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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https://doi.org/10.15414/jmbfs.1846

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). This bacterium can grow in psychrophilic conditions on milk and meat kept in the refrigerator (Doijad et al., 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; Janshiul 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. HlyA is the virulence gene which is produced by 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 (StaA) of L. monocytogenes encoded by the staA gene is responsible for the processing of internalin and inulin in L. monocytogenes. (Doijad 2014) reported the construction of sortase A deletion mutant (LMΔstA) of wild type L. monocytogenes EGDe (LMWT) and found that adherence ability of mutant LMΔstA 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 (StaA) of L. monocytogenes is 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 (O AHL) (Biswa and Doble, 2013). Quzi 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 AHBs but responds to AHB (C6-HSL) molecules by increased exopolysaccharide synthesis and biofilm-forming potential. L. monocytogenes strain BN3 also does not have the potential to produce AHB molecules, but in our previous study we have observed that L. monocytogenes strain BN3 can respond to AHB 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 AHB (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 staA) and gene which has a major role in biofilm formation (staA) in L. monocytogenes in response to AHB 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 staA 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 °C 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, primers used were forward primer 5'-CTTCGGCGGAACTGTAAGC-3' and reverse primer 5'-GCGCTTGCAACGTCTTTTA-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 °C for 30 sec and extension at 72 °C for 30 sec. For srtA gene expression using RT-PCR primers used were Forward primer 5'-GGCGACAGATTACCTGACTG-3' and reverse primer 5'-TGGCTCACCAACTGCGACAA-3' (Eurofins genomics Pvt ltd). qPCR amplification conditions were initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 30 sec, annealing at 53.2 °C 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 bp ampiclon) gene and srtA gene (180 bp ampiclon) 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](image1.png)

**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](image2.png)

**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 agh QS system to control biofilm formation by L. monocytogenes on dairy industry equipment (Naik et al., 2017; Gray et al., 2013), but present study successfully targeting both agh 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.

**Acknowledgment:** Dr. Milind Mohan Naik is thankful to the Science and Engineering Research Board (SERB), Department of Science and Technology, Government of India, for financial support by the Young Scientist Project (File Number: YSS/2014/000258). We are also thankful to Eurofins Genomics Pvt Ltd., Bangalore for their help in real-time PCR study.

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

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