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LISTERIA MONOCYTOGENES STRAIN BN3 RESPONDS TO ACYL HOMOSERINE LACTONE (AHL) BY EXPRESSION OF VIRULENCE GENE (*HLYA*) AND GENE RESPONSIBLE FOR BIOFILM (*SRTA*)

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ABSTRACT

Listeria monocytogenes, a foodborne pathogen and well known causative agent of listeriosis in humans that is also known to form biofilm on utensils used in the dairy industry is well studied for peptide-based Quorum sensing system (*agr* QS system). In the present study, we are reporting *L. monocytogenes* strain BN3 to respond to an acyl-homoserine lactone (AHL) by expression of virulence gene (*hlyA*) and the gene responsible for biofilm formation (*srtA*) using Real-Time PCR technology. It revealed that in *L. monocytogenes*, *hlyA* gene (encodes listeriolysin O) expression increased 0.7-fold in response to 500 nM AHL (C4-HSL treated) as compared to control. Also, in response to 500 nM AHL, (C4-HSL treated) strain BN3 showed 27-fold up-regulation of *srtA* gene (encodes enzyme sortase) as compared to control. Our study confirmed the cross-talk between Gram-positive and Gram-negative bacteria since *L. monocytogenes* strain BN3 responds to C4-HSL, which is normally produced by Gram-negative bacteria. Here, we suggest targeting both *agr* based QS and AHL based QS system together in *L. monocytogenes* to tackle biofilm formed on milk cans in dairy industries and when treating listeria infections.

Keywords: *Listeria monocytogenes*, AHL, virulence gene, biofilm, real time-PCR

INTRODUCTION

Gram-positive *L. monocytogenes* is an emerging food-borne pathogen responsible for listeriosis in humans (**Doijad *et al*., 2011**). Various contaminated foods viz. raw milk, meat, vegetables, ready to eat foods, milking utensils, and fruit juices are the sources of infection to humans by *L. monocytogenes* (**Doijad *et al*., 2010; Naik *et al*., 2017**). This bacterium can grow in psychrophilic conditions on milk and meat kept in the refrigerator (**Doijad 2014**). The infections caused by *L. monocytogenes* show 91 % hospitalization rate, 50 % neonatal death rate (50 %) and 20-30 % death cases (**Babu *et al*., 2017**). Doijad (2014), reported that it is difficult to remove *L. monocytogenes* from the dairy industry clean-in-place (CIP) procedure because *L. monocytogenes* forms a strong biofilm on dairy utensils. The ability of *L. monocytogenes* to form biofilm on food surfaces and utensils is the main reason behind food-borne infection and persistence of *L. monocytogenes* in food industry (**Oliveira *et al*., 2010; Doijad *et al*., 2015; Jamshidi and Zeinali 2019**). Therefore, to control *L. monocytogenes* in food dairy industry, there is a need to control biofilm formation of *L. monocytogenes* on utensils used in food/dairy industry. Hemolysin gene (*hly*) was the first virulence gene detected in *L. monocytogenes* (**Vazquez-Boland *et al*., 2001**) which encodes listeriolysin O (LLO) (**Doijad *et al*., 2010; Doijad *et al*., 2011**). **Garandeau *et al*. (2002)**, reported that the specialized enzyme sortase (SrtA) of *L. monocytogenes* encoded by the *srtA* gene is responsible for the processing of internalin and in virulence. **Doijad (2014)**, reported the construction of sortaseA deletion mutant (LMΔ*srtA*) of wild type *L. monocytogenes* EGDe (LMWT) and found that adherence ability of mutant LMΔ*srtA* was decreased by 28.03 times, whereas biofilm formation potential was decreased by 7.3 times as compared to LMWT. These reports confirmed that sortase enzyme (SrtA) of *L. monocytogenes* are involved in biofilm formation and virulence.

Quorum sensing (QS) molecules such as Acyl homoserine lactones (AHLs) in Gram-negative bacteria and small peptides in Gram-positive bacteria (*agr* QS system) are well known to have a role in virulence gene expression and biofilm formation. The presence of AHL based QS system has never been reported in Gram-positive bacteria and is reported only in Gram-negative bacteria, but there is only one report saying Gram-positive bacteria *Exiguobacterium* sp. isolated from marine water produce C3-oxo-octanoyl homoserine lactone (OOHL) (**Biswa and Doble, 2013**). **Qazi *et al*. (2006)**, reported that although *Staphylococcus aureus* does not have the potential to produce AHL molecules, they can respond negatively to external AHL molecules. They observed that 3-oxo-C12-HSL inhibited *agr* QS expression and antagonized virulence gene expression in *S. aureus*. **Naik *et al*. (2018)**, reported that methicillin-resistant *Staphylococcus sciuri* isolated from the dairy industry does not have the potential to produce AHLs but responds to AHL (C6-HSL) molecules by increased exopolysaccharide synthesis and biofilm-forming potential. *L. monocytogenes* strain BN3 also does not have potential to produce AHL molecules, but in our previous study we have observed that *L. monocytogenes* strain BN3 can respond to AHL molecule (C6-HSL) by enhanced biofilm formation (**Naik *et al*., 2017**). There was a gradual increase in biofilm-forming potential in *L. monocytogenes* strain BN3 with the gradual increase in AHL in concentration-dependent manner. In our recent study we have reported that *L. monocytogenes* strain BN3 responds to AHL (C6-HSL) molecules by enhanced hemolysin production in a concentration-dependent manner as revealed by sheep blood agar well diffusion assay (**Naik *et al*., 2018**). However, this study was performed using a plate assay method, which necessitated further confirmation through gene expression studies. Therefore, in the present investigation, we were interested in testing the expression of virulence genes (*hlyA* and *srtA*) and gene which has a major role in biofilm formation (*srtA*) in *L. monocytogenes* in response to AHL molecule (C4-HSL) by Real-Time PCR (RT-PCR) based gene expression study. For this study we have used *L. monocytogenes* strain BN3, which was previously isolated from the utensils of the dairy industry (**Naik *et al*., 2017**).

Here, we tried to investigate the expression of *hlyA* gene and *srtA* gene which encode for listeriolysin O (hemolysin) and sortase enzyme respectively in *L. monocytogenes* strain BN3 in response to 4 carbon AHL molecules (C4-HSL) (Sigma Aldrich, USA) in Brain heart infusion broth (BHI, 10 ml). We grew *L. monocytogenes* strain BN3 overnight (18 hrs) in BHI broth in the absence (control), and presence of 500 nM C4-HSL (test) and then total RNA was extracted from control and AHL exposed cells. Total RNA extraction was done by isolating RNA using TRIzol reagent (TRIzol-Sigma, USA). The total RNA was treated with DNase to avoid contamination from the genomic DNA, and the total RNA was quantified using NanoDrop 2000 spectrophotometer (Thermo scientific). For the synthesis of cDNA, 500 ng total RNA was used. RNA and Hexa primer (random hexamer), were used for the first-strand cDNA synthesis by Reverse transcription using Verso cDNA synthesis kit (Thermo scientific). Conditions for converting RNA to cDNA are 42 ºC for 30 min (**Varma *et al*.,**

2016). cDNA was quantified using nanodrop spectrophotometry and used as a template to study Real Time-PCR (RT-PCR) based expression of the *hlyA* gene and *srtA* gene. For gene expression study, novel primers were synthesized from the NCBI gene sequences data (primer blast) (Eurofins genomics Pvt ltd). In order to study *hlyA* gene expression using RT-PCR, primes used were forward primer 5'-CTTCGGCGCAATCAGTGAAG-3' and reverse primer 5'-GCGCTTGCAACTGCTCTTTA-3' (Eurofins genomics Pvt ltd). In quantitative PCR (qPCR) amplification was carried out with total volume 20 µl and containing 2 µl of cDNA and 10 µl of super mix containing SYBR Green (Bio-Rad, USA) using Bio-Rad CFX96 system. qPCR amplification conditions were initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 30 sec, annealing at 55.1 ºC for 30 sec and extension at 72 °C for 30 sec. For *srtA* gene expression using RT-PCR primers used were Forward primer 5'-GGCGACAAGATTTACCTGACTG-3' and reverse primer 5'-TAGCTCACCAACTGCGACAA-3' (Eurofins genomics Pvt ltd). qPCR conditions were initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 30 sec, annealing at 53.2 ºC for 30 sec and extension at 72 °C for 30 sec. 16s rRNA gene was used as an internal control (Housekeeping gene) in gene expression studies for the normalization. All the experiments were done in triplicate. Also, we have done PCR amplification of *hlyA* gene and *srtA* gene directly from genomic DNA using above primers.

PCR amplification of *hlyA* gene and *srtA* gene directly using genomic DNA as template confirmed presence of *hlyA* (140 bps amplicon) gene and *srtA* gene (180 bps amplicon) in *L. monocytogenes* strain BN3 and specificity of novel primers synthesized in the present study.

The mRNA expression levels were normalized to that of the housekeeping gene (16s rRNA) and the results were analyzed. The Ct values of the test samples were calculated and the data were expressed in terms of fold change over the BN3-control sample. As seen in figure 1, the results revealed that *hlyA* gene expression in *L. monocytogenes* strain BN3 exposed to 500 nM AHL (C4-HSL treated) increased 0.7-fold as compared to control.

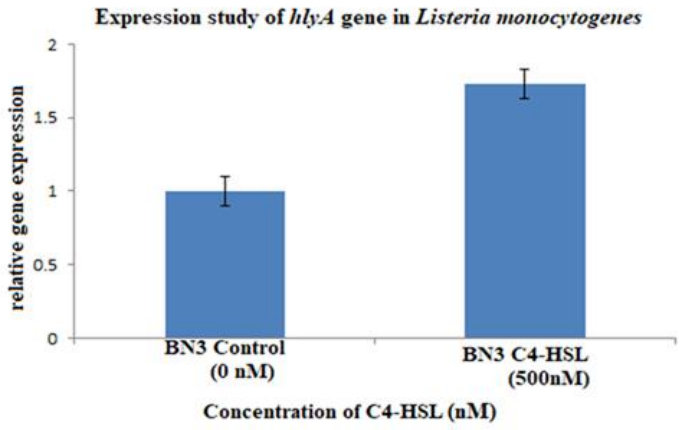

Figure 1 Expression of *hlyA* gene in *L. monocytogenes* strain BN3 in the absence (control) and presence of 500 nM C4-HSL

Further, the case of the *srtA* gene test sample (BN3 exposed to 500 nM C4-HSL) showed 27-fold up-regulation of *srtA* gene as compared to BN3-control sample (Figure 2).

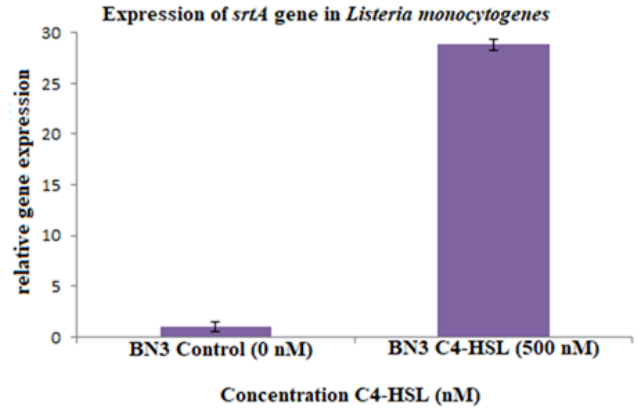

Figure 2 Expression of *srtA* gene in *L. monocytogenes* strain BN3 in the absence (control) and presence of 500 nM C4-HSL

This is the first report on the response of *L. monocytogenes* strain BN3 to 4 carbon AHL (C4-HSL) by enhanced expression of *hlyA* and *srtA* genes which encode for virulence and biofilm formation. It is well known that biofilm formed on utensils used in the dairy industry is a complex biofilm and contains both Gram-negative and Gram-positive bacteria (**Cherif-Antar *et al*., 2016**). In the present investigation *L. monocytogenes* strain BN3 responds to C4-HSL, which is normally produced by Gram-negative bacteria. Therefore, the present study confirmed the cross-talk between Gram-positive and Gram-negative bacteria through AHL (C4-HSL) QS molecule which is not produced by Gram-positive bacteria but produced by Gram-negative bacteria. Researchers across globe target only *agr* QS system to control biofilm formation by *L. monocytogenes* on dairy industry equipment (**Naik *et al*., 2017; Gray *et al*., 2013**), but present study suggest targeting both *agr* based QS and AHL based QS system together in *L. monocytogenes* for best results. This investigation will surely help to understand the interaction between Gram-negative and Gram-positive bacteria when present in the same ecological niche. There is a lot of scope to study receptors for AHL molecules in *L. monocytogenes* for further understanding the communication between Gram-positive and Gram-negative bacteria.

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Author's Contribution All the authors have contributed equally to the work mentioned in this article.

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