# Lead Application for the Stimulation of Fusaricidin Type Compounds by *Paenibacillus polymyxa* SQR-21

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**Summary**: *Paenibacillus polymyxa* strains produce fusaricidin type compounds that are active against a wide variety of Gram-positive bacteria and fungi. Growth and production of fusaricidin type antifungal compounds by *P. polymyxa* SQR-21 were compared in tryptone broth supplemented with three concentrations (200, 400 and 600 μM) of lead. The data revealed that the growth of *P. polymyxa* increased by 7-34 % and fusaricidin type compounds production increased by 15-70 % with the increase in concentration of lead ion ($Pb^{2+}$). The increase in $Pb^{2+}$ concentration, decreased the intracellular carbohydrate contents but increased the intracellular protein and lipid contents, however, higher levels of $Pb^{2+}$ inhibited the intracellular protein and lipid contents. On the other hand, extracellular protein contents were decreased and extracellular polysaccharide contents were increased with the increase in $Pb^{2+}$ contents in liquid culture. In addition, the regulatory effects of lead were also reflected by decrease of total RNA and increase of relative expression of the six module-containing nonribosomal peptide synthetase (*FusA*) when the lead treated experimental samples were compared with the untreated controls. The $Pb^{2+}$ seems to be directly or indirectly correlated with the production of fusaricidin type antifungal compounds. This information will aid in developing fermentation technology for maximum antibiotic production.

## Introduction

*Paenibacillus polymyxa* strains are ubiquitous in nature and known to produce two types of peptide antibiotics. One group comprises the antibiotics active against bacteria, including the polymyxins, polypeptins, jolipeptin, gavaserin and saltavalin [1, 2]. The other group consists of the peptide antibiotics active against fungi and Gram-positive bacteria and includes [3]; gatavalin; antifungal compounds named as LI-F03, LI-F04, LI-F05, LI-F07 and LIF08 and fusaricidins A, B, C and D [4, 5]. In addition, there are many reports in the literature of antimicrobial and antifungal properties of *P. polymyxa* isolates where the nature of the inhibitory agent is undefined [6]. In this experiment, we used *P. polymyxa* strain SQR-21 that was found to produce fusaricidin type of antifungal compounds, composed of a group of cyclic depsipeptides that have molecular masses of 883, 897, 948 and 961 Da, with an unusual 15-guanidino-3-hydroxypentadecanoic acid moiety bound to a free amino group [2]. The fusaricidin biosynthetic gene cluster was cloned and sequenced. It spans 32.4 kb, including an open reading frame, encoding a six-module nonribosomal peptide synthetase. The second, fourth and fifth modules of fusaricidin synthetase contain an epimerization domain, consistent with the structure of fusaricidins [7].

In natural environment, metal-microbe interactions are very important. The role of metal ions in the regulation of bacterial cell growth, nucleic acid synthesis and biosynthesis of metabolites and enzymes is well documented [8]. A taxonomic study of heavy metal tolerant bacteria conducted by Austin *et al.* [9] revealed that metal tolerance in bacteria occurs only among a restricted range of taxa. Other investigations found a relationship between the densities of metal-resistant bacteria and the degree of heavy metal pollution [10]. Much work has been done to investigate lead toxicity in man, animals and plants, but little is known about the effects of lead on bacteria. Abbas and Edwards [11] reported that lead inhibited Actinorhodin synthesis and biomass of *Streptomyces coelicolor*. Lead-sensitive and lead-tolerant bacterial strains have been isolated from the water of the Weser Estuary [12] and from soil environment [13]. The biochemical effects of lead were described by Vallee and Ulmer [14]. Den Dooren de Jong [15] showed with the agar diffusion technique that $10^{-4}$ M of lead nitrate inhibited the growth of *Azotobacter* strains. Tornabene and Edwards [16] found that the cells of *Micrococcus luteus* and *Azotobacter* sp. bound lead bromide mainly into the cell walls and membranes. Later, Tornabene and his coworkers [17] showed that lead, bound in the bacterial cell membrane, interacted with the lipid fraction of the membrane.

Metal ions are found to promote or inhibit the antibiotic production by different microbes [12, 13] but little is known about the effect of lead on antibiotic production and there is no information about the effect of lead on fusaricidin production. As fusaricidin seems to have great potential for industrial uses according to the recent reports on the excellent germicidal activity against pathogenic Gram-positive bacteria and plant pathogenic fungi and thereby it is in increasing demand [4, 5]. Therefore, a study was planned to determine the

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effect of lead ion ($Pb^{2+}$) on growth and fusaricidins production by *P. polymyxa* strain SQR-21. In addition, intra and extracellular chemical composition and the total RNA contents were measured and the fusaricidin synthetase gene expression was evaluated by reverser transcriptase and Real Time PCR assay. It is first report of the study regarding the effect of lead on the fusaricidins produced by *P. polymyxa*.

**Results and Discussion**

*Paenibacillus polymyxa* strain SQR-21 was evaluated for all possible antifungal and antibacterial volatile and non-volatile metabolites and it was found that this strain did not produce any volatile antifungal or antibacterial compound but it produced fusaricidin type compounds that were active against fungi and Gram-positive bacteria. The data of this experiment revealed that increase in the concentration of $Pb^{2+}$ in the liquid culture increased the growth of SQR-21, final pH of liquid culture, production of fusaricidins, intracellular protein and lipid contents, extracellular polysaccharides and the relative expression of fusaricidin synthetase gene while decreased the intracellular carbohydrate, extracellular protein and RNA contents.

The maximum OD was determined at 400 µM $Pb^{2+}$ while maximum antifungal activity was determined at 600 µM $Pb^{2+}$. The increase in OD over untreated control was 07, 21 and 34 %, while increase in the antifungal activity was 15, 51 and 70 % at 200, 400 and 600 µM $Pb^{2+}$, respectively (Fig. 1).

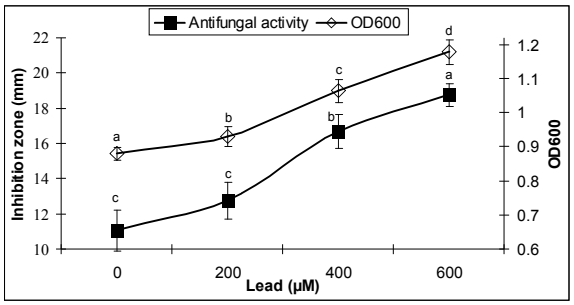


Fig. 1: Effect of different $Pb^{2+}$ concentrations on $OD_{600}$ and antifungal activity of *P. polymyxa* SQR-21 against *Fusarium oxysporum* after four days incubation.

This increase in growth and antifungal activity, more than growth, reflects that $Pb^{2+}$ plays a regulatory role, directly or indirectly, in the production of fusaricidin. It might trigger the expression of proteins involved in the fusaricidin production and the growth of bacteria and transfer the intensions and the sources of cell towards the antibiotic production rather than growth.

Initially, the tryptone medium had pH 7.2 but after four days incubation, *P. polymyxa* cells increased the pH by 8-10 % at all levels of $Pb^{2+}$ (Fig. 2) and among all levels of $Pb^{2+}$, the differences were non-significant. The increase in $Pb^{2+}$ concentration in liquid culture increased the pH although *P. polymyxa* has been found to produce organic acids like acetic, formic and oxalic acid [19]. The acid production by bacteria is normally associated with low nutrient availability as in minimal medium that is low in nutrition. However, in this experiment, we used tryptone broth that has sufficient nutrition for bacterial growth and $Pb^{2+}$ did not influence the acid production by *P. polymyxa*.

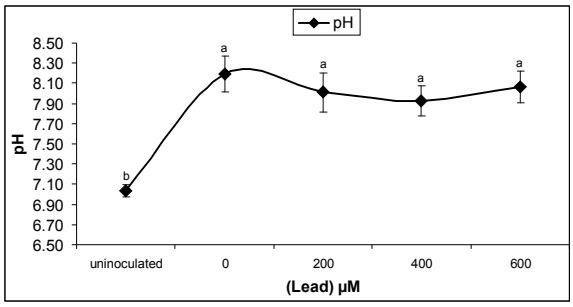


Fig. 2: Effect of different $Pb^{2+}$ concentrations on final pH of *P. polymyxa* SQR-21 liquid culture after four days incubation.

The mechanisms, how $Pb^{2+}$ increased the growth and antifungal activity yet has not been elucidated. Lead is toxic to bacteria as Den Dooren de Jong [15] showed that $10^{-4}$ M of lead nitrate inhibited the growth of *Azotobacter* strains. Capone et al. [20] indicated an initial inhibition by $Pb^{2+}$ followed by a period of stimulation in the case of sulphate reducing bacteria. In our case, the stimulatory effect on growth of *P. polymyxa* could indicate Arndt Schulz effect rather than a requirement for the metal ion and the low toxicity of $Pb^{2+}$ was probably due to the big ion radius and its considerable polarizability [21]. In addition, Some bacteria appear to be capable of immobilizing substantial quantities of $Pb^{2+}$ in their cell walls/cell membranes without injurious effects on cell viability [27] and some strains of *Staphylococcus aureus* contain plasmids that confer resistance to several heavy metals, including $Pb^{2+}$, as well as to penicillin [22].

In our case, lead might be interfering with secondary metabolism more general to enzymes or cellular process. The $Pb^{2+}$ might stimulate the

synthesis of the prepeptide or the activation of the appropriate prepeptide maturation enzymes and the transport out of the cell. Recently, $Ca^{2+}$ binding sites were predicted to be present in *NisP* peptidase, which cleaves the leader peptide from the precursor nisin [23]. Since the precursor is devoid of antibacterial activity [24] so $Pb^{2+}$ might activate the leader peptidase, however, there is no report to support this assumption. The increase in concentration of antibiotic in the medium can be a consequence of the increase in cell wall permeability of SQR-21 promoted by $Pb^{2+}$, which is in agreement with Petit-Glatron et al. [25], who studied the capacity of the cell wall to concentrate $Ca^{2+}$ and proposed that the increased concentration of $Ca^{2+}$ in the microenvironment of the cell wall could play an important role in the last step of the secretion. Another possibility is that the $Pb^{2+}$ activated enzymes whose activity resulted in a change in the regulatory functions of the cell in favor of different secondary metabolites specially fusaricidins.

The liquid culture, used to extract the antifungal compounds and to measure the OD, was also used to estimate the extra and intracellular chemical composition. The data regarding the intracellular chemical composition depicted that increase in the concentration of $Pb^{2+}$ in the liquid culture decreased the intracellular carbohydrate contents (Fig. 3) but increased the intracellular protein (Fig. 3) and lipid contents (Fig. 4). The decrease in the intracellular carbohydrate contents was 11, 15 and 22 %, increase in the intracellular protein contents was 5.5, 9.1 and 1.5 % and in the lipid contents, the increase was 19.5, 10.4 and 3.6 % as compared with control, at 200, 400 and 600 µM $Pb^{2+}$, respectively. All the $Pb^{2+}$ levels showed more protein and lipid contents over control but with the increase in $Pb^{2+}$ concentration from 200 to 600 µM, decrease in the lipid contents was measured. On the other hand, the protein contents were increased from 200 to 400 µM $Pb^{2+}$ while at 600 µM $Pb^{2+}$, decrease in protein contents was measured, but it was still higher than protein contents at 0 µM supplemented $Pb^{2+}$. El-Naggar et al. [26] also reported the increase in protein contents of *Calothrix fusca* and *Nostoc muscorum* at low lead concentrations while increase in lead levels caused reduction in protein contents. In our case, $Pb^{2+}$ seems to increase some enzymes mainly involved in fusaricidin synthesis, growth or other activities related to different cellular processes which resulted in decrease in the intracellular carbohydrate contents and residual energy was being used for the intracellular protein and lipid synthesis.

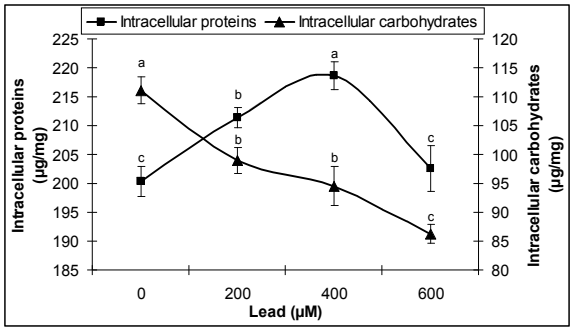


Fig. 3: Effect of different $Pb^{2+}$ concentrations on intracellular protein and carbohydrate contents of *P. polymyxa* SQR-21 after four days incubation.

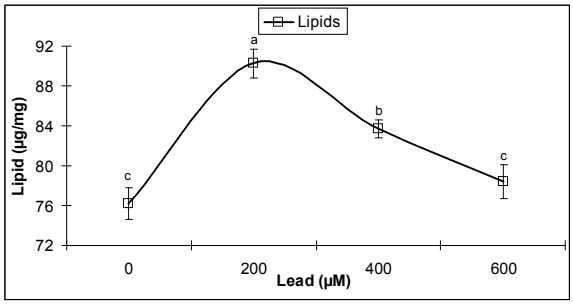


Fig. 4: Effect of different $Pb^{2+}$ concentrations on intracellular lipid contents of *P. polymyxa* SQR-21 after four days incubation.

The data regarding the extracellular chemical composition (Fig. 5) revealed that all levels of $Pb^{2+}$ decreased the extracellular protein contents; however, EPS contents of liquid culture were increased with the increase in $Pb^{2+}$ concentration. The decrease in extracellular protein contents was 18, 47 and 42 % and increase in EPS contents was 11, 7 and 5 % over untreated control at 200, 400 and 600 µM $Pb^{2+}$, respectively. The presence of different metal ions such as $Ca^{2+}$, $Pb^{2+}$ and $Mg^{2+}$ as well as mineral oxides of iron, aluminium and calcium during growth influenced the types and quantity of polysaccharides, proteins and enzymes secreted by the bacteria [27]. Our results showed that extracellular enzymes or other proteineous products were negatively affected by lead; this conclusion was further supported by decrease in RNA contents with the increase in $Pb^{2+}$ concentration. The negative effect of lead on enzyme production has been reported earlier [18]. Haggag [28] reported the increase in the extracellular polysaccharide contents by *P. polymyxa*, which were composed of varying concentrations of glucose, fructose, galactose, mannose and xylose. The EPS aids in the biological uptake of the metal ions

necessary for metabolism and growth. Heavy metals are also known to bind to the cell walls and chelated through the EPS [29]. Therefore, increase in lead concentration in liquid culture might be forcing bacterial cells to produce more EPS to overcome toxic effects of lead by binding with cell wall or precipitation by chelation. In addition, the cells of Gram-positive bacteria like *P. polymyxa* are rigid and relatively insensitive to shear forces because of their thick cell wall. The exopolymers or exopolysaccharides that they form might play a crucial role in metal biosorption and precipitation as *P. polymyxa* strains have been used in the biosorption of copper [30]. The mechanisms of biosorption may involve intracellular uptake and storage via active cationic transport systems, surface binding, or some undefined mechanisms [31]. The biological and chemical characteristics of these uptake processes are important as an aid in the understanding of the role of metallic ions in basic cellular functions.

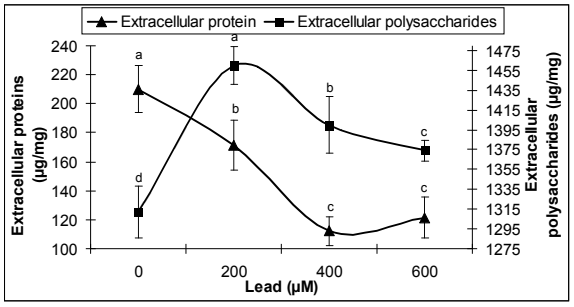


Fig. 5: Effect of different $Pb^{2+}$ concentrations on extracellular protein and polysaccharide contents of *P. polymyxa* SQR-21 after four days incubation.

The results of the RNA contents (Fig. 6) and the relative expression of fusaricidin synthetase gene (*fusA*) of *P. polymyxa* SQR-21 (Fig. 7) revealed that total RNA contents were decreased while the relative expression of *fusA* gene was increased with the increase in concentration of $Pb^{2+}$ in the liquid culture. After RNA extraction, DNase treatment was carried out to degrade genomic DNA and it was confirmed by RT-PCR. No DNA contamination was observed after DNase treatment as shown in Fig. 7. Total RNA contents were measured prior to cDNA synthesis. For RT- and Real Time-PCR, 16S rRNA was used as positive control. The intensity of amplified *fusA* gene bands also verified increase in the gene expression with increase in the concentration of $Pb^{2+}$ in liquid culture. The decrease in total RNA contents was 47, 34 and 9 % and increase in the relative expression of *fusA* gene was 17.7, 26.9 and 81.6 % over untreated control, at 200, 400 and 600 µM $Pb^{2+}$, respectively. Although there was decrease in total RNA contents, at all levels of $Pb^{2+}$ as compared with untreated control but from 200 to 600 µM $Pb^{2+}$ total RNA contents were increased.

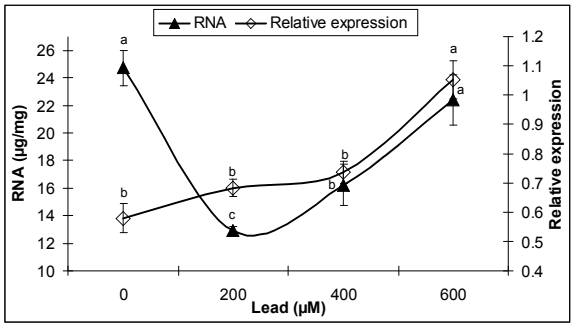


Fig. 6: Effect of different $Pb^{2+}$ concentrations on total RNA contents and relative expression of fusaricidin synthetase gene (*fusA*) in *P. polymyxa* SQR-21.

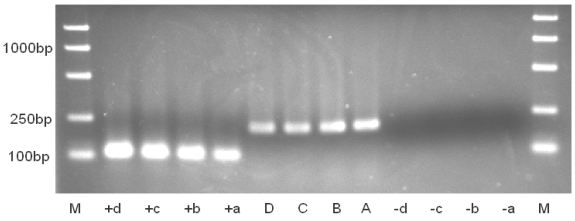


Fig. 7: RT-PCR products originating from cDNA, after extraction of total RNA of *P. polymyxa* SQR-21 grown in submerged culture treated with four concentrations of $Pb^{2+}$(a = 0, b = 200, c = 400, d = 600 µM $Pb^{2+}$), M = DNA marker, A to D= positive control 16S rRNA gene, +a to +d = *fusA* gene (+RT), -a to –d = negative controls for *fusA* gene (-RT).
(+RT = Reverse transcriptase reaction product of RNA extracted from *P. polymyxa*, -RT = RNA extracted from *P. polymyxa*)

Inorganic Pb inhibited the growth and photosynthesis of marine algae [32], nitrogen fixation by cyanobacteria [33] and germination of spores and mycelial growth of fungi [34]. The microbiota of soils and of the leaf surface of plants from sites heavily contaminated with Pb and other heavy metals had lower species diversity than did soils and plants from non-contaminated sites [35]. It is clear from above discussion that lead is toxic for most of the bacteria, fungi and algae but not for *P. polymyxa* because it has some mechanism to cope with lead toxicity specially the production of EPS. Variable concentration dependent effects of $Pb^{2+}$ on growth and antibiotic production by different

microorganisms have been reported but such information was not available for *P. polymyxa* before. It is preliminary but the first report of the effect of $Pb^{2+}$ on the fusaricidin production by *P. polymyxa*. Although $Pb^{2+}$ exerted negative effects on extracellular protein and intracellular carbohydrate contents but it increased the antibiotic production up to 70 % in the liquid culture. The $Pb^{2+}$ levels used here do not exist under natural conditions but our research gives useful information about the effects of $Pb^{2+}$ on the overall metabolic processes of *P. polymyxa* mainly fusaricidin production. This information will aid in developing fermentation technology for maximum antibiotic production. As fusaricidin seems to have great potential for industrial uses according to the recent reports on the excellent germicidal activity against pathogenic Gram-positive bacteria and plant pathogenic fungi and thereby it is in increasing demand [2, 4]. However, more research is needed at the cellular level, to evaluate the behavior of metal ions alone and in the combinations on antifungal compounds production by *P. polymyxa*.

## Experimental

### *Bacterial and Fungi Cultures*

A chitinase deficient and fusaricidin producing strain of *Paenibacillus polymyxa* SQR-21 and a tested pathogenic strain *Fusarium oxysporum* f. sp. *cucumerinum* (*F. oxysporum*) were provided by Soil-Microbe-Interaction Laboratory, Nanjing Agriculture University, Nanjing, China. The bacterial culture was maintained on potato dextrose agar (PDA) plates and was stored at -80 °C in tryptic soya broth (TSB) containing 20 % glycerol for further use. The fungal pathogenic strain was maintained by cultivation on PDA plates for 3 d at 28 °C and then the plates were sealed with parafilm and stored at 4 °C. The pathogen was subcultured onto a fresh PDA plate after one month.

### *Metal Ion Media Preparation and Antifungal Activity Assay*

Liquid-culture experiments were performed in 100 mL of tryptone broth (tryptone; 10, NaCl; 5 and sucrose; 10 g/L; pH 7.2) in 500 mL Erlenmeyer flasks. Initial $Pb^{2+}$ contents in tryptone broth were 0.8 µM, determined by Spectra AA, 220 FS atomic absorbance spectrometry. After sterilization, the cultures were supplemented with $Pb(NO_3)_2$; three concentrations of $Pb^{2+}$ (200, 400 and 600 µM) were considered. Each experiment had three replicates including control cultures without supplemented $Pb^{2+}$. For the isolation of antifungal compounds, SQR-21 strain was pre-inoculated in tryptone broth overnight at 37 °C. After adding $Pb(NO_3)_2$, tryptone broth was inoculated with 100 $\mu$L of over night culture of SQR-21 and incubated in an shaking incubator (170rpm, 37 °C). After 4 days, $OD_{600}$ and pH was determined and liquid cultures were centrifuged at 12000 × g for 10 min to remove cells. The supernatants were pooled and active compounds were extracted twice with an equal volume of n-butanol. The extracts were concentrated by using a rotary evaporator and the residues were dissolved in methanol. These extracts were used to determine antifungal activity by agar diffusion assay using *F. oxysporum* as test pathogen. After three days, the diameter of inhibition zone was measured. The fusaricidin type compounds are not available commercially so we used the size of inhibition zone for the estimation of their production. The lyophilized cell pellets were used to measure cellular dry weight.

### *Extracellular and Intracellular Chemical Composition*

The bacterial liquid culture samples (2 mL) were centrifuged (12,000 x g) for 10 min. The pellets were suspended in 2 mL of deionized water for washing and centrifuged three times. These pellets were used for the determination of total intracellular protein, carbohydrate and lipid contents. For total protein contents, the rinsed cells were resuspended in deionized water and incubated in 1N NaOH at 90 °C for 10 min to solubilize cellular protein. Proteins were measured by the method of Bradford [36] with bovine serum albumin standards ranging from 10-100 µg $mL^{-1}$. Total carbohydrate was estimated in rinsed-cell samples by the phenol-sulfuric acid method [37]. The lipid contents of bacterial cells were calculated by the phosphoric acid–vanillin reagent method of Izard and Limberger [38] with Triolein standards ranging from 10 to 100 µg. The cell free liquid culture was used for the estimation of extracellular protein and polysaccharide (EPS) contents by the above-described methods. Before assaying protein, the resulting EPS solution was dialyzed using a membrane of 1000 MW cut off against ultra pure water for 2 days at 4 °C to remove the small molecules and entrained media residues.

### *RNA Extraction and Primers Design*

Total RNA was isolated by using the Trizol reagent method (Invitrogen™, Shanghai) according to manufacturer's instructions. To remove DNA contamination, 10U DNase1 (Takara, Dalian) along with 20U RNase inhibitor (Takara, Dalian) (37°C, 40 min) were used in the reaction mixture of 50 µL

containing 20-50 µg RNA. RNA was estimated by determining the absorbance at 260 nm. Specific primers for fusaricidin synthetase gene (*fusA*) (111bp) and 16S rRNA gene (16s) (210bp) were designed by using primer premier 5 software (PREMIER biosoft international). The designed primers were as follows, fusA1, 5' GCAGAGGATGATAGTGTTGGTC 3', fasA2, 5' CAGCACATCATGCGTTCC 3', 16s1, 5' CATTCATCGTTTACGGCGT 3' and 16s2, 5' TGTTAATCCCGAGGCTCACT 3'.

*Reverse Transcription and Real Time PCR Assay*

For the synthesis of first strand cDNA, 3µg of total RNA, 200U of RevertAid$^{TM}$ M-MuLV reverse transcriptase (Fermantas), 20U RNase inhibitor (TaKaRa, Dalian), 0.2 µg of Random hexamer primer and 1mM dNTP in the total volume of 20 µL were used. Reverse transcription (RT) was performed using the following parameters, denaturation for 5 min at 65 $^{\circ}$C; (tubes were then chilled on ice for 2 min, before the addition of enzymes), incubation for 60 min at 42 $^{\circ}$C and inactivation for 5 min at 95 $^{\circ}$C. Target genes from cDNA were amplified separately using 3µl aliquots of RT product as template and 30 pmol of each primer pair (fusA1 and fusA2, 16s1 and 16s2). Reaction mixtures for PCR contained 2.5 U Taq polymerase (TaKaRa, Dalian), 20 nmol of dNTP and 100 nmol $Mg^{2+}$. The PCR conditions were as follows**,** after initial 5 min incubation at 95 $^{\circ}$C, the mixture was subjected to 30 cycles, each including 30s at 94 $^{\circ}$C, 30s at 58 $^{\circ}$C and 1 min at 72 $^{\circ}$C. A final extension was performed at 72 $^{\circ}$C for 2 min. Amplified products were separated by agarose gel electrophoresis (2 % (w/v agarose), stained with ethidium bromide and viewed under UV light to check band intensity and cDNA quality.

Singleplex relative Real Time PCR was performed using an iCycler MyiQ$^{TM}$ single color Real Time PCR detection system (BioRad). Reactions were performed in a 20 µL volume reaction mixture containing 1 mM primers, 3µL cDNA and 10µL of SYBR®*Premix Ex Taq*™ (Perfect Real Time) (TaKaRa, Dalian) including TaKaRa *Ex Taq*™ HS and SYBR® Green I, dNTP and buffer. The PCR protocol included a 10 min denaturation step at 95 $^{\circ}$C followed by 40 cycles with 95 $^{\circ}$C for 30 s, 58 $^{\circ}$C for 30 s and 72 $^{\circ}$C for 1 min. Detection of the fluorescent product was carried out at the end of the 72 $^{\circ}$C extension period (2 min). After the PCR, these samples were heated from 58 to 95 $^{\circ}$C. When the temperature reached the Tm of each fragment, there was a steep decrease in fluorescence of the product. The 2 µL cDNA of each treatment were mixed together to prepare standard curve that was used to compare the unknown samples to get relative expression data. The whole experiment was repeated twice.

*Statistical Analysis*

The values were represented as the means of three replicates along with standard deviation (SD). Differences were assessed with one-way ANOVA. Duncan's multiple range test was applied when one-way ANOVA revealed significant differences ($P \leq 0.05$). All statistical analysis was performed with SPSS BASE ver.11.5 statistical software (SPSS, Chicago, IL).

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