii) general inability for AHL quantitation. Therefore, more sensitive methods than biosensors should be used to understand QS-signals detection and their roles in natural bioprocesses.

Multicultural biofilms are inter-kingdom structures, bacteria can easily contact to each other and become stronger, more resistant to disinfectants and/or antibiotics. Also, QS-signals' genes can transfer to microorganisms to microorganisms and become inter-communicative in biofilm structure and easily cross-talk with possible hosts (Verbeke *et al.* 2017). Therefore, we conducted our research to examine the presence of AHL and AI-2 signals of both Gram-negative and Gram-positive isolates to discover how the QS-systems work in a biofilm structure. LC-MSMS method is chosen for more precise signal detection. It has the capability of high degree separation, chromatographic separation and molecular fragmentation via mass spectroscopy; also, its success on detection the same target substances compared with HPLC (Barriuso *et al.* 2008, Hong *et al.* 2012, How *et al.* 2015, Mesa *et al.* 2019, Naik *et al.* 2017, Naik *et al.* 2018, Patel *et al.* 2016, Saurav *et al.* 2016, Tang *et al.* 2013, Verbeke *et al.* 2017, Zhao *et al.* 2016, Zimmer *et al.* 2014).

Biosensor strains and HPLC methods did not detect the signal presence of *O. anthropi*, *M. haemolytica* and *P. stutzeri*. The results suggest that the strong biofilm producer isolates were defined as high-chain AHL producers. This result confirms the correlation between biofilm production and the presence of QS-signals. Previous studies indicated that MRM values of AHL and

DFD signals correlate with the study's results (Churchill *et al.* 2011, Saurav *et al.* 2016, Niu *et al.* 2008, Zhao *et al.* 2016). HPLC method did not detect the C14-HSL signals of *O. anthropi*, *M. haemolytica* and *P. stutzeri* while these signals were detected by the LC-MSMS method. The reason for this might be that the LC-MSMS method is more sensitive in detection and that the signal might be very concentrated.

There is limited published data which reports AHL producer Gram-positive bacteria. Biswa & Doble (2013) reported an AHL-producer Gram-positive bacteria, *Exiguobacterium* sp, which was isolated from marine. Their findings suggested that oxo-octanoyl homoserine lactone signals were detected by using the GC-MS method. The *ExgR* gene, which is similar to the *LuxI* gene of *P. putida*, was determined by molecular methods. Highlander *et al*. (2007) and Kunst *et al.* (1997) reported AHL-producer genes from *Staphylococcus* sp. and *Bacillus* sp. as Gram-positive bacteria in full genome studies. They suggested that this situation might happen due to the horizontal gene transfer.

Rajput & Kumar (2017) reported *LuxI/LuxR* genes which are responsible for AHL production by Gram-positive bacteria (mainly *Firmicutes* and *Actinobacteria*) and also, reported presence of AHL-producer genes *LuxI* and *LuxR* in *Firmicutes* and *Actinobacteria* sp. genome. In addition, horizontal gene transfer from Gram-negative bacteria were inferred using molecular methods. Naik *et al.* (2017) studied the AHL signal presence of biofilm positive *L. monocytogenes* isolated from dairy process lines. N-hexanoyl-homoserine lactone molecule (C6-HSL) was detected from *L. monocytogenes* strain BN3 by chrome azurol S (CAS) agar test. The same study also suggested that the molecule C6-HSL has an important role in biofilm formation by the strain. It provides support in the production of haemolysin and virulence of the same bacteria. Our research indicates that biofilm positive and all Gram-positive isolates, isolated from dairy process lines, were AHL-producers. This is in line with the findings of aforementioned studies (Naik *et al.* 2018).

These results demonstrated that the bacterial communication system is a complex system and indicator microorganism method may not be efficient to detect the presence of QS signals. Due to horizontal gene transfer in multicultural matrices, signals may be lowly concentrated and may not be deviated by their Gram reactions and species. Quorum quenching (interrupting the communication between microorganisms) becomes a remarkable factor for blocking the biofilm formation and resistance gaining. In conclusion, defining the profile and chemical composition of QS-signals are essential for interrupting bacterial communications completely. If the communication between microorganisms cannot be interrupted completely, microorganisms may continue to gain resistance.

Acknowledgement

I am thankful to laboratory members of Assoc. Prof. Dr. Nükhet N. Demirel Zorba in Food Engineering Department, Çanakkale Onsekiz Mart University, Çanakkale, Türkiye for their kind support. I am also thankful to Çanakkale Onsekiz Mart University Science and Technology Application and Research Center for their cooperation in LC-MSMS study. I express my sincere thanks to Prof. Dr. Ji Hyang Kweon and Assist. Prof. Dr. Harshade Lade and their research team, Water Treatment and Membrane Laboratory, Department of Environmental Engineering, Konkuk University, Seoul, Korea for their kind cooperation in HPLC study.

Ethics Committee Approval: Since the article does not contain any studies with human or animal subject, its approval to the ethics committee was not required.

Data Sharing Statement: Data available on reasonable request.

Conflict of Interest: The author has no conflicts of interest to declare.

Funding: This research was supported by the Scientific Research Project Commission of Çanakkale Onsekiz Mart University (FBA-2018-1438).

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