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Modulation of quorum sensing genes expression in *Pseudomonas aeruginosa* clinical strains by supernatants of *Bifidobacterium sp.* probiotic strain

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ABSTRACT

Although *Pseudomonas aeruginosa* is not generally considered as an important cause of diarrhea, several cases produced by *P. aeruginosa* have been reported. We experienced tow cases of nosocomial diarrhea presumably caused by *P. aeruginosa*, which was the predominant organism isolated from the respective stool cultures. Cell-to-cell communication system, known as quorum sensing (QS), allows a coordinated expression of tissue-damaging factors in *P. aeruginosa* strains, populating the enteric mucosal tissue. There are studies regarding the use of probiotics in enteric and diarrheal diseases targeting, many probiotics being effective for preventing and treating acute gastroenteritis and persistent diarrhea. The purpose of this study was to investigate the QS genes expression level of *P. aeruginosa* strains recently isolated from digestive tract infections, under the influence of potential probiotic *Bifidobacterium sp.* strains isolated from infants faeces, using the qRT-PCR method.

Keywords: *P. aeruginosa, qRT-PCR method, quorum sensing, supernatants, Bifidobacterium sp.*

1. INTRODUCTION

The pathogenesis of intra-abdominal infections often begins with altered intestinal microbiota [1]. Digestive resident microbiota is very complex and includes aerobic and anaerobic bacteria, fungi and protozoa, being normally present only at the extremities of the digestive tract, especially in the terminal area [2].

The microbial colonization of the infant gut begins immediately after birth and is essential for the development of the intestine and immune system and later well-being [3]. The mechanisms by which probiotic bacteria restore the homeostasis of the intestine after a prolonged treatment with antibiotics or a immunological imbalance consist in optimizing the functionality of the intestinal barrier, the lower production of pro-inflammatory cytokines and prevention of epithelial cell apoptosis induced by cytokines [4]. It has been shown that probiotic bacteria synthesize signaling molecules, there target being not only the microbial populations, but also the intestinal epithelial cells with immune function (lymphocytes, dendritic cells) [5].

Pseudomonas aeruginosa as a cause of infectious diarrhea is rare[6,7]. It usually represents a nosocomial infection in an immunocompromised host. The production of most of the extracellular virulence factors in *P. aeruginosa* is controlled by at least two well-defined, interrelated quorum sensing systems (QS) *las* and *rhl*, which are playing a key role in the pathogenesis of *P. aeruginosa* [8, 9].

The two systems of cellular signaling are involved in controlling theexpressionandcoordinatedproduction of (elastase, alkalineprotease), secondary metabolites (piocianin, cyanides, hydrogen, pioverdin) andtoxin (exotoxin), and the development of biofilms, which cause extensive tissuedamage, invasion ofthebloodstream, andhencesystemicspread.

These QS systemsexert their action by small diffusible signal molecules called *N*-acylhomoserine lactones (AHLs) [10, 11]. Each QS system consists of two components, the autoinducer synthases (LasI and RhlI, respectively) and their cognate transcriptional regulators (LasR and RhlR, respectively). LasI is the synthase for the autoinducer N-(3-oxododecanoyl) homoserine lactone (3OC12-HSL), while RhlI synthesizes the autoinducer N-butyryl homoserine lactone (C4-HSL). Each type of autoinducer (AI) interacts with his cognate transcriptional regulator to induce the expression of target genes. At high cell density, 3OC12-HSL and C4-HSL reach critical levels and activate their regulators, which in turn enhance the transcription of different virulence genes [12].

At high cell density, $3OC_{12}$-HSL and C_4-HSL reach critical levels and activate their regulators, which in turn enhance the transcription of different virulence genes [12, 13, 14]. Therefore, functional quorum sensingrequires transcription of the autoinducer synthase (*lasI, rhlI*)and response regulator (*lasR, rhlR*) and production of autoinducers. The *las* system has been shown to

modulate expression of *lasI*itself, *lasB*(elastase), *lasA*(Staphylolytic protease), *apr*(alkaline protease), the *xcp*secretion pathway, twitching motility and *rhlR.* The *rhl*system modulates expression of *rhlI*itself, *rhlAB*(rhamnolipid biosynthesis), *lasB*(elastase), *apr*(alkaline protease), twitching motility, *rpoS* expression (encoding stationary-phase sigma factor) and production of secondary metabolites (pyocianin and hydrogen cyanide) [15, 16].

These QSs besides regulation of the coordinated production of virulence factors, coordinate different bacterial physiological processes, including: metabolic pathways dependent on cellular density, the ability to form biofilms, and motility [16].

The generally symbiotic relationships can sometimes turn from mutualistic to parasitic, causing opportunistic endogenous infections and being responsible of the generation of carcinogenic compounds [17]. Currently, antibiotic treatment of gastrointestinal infections is designed to cover a broad spectrum and often polymicrobial etiology (ex. penems, third generation cephalosporins), being associated with disbiosis, which could be prevented or diminished by using probiotics [18]. *P. aeruginosa* strains were used further to investigate the effect of a probiotic *Bifidobacterium sp.* strain on the expression of quorum sensing genes.

2. EXPERIMENTAL SECTION

2.1. Microbial strains. In this study there were used 12 strains of *Bifidobacterium sp.* and 2 strains of enteropathogenic *P. aeruginosa* isolated from faeces, from patients with ages between 3 days and 5 years. Most of the *Bifidobacterium sp*. strains were isolated from healthy patients. Isolation was performed on Selective Agar Bifidobacterium for *Bifidobacterium* strains and CLED (cysteine-, lactose-, and electrolyte-deficient) agar for *P. aeruginosa*. For the anaerobic bacteria, the plates were incubated in anaerobic conditions, for 24-48h. Isolated strains were identified by MALDI-TOF automated method (Matrix Assisted Laser Desorption of / ionization time of flight) using an analyzer Microflex [19].

2.2. qRT-PCR for determination of supernatant probiotic influence on *P. aeruginosa* QS genes expression level. We used two strains of *P. aeruginosa* isolated from the faeces and one *P. aeruginosa* PAO1 control strain. The *Bifidobacterium breve* strains were cultivated on nutrient blood agar 5%, for 24 hours, in anaerobically condition. This strain was inoculated in thyoglycollate medium and incubated for 24 hours, at 37^0 C in anaerobic condition. Subsequently, the logarithmic phase cultures were centrifuged at 6000 rpm for 15 minutes, in order to obtain the cell-free supernatants, prospected to contain the highest amounts of QS soluble mediators (autoinducers). The enteropathogenic strains were cultivated on nutrient agar, for 18 h in order to prepare bacterial cells suspensions in PBS (phosphate buffered saline), with standard density 0.5 McFarland (corresponding to 10^7 CFU/mL). The enteropathogenic bacterial strains were cultivated for 18 h in the presence of *Bifidobacterium breve* supernatant (ratio 1:9).

2.3. RNA isolation. The total RNA was extracted from the analyzed strains grown in normal conditions, respectively in the presence of the sterile probiotic supernatant. RNA isolation was performed using Trizol reagent, according with manufacturers' procedure. Briefly, after addition of chloroform and 1-bromo-3-chloropropane, the mixture separates into three phases: top aqueous phase (RNA), interphase (DNA) and organic phase (proteins). The total RNA was precipitated with isopropanol and washed with ethanol. The concentration and purity of RNA samples was determined using NanoDrop spectrophotometer (UV-Vis Spectrophotometer, Thermo Scientific).

2.4. cDNA synthesis. The total RNA was revers-transcribed to DNA using Hight-Capacity cDNA Reverse Transcription Kits (Applied Biosystems) according to the protocol to use. Briefly, 2µg total RNA was combined with revers-transcription reaction mixture containing Revers-transcriptase MultiScribe™ and RT master mix (RT buffer, dNTP Mix (10 mM), random primers) and then introduced DNA Engine Dyad Peltier device Bio Rad Thermal Cycler. The reaction mixtures were incubated for 10 min at 25°C and then at 37°C for 2 h. Revers-transcriptase MultiScribe™ was inactivated by heating the reaction mixtures at 85°C for 5 min.

The cDNAs was conserved at – 20°C until performing the real-time PCR experiments for the quantification of *lasI, lasR, rhlI* and *rhlR* gene sexpression in *P.aeruginosa* cultures grown in the presence of probiotic cultures filtrates (samples) respectively in those grown in normal conditions (controls).

2.5. Real-time qPCR. The relative quantification of QS genes expression by real-time qPCR assays was performed with obtained cDNA, TaqMan probes, primers specific for detection of QS and *rplU* genes previously described [15,16] using Universal PCR Master Mix TaqMan®Fast (Applied Biosystems). The housekeeping gene *rplU* was used as the normalizing gene, being a ribosomal gene which is present in thebacterial cells in reasonably constant levels under normal growth conditions [16]. All real-time PCR reactions were run in triplicate for each analyzed gene on ABI 7300 Real Time PCR System (Applied Biosystems). Controls run without reverse transcriptase were used to check for the absence of contaminating DNA in all samples.

The program of amplificationused in real-time qPCR reactions for all genes was represented by 1 cycle for DNA-polymerase activation at 95 °C, 10 min, followed by 40 cycles, consisted from denaturation 95°C, 15 sec, primer annealing and extension at 60°C, 1 min. The fluorescence data were aquired at the end of the primer extension step of each amplification cycle. The obtained results of relative quantification of target gene (l*asI, lasR, rhlI, rhlR*) versus the reference gene (*rplU*) were interpreted using 7300 System SDS Software.

3. RESULTS SECTION

3.1. The QS genes expression in *P. aeruginosa* strains grown in the presence of probiotic culture filtrate. The results of the real-time qPCR assays showed that all *P. aeruginosa* analyzed strains (samples and controls) possess all quorumsensing tested genes (*lasI, lasR, rhlI* and *rhlR)*. Relative quantification by real-time PCR of *P. aeruginosa* QS gene expression, after growing in the presence of probiotic strains supernatant, compared to control, was performed according to the formula of Pfaffe. The normalized values of expression level of each QS gene are shown in figures 1-3.

The results of the relative quantification of each QS gene expression by real time RT-qPCR showed that, for *P. aeruginosa* strain grown in the presence of the probiotic supernatant, the level of QS genes expression was different than the controls.

In case of *P.aeruginosa* 37 strain, the level of *lasR* and *rhlR* genes expression increased with 0.151 and 0.299 respectively, compared with the level of the same genes in the control, whereas for *P.aeruginosa* 4 strain, the expression level increased with 0.671 for *lasR* gene and with 0,979 for *rhlR* gene respectively, comparatively with controls. The expression level for *lasI* gene was reduced in both *P.aeruginosa* 37 and 4 strains grown in the presence of probiotic with -0.172 and -0.126 respectively, comparing with controls. The expression level for*rhlI* gene was reduced only in *P.aeruginosa* 37 strain grown in the presence of probiotic, with -0.444, while in *P.aeruginosa* 4 strain grown in the presence of the probiotic, the expression level of the same gene was increased with 0.023 comparing with the control.

Finally, for the last analysed, *P.aeruginosa* PAO1 standard strain, grown in the presence of probiotic culture filtrate, the expression level of *lasI, lasR* and *rhlI* genes was reduced with -0.427, - 0.286 and -0.563, respectively, whereas the expression level of *rhlI* gene increased with 1.064 comparatively with control.

Recent studies have shown that *Bifidobacterium breve* strain isolated from faeces presented a potential probiotic effect, revealed by the ability of the entire culture or cell-free supernatant to modulate growth and to inhibit the mobility expression of *P. aeruginosa* strains, when it was used integral bacterial culture of the intestinal pathogens and thus may represent an effective approach to prevent or treat bacterial enteric infections. In most cases, the influence of *B. breve* strain consisted of reducing the logarithmic phase, representing the rate of multiplication, and shortening the total length of the growth curve, with an entry earlier phase of decline and inhibiting the ability of joining the cell substrate [21].

The results of the relative quantification of each QS gene expression by real time RTqPCR showed that for all *P.aeruginosa* strains grown in the presence of probiotic culture filtrate, the expression level of *lasI* gene was reduced, comparatively with controls, whereas the expression level of *rhlI* gene decreased only for two strains and for *lasR* gene only for one strain.

These results showed that in the probiotic culture filtrate is accumulating soluble molecules with inhibitory activity on the QS genes expression. Other studies have shown that plurifactorial anti-infective action of probiotics is also due to the modulation of virulence factors and antibiotic susceptibility expression in different enteropathognic strains [22]. Also, the organic acids secreted by some probiotics could act as quorum sensing inhibitors [16].

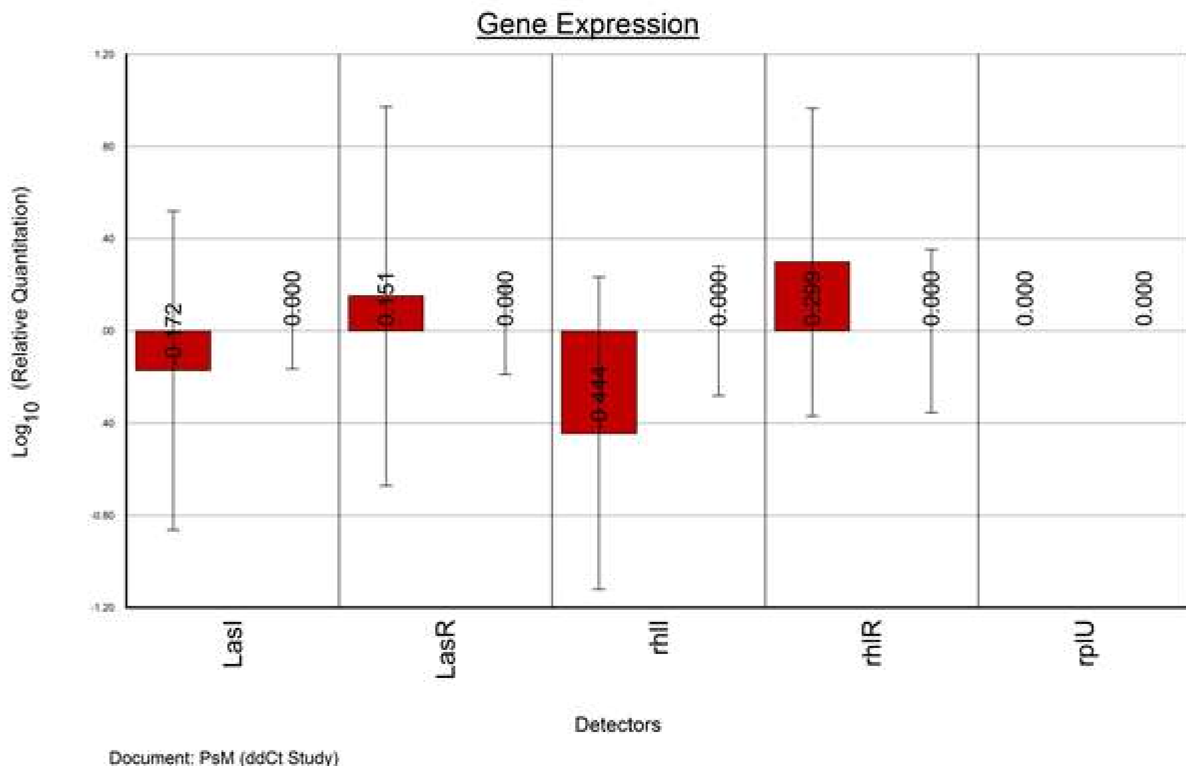

Figure 1. The expression level of QS genes in *P.aeruginosa 37* strain grown in the presence of probiotic filtrate comparatively with control (the same strain grown in normal condition).

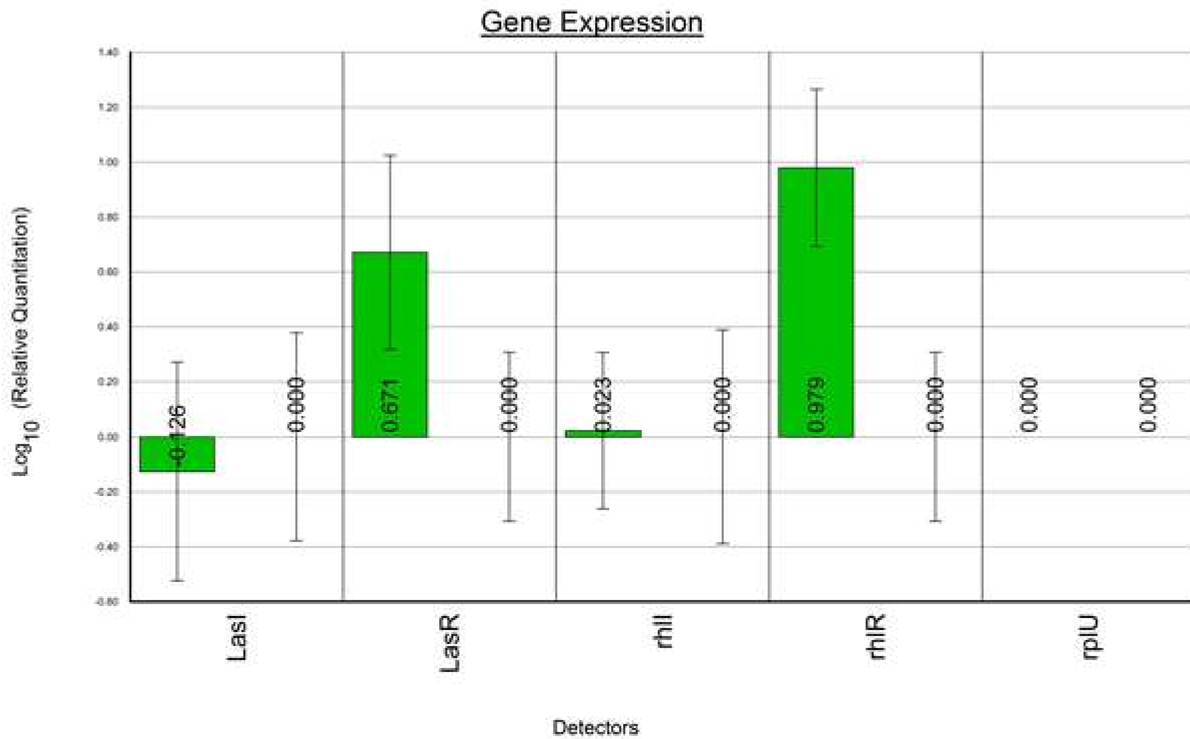

Figure 2. The expression level of QS genes in *P.aeruginosa 4* strain grown in the presence of probiotic filtrate comparatively with control (the same strain grown in normal condition).

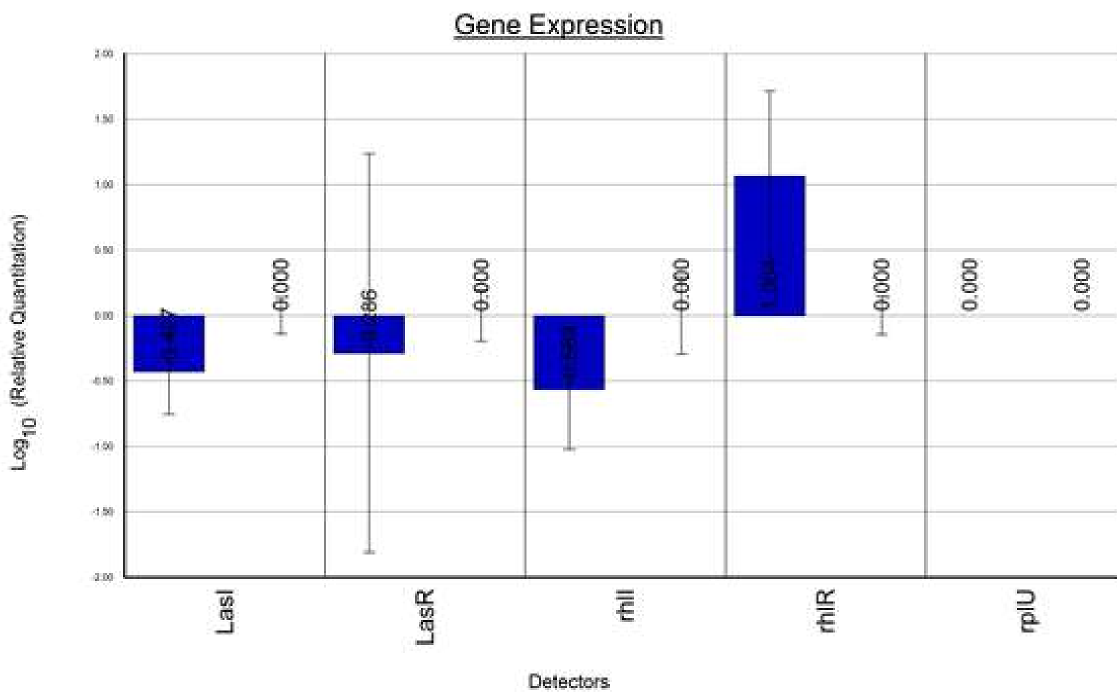

Figure 3. The expression level of QS genes in *P.aeruginosa PAO1* standard strain grown in the presence of probiotic filtrate comparatively with control (the same strain grown in normal condition).

4. CONCLUSIONS

The results of this study indicate that *Bifidobacterium breve*s trains recently isolated from faeces presented a significant probiotic potential, demonstrated by the ability of culture and the sterile supernatant to different *B. Breve* strains to inhibit the quorum-sensing genes expression level, when *P. aeruginosa* enteropathogenic strains were co-cultivated in the presence of the two culture fractions.

This study states that probiotic cultures could be used in the development of anti-pathogenic strategies, due to their inhibitory effect on *P. aeruginosa* QS genes expression. However, further studies should be focused on the characterization of the probiotic supernatant filtrate and on the isolation of the soluble molecules responsible for inhibitory activity of QS, and indirectly of virulence factors.

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