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SHORT COMMUNICATION

The First report on Acyl homoserine lactone (AHL) enhanced expression of virulence factor, hemolysin in Gram positive, *Listeria monocytogenes* strain BN3 isolated from dairy industry

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Abstract: Gram-positive, *Listeria monocytogenes (Lm)* are well-known bacterial pathogens causing listeriosis in humans. *Lm* is known to control its gene expression encoding virulence factors via *agr* peptide based quorum sensing (QS). In our previous study we reported that *Lm* strain BN3 isolated from milk collecting utensils in dairy industry, do not produce Acyl homoserine lactone (AHL) molecule, which is typical autoinducers in Gram-negative bacteria. But *Lm* strain BN3 was found to respond to AHL molecules by enhanced biofilm formation in concentration dependent manner. In the present study, response of *Listeria monocytogenes* strain BN3 to *N*-hexanoyl-homoserine lactone (C6-HSL) by production of virulence factor, hemolysin, was investigated using sheep blood agar well diffusion assay. Increased hemolysin production was observed in response to increasing concentration of AHL molecules (100 nM, 250 nM and 500 nM) as seen by blood hemolysis on blood agar plate. This is the first report on expression of virulence factor (hemolysin production) by *Lm* in response to AHL molecules which are usually produced by Gram-negative bacteria. This study will help to further investigate cross-talk between Gram-positive and Gram-negative bacteria, which share common econiche in the dairy industry.

Keywords: *Listeria monocytogenes,* · Quorum sensing, · AHL· Cross talk,· Hemolysis

Strongest and most imperative gesture ever evolved in every living creature is communication. Microorganisms can communicate with the members in its population and also with the population of other species through a population density dependent quorum sensing (QS) mechanism. Using QS bacteria can modulate the expression of specific genes in a population density-dependent manner (Waters and Bassler, 2005; Ng and Bassler, 2009). Bacterial cells communicate with each other by releasing, sensing and responding to small diffusible signal molecules called autoinducers (AI) that increase in concentration as a function of cell density (Waters and Bassler, 2005; Naik et al. 2017). QS in Gram-negative bacteria *Vibrio fischeri* described in the bioluminescent marine symbiont (Squid), is mediated by the autoinducer 3-oxo-C6-HSL (AHL) (March and Bentley, 2004; Waters and Bassler, 2005) whereas Gram-positive bacteria have *agr* QS system which uses small post-translationally processed peptide as a signal molecule (Naik et al. 2017). Gram-positive bacteria usually do not produce AHL molecules except for one reported marine bacteria *Exiguobacterium* sp. AT1b which produces C3-oxo-octanoyl homoserine lactone (Biswa and Doble, 2013).

Gram-positive, *Listeria monocytogenes (Lm)* are well-known bacterial pathogens causing listeriosis both in humans and animals (Doijad et al. 2011; Rantsiou et al. 2012; Tirumalai and Prakash, 2012; Naik et al. 2017). *Lm* is known to synthesize several virulence factors such as invasion-associated proteins, internalin A, listeriolysin O (LLO), biofilm formation, Phosphatidyl-inositol Phospholipase (Rieu et al. 2007; Riedel et al. 2009; Naik et al. 2017), and various transcription factors (PrfA, spxA1, and spxA2) (Milenbachs et al. 2014; Rantsiou et al. 2012; Whiteley et al. 2017). Virulence factors encoding genes are under the control of QS mechanism involving *agr*-peptide-sensing system as a complex regulatory network for coordinating gene expression (Garmyn et al. 2009; Saleh, 2011; Garmyn et al. 2011). The virulence potential of an *agrD* deletion mutant was investigated by Riedel et al. (2009), which confirms the role of the *agr* quorum sensing system in the virulence of *L. monocytogenes*.

3-oxo-C12-HSL was reported to inhibit *agr* QS expression in *S. aureus* (Qazi et al. 2006). In our recent study (Naik et al. 2017), it was reported that *Lm* strain BN3 responds to *N*-hexanoyl-homoserine lactone molecule (C6-HSL) by gradual increase in their biofilm-forming potential with the gradual increase in AHL (C6-HSL) concentration (250 nM, 500 nM and 1 µM). These

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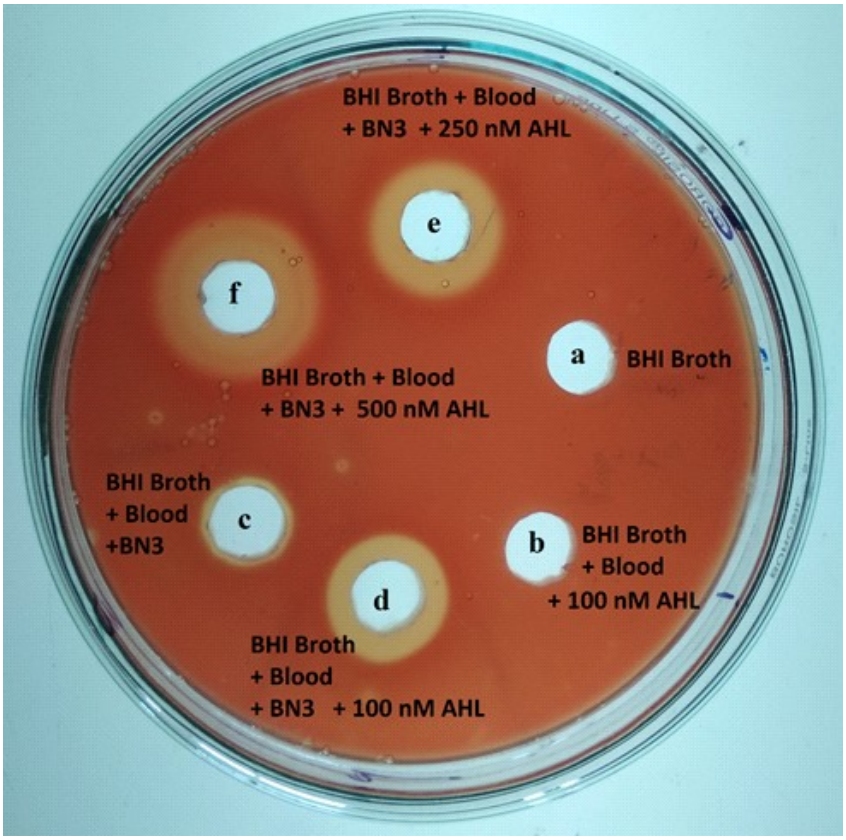

Figure. 1A. Hemolysis on 2% sheep blood agar plate after adding culture filtrate from (a) BHI broth (b) BHI broth + 0.1% blood+ 100 nM AHL (c) BHI broth + 0.1%Blood + *Lm* BN3 (d) BHI broth + 0.1% Blood + 100 nM AHL+ *Lm* BN3 (e) BHI broth + 0.1% Blood + 250 nM AHL+ *Lm* BN3 (f) BHI broth + 0.1% Blood + 500 nM AHL+ *Lm* BN3.

reports provide the evidence of cross talk between Gram-positive and Gram-negative bacteria present in a same environmental niche. This short communication insists on the enhancement of hemolysin production by *Lm* strain BN3 in response to increasing concentration of N-hexanoyl homoserine lactone (C6-HSL) which is one of the typical AI in Gram-negative bacteria. To the best of our knowledge, this is the first report on expression of virulence factor (hemolysin production) by *Lm* in response to AHL (C6 HSL) molecules using sheep blood agar well diffusion assay. *Lm* strain BN3 used in the present study was isolated from milk utensils used by the milk collection centre/society at Usgao, Goa and identified using 16S rRNA gene sequencing with GenBank accession number MF095110 (Naik et al. 2017).

In order to check the response of *Lm* strain BN3 to AHL (C6-HSL) molecules, we exposed *Lm* strain BN3 to varying concentrations of AHL (100 nM, 250 nM and 500 nM) in 50 ml BHI broth (Brain heart infusion broth) containing 0.1 % sheep blood. In this experiment three controls were also maintained: (1) Only BHI broth without *Lm* strain BN3 inoculation (media control); (2) BHI broth with 0.1% sheep blood + 100 nM AHL without BN3 inoculation (AHL control) and (3) BHI broth containing 0.1% sheep blood and inoculated with *Lm* strain BN3 culture (positive control). All the six flasks were incubated at 37 ºC with constant shaking at 150 rpm for 48 h. After 48 h content from flasks was centrifuged at 10000 rpm under sterile condition and the supernatant was filter sterilized using 0.2 µm nitrocellulose filter. Resultant filtrate was then tested for hemolysin production on 2% sheep blood agar plates by well diffusion assay. Sterile 2% sheep blood agar plates were prepared using BHI agar. On solidification of agar in the plates, six wells (6 mm diameter each) were bored in the agar using cork borer under sterile condition. Above obtained filtrate (50 µL) form 6 flasks were added to each well respectively. The filtrate was allowed to diffuse in the sheep blood agar and the plate were incubated at 37°C for 6 h. Zone of clearance due to hemolysis surrounding the well was measured, which was correlated with the hemolysin production in the culture supernatant. All the experiments were done in triplicate and the standard deviation was calculated.

Lm is known to produce hemolysin as one among several other virulence factors (Tirumalai and Prakash, 2012). Weak Hemolysis zones were observed on 2% sheep blood agar plates with the filtrate obtained from the culture supernatant of the *Lm* strain BN3 grown in BHI broth having 0.1% sheep blood since weak β-hemolysis is characteristic of *Lm* (Fig. 1A). The hemolysis zones were not seen on sheep agar plate using filtrate of (a) Plain BHI broth and (b) BHI broth + 0.1% blood+ 100 nM AHL without inoculum, thus proving that plain BHI and AHL alone are not responsible for blood hemolysis. The hemolysis zones were seen surrounding the wells where the filtrate obtained from *Lm* strain

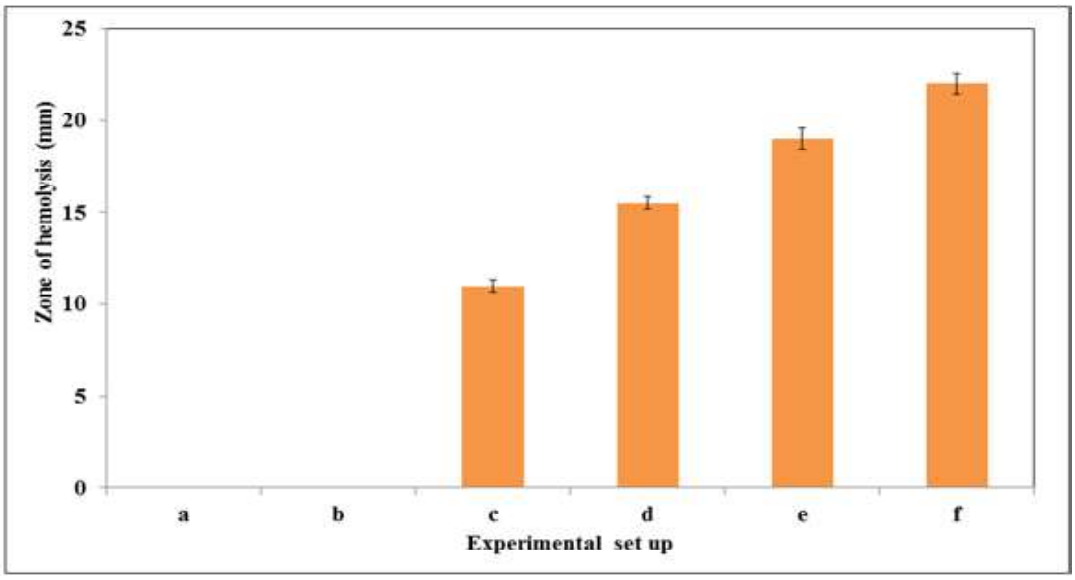

Figure. 1B Graph of Hemolysis observed on 2% sheep blood agar plate after adding culture filtrate from (a) BHI broth (b) BHI broth + 0.1% blood+ 100 nM AHL (c) BHI broth + 0.1%Blood + *Lm* BN3 (d) BHI broth + 0.1% Blood + 100 nM AHL+ *Lm* BN3 (e) BHI broth + 0.1% Blood + 250 nM AHL+ *Lm* BN3 (f) BHI broth + 0.1% Blood + 500 nM AHL+ *Lm* BN3.

BN3 culture grown in presence 100 nM, 250 nM and 500 nM, C6-HSL molecules. It can be seen in figure 1A that, hemolysis zone size increased with increasing concentration of C6-HSL(100 nM, 250 nM and 500 nM). This revealed that, under the presence of AHL molecule, *Lm* strain BN3 not only secreted more protein hemolysin, but the concentration of hemolysin increased with increasing concentration of AHL. As compared to positive control (i.e. BN3 without C6-HSL), hemolysin production by *Lm* strain BN3 was increased to 31.4%, 62.8% and 88.5% in presence of C6-HSL, in a concentration dependent manner, i.e. in presence of 100 nM, 250 nM and 500 nM C6-HSL molecule respectively (Fig. 1B).

The discovery of the QS system in bacteria and its role in cell behaviour has made it a target for microbiologists to understand bacterial behaviour and adaptation (Saleh, 2011). *Lm* strain BN3 was isolated from milk collecting utensils in dairy industry (from complex biofilm by taking swabs) where both Gram-positive and Gram-negative bacteria share common econiche. Gram-negative and Gram-positive bacteria interact with each other when present in the same ecological niche, this is evident by a response of *Lm* to AHL by increasing biofilm formation in concentration dependent manner (Naik et al. 2017). In the present study *Lm* strain BN3 showed enhance production of virulence factor (hemolysin) in response to C6-HSL. As explained by Decho et al. 2010, when a signal molecule travel through extracellular environments, there could be geochemical and biological modifications in the signals which could disrupt intended communication through signals due to non-recognition. However, if such a modified signal is able to interact with a different receptor then further environmental information can be gained by the receiving cells. Similar geochemical and biological modifications may have happened to AHL released by Gram-negative bacteria when these modified signals interact with different receptors present on *Lm*, then the *Lm* BN3 responded by forming enhanced biofilm or expression of virulent genes like hemolysin may be as immune mechanism towards Gram-negative bacteria since both share common econiche (biofilm on milk utensil). This hypothesis is supported by a report where Gram-negative bacteria produce *N*-(3-oxododecanoyl) homoserine lactone which is bactericidal agent on Gram-positive bacteria (Kaufmann et al. 2005), therefore Gram-positive bacteria must have evolved in response to AHL as immune mechanism towards Gram-negative bacteria.

Conclusions

The present study confirms the cross talk between Gram-positive and Gram-negative bacteria through AHL molecule produced by Gram-negative bacteria in Dairy industry i.e. expressing virulence factor (hemolysin).

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