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Quorum sensing in *Enterococcus faecium*, *Enterococcus faecalis* and *Bacillus cereus* strains isolated from ricotta processing

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ABSTRACT: *The quorum sensing phenomenon is a process of intra- and inter-species microbial communication involving the production and detection of extracellular signaling molecules. The autoinducer AI-2 has been proposed to serve as a 'universal signal' for interspecies communication. This study aimed to evaluate the capability of Enterococcus faecium, Enterococcus faecalis, and* Bacillus cereus *strains isolated from ricotta processing to produce quorum sensing signalling molecules (AI-2). The strains were evaluated for the presence of the luxS gene using the polymerase chain reaction. AI-2 quorum sensing signalling molecules were measured in relative light units (RLUs) using a luminometer. A total of 74% of E. faecium, 91% of E. faecalis, and 95% of B. cereus isolates were positive for* lux*S gene. In addition, the induced bioluminescence in Vibrio harveyi BB170 was observed in all strains, indicating the presence of the AI-2 autoinducer.*
Key words: *luxS*, *AI-2*, *Vibrio harveyi*, *bioluminescence*, *Enterococcus spp.*, *Bacillus cereus.*

Quorum sensing em cepas de *Enterococcus faecium*, *Enterococcus faecalis* e *Bacillus cereus* isoladas do processamento de ricota

RESUMO: *O fenômeno quorum sensing corresponde a um processo de comunicação intra e interespécies microbianas e é mediado por sinais químicos extracelulares, denominados moléculas sinalizadoras ou auto indutoras (AI). A molécula AI2 está envolvida na comunicação interespécies, denominada sistema "universal" de comunicação. Este estudo teve como objetivo avaliar a capacidade de Enterococcus faecium, Enterococcus faecalis e Bacillus cereus isolados do processamento de ricota em produzir moléculas sinalizadoras de Quorum sensing (AI-2). Os isolados foram avaliados quanto à presença do gene luxS utilizando a reação em cadeia da polimerase (PCR). As moléculas sinalizadoras (AI-2) foram medidas em unidades relativas de luz (RLU) através de um luminômetro. Um total de 74% dos isolados de E. faecium, 91% de E. faecalis e 95% de B. cereus foram positivos para o gene* lux*S. Além disso, todos os isolados apresentaram capacidade de induzir o fenômeno de bioluminescência em Vibrio harveyi BB170, indicando a presença de auto indutores AI-2.*
Palavras-chave: *luxS*, *AI-2*, *Vibrio harveyi*, *bioluminescência*, *Enterococcus spp.*, *Bacillus cereus.*

Quorum sensing phenomenon is a process of intra- and inter-species microbial communication and it is measured by extracellular chemical signals known as signal molecules or autoinducers (AI) (READING & SPERANDIO, 2006). These molecules are produced by bacteria during their growth and are released into the environment. The extracellular accumulation of these signals indicates the presence of a relatively dense population, leading the bacteria to initiate a coordinated behavior (GRIFFITHS, 2005). This mechanism allows cells to control many of their functions, including virulence gene expression, plasmid transfer, toxin production, exopolysaccharide production and sporulation, and biofilm formation. Many bacteria, including various clinically important pathogens can use a molecule denominated as the autoinducer AI-2 for intercellular communication (BASSLER, 2002).

This study aimed to evaluate the capability of *E. faecium, E. faecalis*, and *B. cereus* strains isolated from ricotta processing to produce quorum sensing signalling molecules (AI-2). In total, 53 *E. faecium* isolates (43 from ricotta and 10 from environmental surfaces), 46 *E. faecalis* isolates (22 from ricotta, 12 from raw milk, 1 from cheese whey, and 11 from environmental surfaces) (FERNANDES et al., 2015a), and 38 *B. cereus* isolates (1 from raw milk, 2 from pasteurized milk, 2 from cheese whey, 18 from ricotta, and 15 from environmental surfaces) (FERNANDES et al., 2014) were evaluated in regards

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to the *luxS* gene. All strains were characterized in our previous studies as pathogenic, because they have virulence genes and are resistant to different antimicrobials. For more details, see FERNANDES et al. (2014) and FERNANDES et al. (2015a).

Table 1 shows the primers (Invitrogen, Life Technologies, Carlsbad, USA) used for amplification of the *luxS* gene. Primers were designed for *luxS* gene identification based on the sequences of *E. faecalis* ATCC 29212, *E. faecium* ATCC 51299 and *B. cereus* ATCC 14579, which are deposited in GenBank at the National Center for Biotechnology Information (NCBI) in Fasta format (GenBank access number 004722). Genomic DNA from pure cultures was extracted according to the methods described by FURRER et al. (1991). The PCR mixture (25 µl final volume) contained 0.2µl of Taq polymerase (Invitrogen, Life Technologies, Carlsbad, USA) (1 U/µl), 2.5µl of 10X buffer (Invitrogen, Life Technologies, Carlsbad, USA) (200mM Tris HCl pH 8.0, 500mM KCl), 1.5µl of 50mM $MgCl_2$ (Invitrogen, Life Technologies, Carlsbad, USA), 0.5µl of 10mM dNTPs (Invitrogen, Life Technologies, Carlsbad, USA), 1µl (12.5mM) of each primer, 1µl of extracted DNA and sterile Milli-Q water. Thermocycler parameters were as follows: an initial cycle of 94°C for 3min, 35 cycles of 94°C for 30 sec, 60°C for 1min and 72°C for 1.5min, a final extension step of 72°C for 7min and then holding at 4°C. Electrophoresis of the PCR products was performed on 1.5% agarose gels (Invitrogen, Life Technologies, EUA), which were stained with ethidium bromide. Visualization of the PCR products was performed using a UV transilluminator. The *E. faecalis* ATCC 29212, *E. faecalis* ATCC 51299, and *B. cereus* ATCC 14579 were utilized as positive control. As negative control, sterile Milli-Q water was used.

Biofilms are composed of aggregated cells and, consequently, they become a suitable environment for the quorum sensing phenomenon (BASSLER, 2002). Therefore, we selected 4 *E. faecium* strains, 4 *E. faecalis* strains, and 4 *B. cereus* strains from the previously mentioned strains for detection of the AI-2 signalling molecules. These strains are known to be able of forming biofilm on stainless steel surfaces in our previous studies (FERNANDES et al., 2014; FERNANDES et al., 2015b).

All assays were performed in triplicate, according to TAGA & XAVIER (2011). The strains were activated in Luria-Bertani (LB) broth (Difco, Becton, Dickinson and Company) supplemented with 0.5% glucose with continuous stirring at 175 rpm and 30°C for 24h. Cultures were then diluted 1:100 in LB broth (supplemented with 0.5% glucose) and incubated again for 6 hours (to mid-exponential phase) under the same conditions. Aliquots (1mL) of bacterial culture were transferred to Eppendorf® tubes and centrifuged (Eppendorf® Centrifuge 5415D, Hamburg, Germany) at 15000 x g for 5min. The supernatants were filtered through a 0.22-µm membrane (Millex® GV, Merck Millipore Ltd., Co. Cork, Ireland), and the filtered samples were immediately subjected to the assay. The *V. harveyi* BB170 biosensor was activated in Autoinducer Bioassay (AB) medium (SURETTE & BASSLER, 1998) and incubated at 30°C for 16h with continuous stirring at 175 rpm. The culture was then diluted 1:5000 in AB medium. The strains *Salmonella* Typhimurium ATCC 14028 and *Escherichia coli* O157:H7 were used as positive controls. As negative controls, sterile AB and LB culture media were used (TAGA & XAVIER, 2011). For detection of AI-2, 10µl of filtered culture supernatant and 90µl of the *V. harveyi* BB170 dilution were combined in microwell plates (Greiner Bio One GmbH, Frickenhausen, Germany). Plates were incubated at 30°C with continuous stirring at 300 rpm, and bioluminescent activity was measured in relative light units (RLU) using a luminometer (FLUOstar Omega Microplate Reader, BMG Labtech, Offenburg, Germany) at

Table 1 - Primer sequences used to detect the *luxS* gene.

Microorganism	Sequence of oligonucleotides (5' to 3')	Product size (pb)	Reference
E. faecalis	CACCATATGTTCGCCTTGCT ATAAAAACCAGTGCGGCAAC	203	Present study*
E. faecium	GAGCACTTGACTGCCGAACT GCCACATTGTGTTTCATTGC	198	Present study*
B. cereus	CCCTTTCACAGGCAGTTTTC GATCATACGATTGTAAAGGCACC	450	Present study*

*GenBank access number: 004722.

15min intervals for at least 5 hours. All assays were performed in triplicate, and the results correspond to the induction of bioluminescence as determined by the following equation.

Eq. 1:

Induction of bioluminescence = RLU of the sample/ RLU of negative control.

Possible cell-to-cell communication between the *E. faecium, E. faecalis,* and *B. cereus* was evidenced by the presence of the *luxS* gene and by a positive bioluminescence reaction. Of the 53 *E. faecium* isolates, 74% (39) were positive for the *luxS* gene. Among them, 68% (29/43) of the ricotta isolates were positive for the *luxS* gene, while 100% (10/10) of the isolates from the environment were positive for this gene. For *E. faecalis*, 91% (42/46) of the isolates were positive for the *luxS* gene. All raw milk (12/12) and cheese whey (1/1) samples contained the *luxS* gene, and 90% (10/11) of samples from the environment and 86% (19/22) of ricotta samples were positive for this gene. Additionally, 95% (36/38) of the *B. cereus* isolates were positive for the *luxS*, while all isolates from ricotta (18/18) and raw (1/1) and pasteurised milk (2/2) were positive for this gene. Furthermore, 94% (14/15) of isolates from the environment and 50% (1/2) of isolates from cheese whey were also positive for the *luxS* gene. Therefore, this study proves the existence of the *luxS* gene in *E. faecium*, *E. faecalis* and *B. cereus* strains isolated from various locations, ranging from the raw material to the final product. The majority of *B. cereus*, *E. faecalis,* and *E. faecium* strains presented the *luxS*, demonstrating the possibility of producing the Al-2 autoinducer (XAVIER & BASSLER, 2003). The autoinducer AI-2 is synthesized by the enzyme *luxS*, which is involved in the metabolism of S-adenosylmethionine (SAM), converting it into ribosylhomocysteine S-homocysteine (SRH) and 4,5-dihydroxy-2,3-pentanedione (DPD). The DPD is a very unstable compound that reacts with water and cyclizes to form several furanones, one of which is thought to be the precursor of AI-2 (SCHAUDER et al., 2001). Results indicating the presence of the Al-2 quorum sensing signaling molecules by detecting the presence of the *luxS* gene were corroborated by confirmation of the capacity to induce the phenomenon of bioluminescence in *V. harveyi* BB170. Normalized relative light unit (RLU) levels ranging from 0.89 to 1.59, were obtained. According to figure 1, *E.*

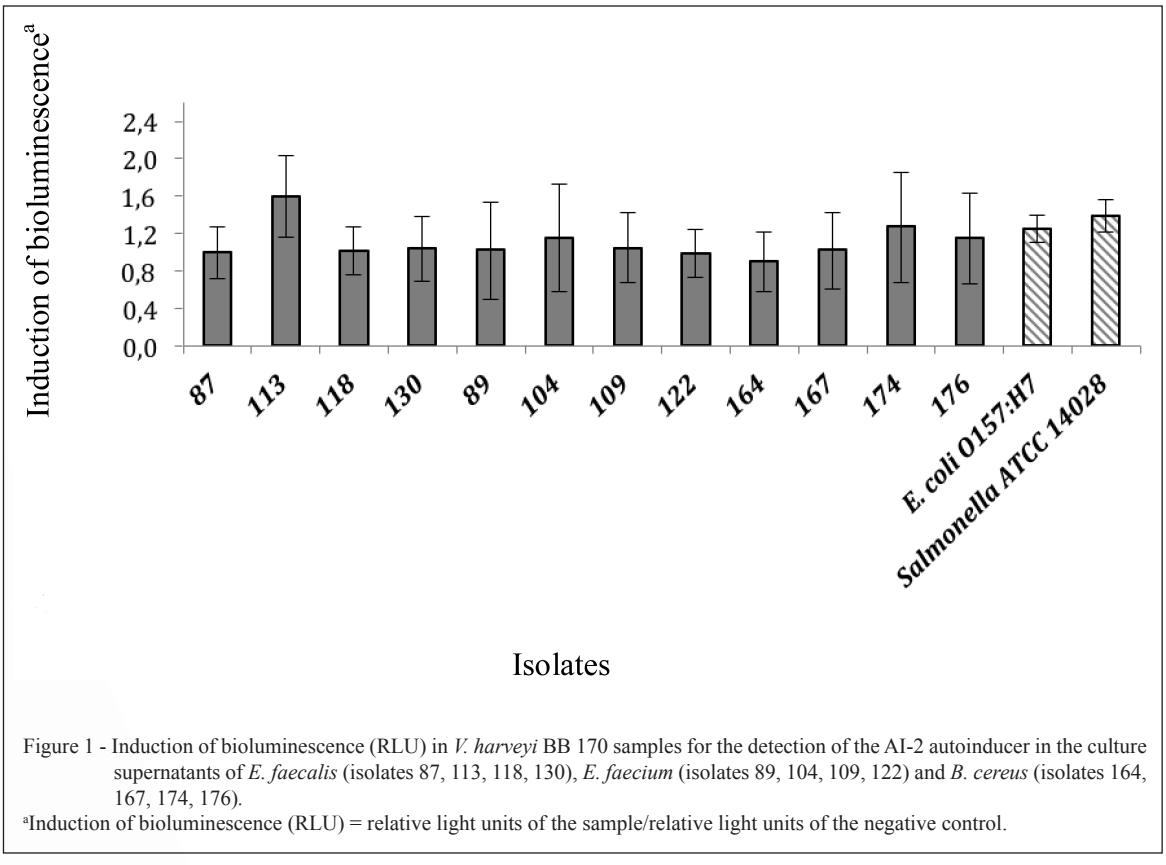

Figure 1 - Induction of bioluminescence (RLU) in *V. harveyi* BB 170 samples for the detection of the AI-2 autoinducer in the culture supernatants of *E. faecalis* (isolates 87, 113, 118, 130), *E. faecium* (isolates 89, 104, 109, 122) and *B. cereus* (isolates 164, 167, 174, 176).

[a]Induction of bioluminescence (RLU) = relative light units of the sample/relative light units of the negative control.

faecalis isolate 113 and *B. cereus* isolate 174 stood out for showing the highest induction activity as compared to the other samples tested. These results are in accordance with TAGA & XAVIER (2011), whose reported a bioluminescence induction index of 1.8 after a 6-h culture of *S.* Typhimurium. When calculating AI-2 activity, the possible presences of AI-2 exogenous molecules (produced by the test strains) and of AI-2 endogenous molecules (produced by *V. harveyi*) have been taken into consideration. Endogenous AI-2 was not a target of this study but could cause an error in interpretation, since after a few hours, an accumulation of endogenous AI-2 can occur due to the increase in the population of *V. harveyi* BB 170, inducing bioluminescence once again. Thus, when calculating AI-2 activity, the light was measured the instant immediately prior to the start of light production by *V. harveyi* (TAGA & XAVIER, 2011). In this study, the 5-h time point was selected to monitor the effects of exogenous AI-2 (SHAO et al., 2012). Confirmation of the presence of the *luxS* gene associated with a positive response for the phenomenon of bioluminescence with *V. harveyi* BB170 for most isolates tested in this study, corroborates with the hypothesis of the presence of the AI-2 autoinducer and the existence of the phenomenon of quorum sensing. This phenomenon may favor the adaptation and survival of microorganisms in both food and biofilms. The *E. faecium*, *E. faecalis* and *B. cereus* strains analyzed in this study are able of forming biofilm. According to SHAO et al. (2012) and AUGER et al. (2006), AI-2 has an important role in biofilm formation by *E. faecalis* and *B. cereus*, respectively. AUGER et al. (2006) showed that an increase in the level of AI-2 in the medium resulted in a decrease in the density of the biofilm formed by *B. cereus*. This result indicated that the presence of AI-2 can also cause the detachment of a large proportion of the cells in the biofilm. Cells detachment or parts of biofilms may lead to the contamination of the food or to the colonization of other regions, resulting in new biofilms (SIMÕES et al., 2010). These facts indicated that AI-2 may be related to the regulation of important phenotypes in food contamination. Further research should be conducted to promote understanding of the way bacteria communicate. The knowledge of the mechanisms of survival and interaction of microorganisms in a biofilm and its relation with the quorum sensing system can help in the development of measures of control and elimination of these biofilms in the food processing environment, providing food safety.

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