The Effect of *Bifidobacterium breve* and *Lactobacillus salivarius* on the Gene Expression Involved in Patulin Biosynthesis Produced by *Penicillium expansum* .

Sumaiya N. Hawar
Sahar S. Karieb
Dept. of Biology/College of Education For Pure Science(Ibn Al-Haitham) /University of Baghdad

Received in :18/ December /2014 , Accpted in: 2/February /2015

Abstract

Penicillium expansum produced the toxin patulin in pome fruits. To evaluate the molecular mechanism by which the treatment of probiotic bacteria *Bifidobacterium breve* and *Lactobacillus salivarius* could modulate patulin production, three genes involved in the biosynthesis of patulin were measured using real time-PCR technique. The result of this study found that the supplementation with *B. brave* and *L. salivarius* down-regulate the relative expression of 6-methylsalicylic acid synthase (msas), ATP binding cassette transporter (ABC) and putative cytochrome P450 monooxygenases (*P450-1*) as well. Although, there is no effect of some strains on this expression. However, these finding suggested that these bacteria decreased patulin production through effect on the genes responsible for its biosynthesis which can be the main mechanism by which patulin reduced in the presence of these bacteria.

Key word: Patulin, lactic acid bacteria, *Penicillium expansum* and gene

Introduction

The majority of important toxigenic species of *Penicillium* and food spoilage species are found in subgenus *Penicillium* [1]. *P. expansum* is the cause of blue mold widely distributed and is broad- spectrum pathogen on fresh fruits especially pomaceous fruits and is dominating *Penicillium* to apples [2 ; 3], it also occurs on nuts, oil seeds dried meat and soil [4], *P.expansum* is a post-harvest pathogen causing a large part of the economic losses that occur during storage and shipment of apples & deciduous fruits [5].*P. expansum* also has potential public health significance, since it produces the mycotoxin patulin, especially in apple productes .

Patulin($C_6H_7O_4$) is a low molecular weight ,produced by fungi belonging to several genera including *Penicillium*, *Aspergillus* and *Byssochlamys.* PAT has been found as a natural contaminant of many moldy fruits , vegetables, cereals & other foods [6 ; 7] but generally the major source of PAT consumption is apples with blue rot and apple cider or juice pressed from moldy fruits . Preceding research highlighted the toxic effects of PAT towards human exposed to the toxin via consumption of contaminated products [8]. Studies also revealed mutagenic, genotoxic, immunosuppressive effect of PAT [9], neurotoxic effects on rodents [10] and teratogenic effect on chickens [11].

Several strategies, including chemical, physical and biological control methods have been investigated to manage mycotoxins generally in foods. Among these, biological control of different mycotoxin, such as aflatoxins, ochratoxin, deoxynivalenol, fusarenon & patulin. Concerning the activity of bacteria and lactic acid bacteria towards PAT ,[12] reported the inhibitory activity of cell free supernatants of *Lactobacillus casei* strains on the growth of *Penicillium* spp. and production of toxins (patulin & citrinin).

Materials and Methods

2.1. chemicals

Patulin (98%) was obtained from Sigma Aldrich (Poole, UK), suspended in methanol at 5 mg /ml and stored at -20 °C.

2.2. *P. expansum* and Lactic acid bacteria (LAB) culture

P. expansum (297959)was obtained from CABI (UK) and subcultured on potato dextrose agar (PDA) (Oxoid, UK) at 25 °C. *P. expansum* produce 58.4± 1.0 µg ml^{-1} of patulin offer growing on Lablemco-Tryptone Broth (LTB) that supplementation and with 0.01 % manganese over 10 d. and patulin level would be raised to 347.5± 9.3 µg ml^{-1} .

Two probiotic LAB strains were originally isolated from a range of materials and kept in the University of Plymouth culture collection. These included *Bifidobacterium breve* and *Lactobacillus salivarius* .Strains were grown routinely in MRS media at 37°C /5%CO2/Ph6.2 and stored long term at ¬8°C 15%glycerol.DNA was extracted from confluent cultures using a Wizard SV genomic DNA kit(Promega,WI,USA),PCR undertaken with 9F-1507R primers and the product commercially sequenced before BLAST searching.

2.3. Evaluating the efficacy of LAB interaction in different *P.expansum* contamination scenarios

Three different treatments were conducted using Lablemco-Tryptone Broth(LTB) supplemented with 0.01% manganese as follows: Group-B: 1 ml of *P.expansum* spore suspension(106 spores ml^{-1}) and 1 ml of LAB suspension (10^7 CFU ml^{-1}) were inoculated simultaneously into 100 ml flasks and incubated for10 days at 25°C . Group-C: 100 ml of LTB was first inoculated with 1 ml of *P.expansum* spore suspension (10^7), this culture incubated for 4 days at 25°C, then 1ml of 1 ml LAB (10^7) cells was added to this and incubated for a LAB suspension(10^6) and incubated for 48h at 37°C ,after that 1 ml of spore

suspension(10^6) was added and incubation was then continued for 8 days at 25°C. Group A control treatment consisted of 1 ml spores only (10^6) for 10 days. On completion, all flask contents were filtered and mycelial dry weight derived as before patulin was extracted from the spent LTB broth by extraction with double sample volume of ethyl acetate, dried over anhydrous Na_2SO_4, evaporating to dryness with a rotary evaporated at 48°C , redissolved in 2 ml chloroform, evaporated under N2, and serially diluted in 1% CAN for HPLC analysis [13].

2.4. Gene expression analysis using quantitative reverse transcription polymerase chain reaction (qRT-PCR)

To determine whether the application of LAB affects the expression of isoepoxydon dehydrogenase (IDH), a key enzyme in the patulin synthesis pathway, qRT-PCR was undertaken on mycelial extract at the end of incubation.

2.5. RNA extraction and cDNA formation

Total RNA was extracted using 100 mg of fungal mycelium homogenized in liquid nitrogen according to the manufacturer's protocol. RNA extracted using GenElute™ mammalian total RNA miniprep kit (Sigma, Poole, Dorset, UK), which utilizes a column based technique to isolate and purify RNA. All consumables and reagents used were free of DNAase and RNAase. RNA and DNA quantity and purity were measured using a nandrop spectrophotometer (ND-1000) and A260:A280 was used to assess sample purity. Complementary DNA was generated as follows: 1 µg RNA sample was denatured at 70 °C for 10 min in the presence of dNTPs and random nonamers (GeneAmp PCR System 9700), then 1 unit of MMLV-reverse transcriptase was added to each reaction followed by incubating 10 min at 21 °C, 37 °C for 50 min and 94 °C for 5 min. RTs were stored at 4 °C until used. Quantitative RTPCR was performed using a Step One PCR system (Applied Biosystems, UK) using the DNA-binding dye SYBR green for detection of PCR products. Primer sequences were designed using the NCBI website and according to the mRNA sequence of each gene published in the same website using primer blast software. Designed primers were purchased from Eurofins MWG Operon (Germany). Primers used for 6-methylsalicylic acidsynthase (*msas*): forward GCCTTCTTCCTGCGTCGTTGCT, reverse TCCGAGTGCGTCTCGAAGAGGA (GenBank DQ084387.1); cytochrome P450 monoxygenase (p4502):forward TCGCCCGCGTCTCAAATCGG , reverse GCAGGAAATACGTTGCAAGGCCA (GenBank DQ084390.1); gene coding an ATP-binding cassette (ABC) transporter: forward CCACGGCGGTCGAATTCATGG, reverse GTCCCGGGCTGGGCCATAGTA and forward b-tubulin ATG GTA CCT CCGACC TCC AGC, reverse CGG CAC GGG GAA CGT ACT TGT acted as a reference housekeeping gene control. Product size and primer specificity was then confirmed using agarose gel electrophoresis. Reaction for QPCR contains the following, 2µl of external plasmid standard or cDNA added to a final reaction volume of 25µl which contained 0.05U/µl Taq, SYBR green, PCR buffer (300 nM) reference dye (Rox) and specific sense and antisense primers (0.2µM).

The progress of the PCR amplification was monitored by real-time fluorescence emitted from SYBR Green during the extension time. Reaction conditions were 94°C for 2 minutes, followed by 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. The data were analyzed based on the differences between the reference (control group) and the treatment groups using a comparative Ct analysis according to the following formula: [14].

$\Delta C_{t\ sample} = C_{t\ sample} - C_{t\ reference\ gene}$

$\Delta\Delta Ct = \Delta C_{t\ sample} - \Delta C_{t\ reference\ control}$

Amount of target (RQ) = $2^{-(\Delta\Delta Ct)}$

Where Ct is a threshold cycle.

Statistical analysis

Differences between groups were assessed using SPSS program 18 (IBM SPSS Statistics software). Data were expressed as mean ± standard error (S.E.) using Student's T-test. A difference of $P<0.05$ was considered statistically significant.

Results

Patulin production is controlled by network of transcriptional factors. Therefore, to evaluate the effect of probiotic bacteria on *P.expansum* production, the expression gene involved in patulin biosynthesis was assessed using real time quantitative PCR technique.

Genes responsible for patulin production by *P.expansum* was affected by the treatments with bacteria found in the recent study. As shown previously, the treatment of fungi with several strains of bacteria modulated Idh expression; one of the genes translates for patulin , however three genes also are implicated patulin biosynthesis. The recent study found that 6-mas levels significantly down-regulated in the presence of bb, sb and bc1 ($p<0.005$), while this expression had up-regulated in the presence Sc1 bacteria (Figure 1, 2).

P450 levels also had been shown significantly decreased by Sb, bc1 and Sb1 bacteria ($p<0.005$), while this expression increased by bb bacteria treatment but still not significant versus control group (Figure 3, 4). Additionally, the expression of ABC not affected by bb bacteria treatment, while its levels were decreased in the presence of Sb, bc1 and SC1 bacteria in comparison to control group ($p<0.005$) (Figure 5, 6). All demonstrated data found the treatment with specific bacteria could modulate patulin expression by *P.expansum* which can affect on the growth of this fungus.

Discussion

Patulin production is controlled by network of transcriptional factors including genes. Therefore to evaluate the effect of probiotic bacteria on *P.expansum* production ,the expression of three gene 6-msas, p450 and ABC involved in patulin biosynthesis was assessed using real time quantitative PCR technique based on the results. The main objective of the present investigation was to gain insight into the mechanism by which probiotic bacteria reduce or transform patulin accumulation. This assumption was also supported by previous evidence that the expression of genes of secondary metabolism is strictly controlled by nutrients, inducers, products, metals, and growth rate, and in most cases, regulation is at transcription levels [6]. This assumption was also supported by [15]. The msas gene belongs to the polyketide synthase family. Although polyketide biosynthesis has been the focus of intensive research over the past decade, relatively few polyketide synthase gene clusters have been described in fungi [16].The best understood system is the one responsible for the biosynthesis of 6-msas, which is the first step in the patulin synthetic pathway. The peab1 gene encodes an ATP-binding cassette (ABC) transporter that functions as an efflux pump. Its function is of particular interest since it can play a significant role in protecting plant pathogens from synthetic fungicides or from plant defense compounds.

The cytochrome monooxygenases p450-1 and p450-2 seemed to be the best candidates for explaining the effect of bacteria on patulin biosynthesis. [17] showed that the expression of idh gene, 6-msas gene and cytochrome P450 was to be higher under patulin permissive condition, indicating for the first time that regulation of biosynthetic in *P.expansum* is mediated at the level of gene transcription. Transcription regulation of mycotoxin biosynthetic genes under different physiological conditions is quite common in mycotoxigenic fungi, for example for aflatoxin and sterigmatocystin production in *Aspergillus parasiticus* and *A. nidulans* [10 ; 5] . This suggested to us that other genes involved in patulin biosynthesis . [18] who was reported that the absence of genes in the patulin producing fungus *Byssochlamys fulva* resulted in its inability to produce toxin, the result of [15] provide evidence that quercetin and umbelliferone (phenolic compounds) do not seem to affect primary fungal

metabolism, but reduce patulin production by acting on its biosynthetic pathway [19] found that idh mRNA expression significantly reduced in the group inoculated with *B. breve* and *Lb. salivarius* at the same time with *P.expansum*. Furthermore, idh expression also suppressed in the group treated with *B. breve* bacteria after 4 days of *P. expansum* growing .

References

1. Pitt , J. I. & Hocking A.D. (1997) . Fungi and food spoilage . Blackie Academic and professional , London.
2. Abrunhosa, L.; Paterson, R. P. M. & Kozakiewicz, Z. (2001). mycotoxin production from fungi isolated from grapes. Lett. Appl. Microbiol., 32: 240-242.
3. Arici, M. (2000). Patulin production of *Penicillium* Isolates from fermented olives in a synthetic medium. Ernahrung., 24: 257-259.
4. Barkai-Golan, R. (2008). *Penicillium* mycotoxins. In: Mycotoxins in fruits and vegetables (Barkai-Golan, R. & Paster, N., eds.), Chapter7, pp.153-1583.
5. Bhatnagar, D. ; Ehrlich, K. & Cleveland, T.E. (2003). Molecular genetic analysis and regulation of aflatoxin biosynthesis. Appl. Microbiol. Biotechnol., 61:83-93.
6. Demain, A.L. (1996). Fungal secondary metabolism: regulation and function. In: Sutton, B. (Ed). Century of Mycology . Combridge , M.A. Cambridge university press, pp. 233-254 .
7. Frisvad, J. C. (1981). Physiological criteria and mycotoxin production as aids in identification of common asymmetric penicillia. Appl. Environ. Microbiol., 41:568-579.
8. Frisvad, J. C. and Samson, R. A. (2004). Polyphasic taxonomy of *Penicillium* Sub genus *Penicillium*: Aguide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. Studies in Mycology, 49:1-52.
9. Gourama, H. (1997). Inhibition of growth and mycotoxin production of *Penicillum* by *Lactobacillus* species. Lebensmittel-Wissenshaft und-Technologie, 30:279-283.
10. - Liu, B. & Chu, F.S.(1998). Regulation of aflR and its product, AflR, associated with aflatoxin biosynthesis. Appl. Environ. Microbiol., 64:3718-3723.

11. Mahfoud, R. ; Maresca, M. ; Garmy, N. & Fantini, J. (2002).The mycotoxin patulin alters the barrier function of the intestinal epithelium: mechanism of action of the toxin and effect of glutathione. Toxicology and Applied Pharmacology,181:209-218.
12. Pfeiffer, E. ; Gross, k. & Metzler, M.(1998). A neuploidogenic and clastogenic potential of the mycotoxins citrinin and patulin. Carcinogenesis, 19:1313-1318.

13. Pitt , J. I. & Leistner, L. (1991) . Toxigenic *Penicillium* species . 91 – 99 . In: Doyle, M.P. ; Beuchat L.R. & Montville T.J. (ed) . Food Microbiology and Frontiers. ASM press, Washington D.C., 768.
14. Karieb, S. & Fox, W. (2011). Phytoestrogens directly inhibit TNF-α-induced borne resorption in RAW264.7 cells by suppressing c-fos-induced NFATc1 expression. Journal of Cellular Biochemistry, 112:476-487.
15- Sanzani, S.M. ; Schena, L. ; Nigro, F. ; Girolama, A. & Ippolito, A. (2009). Effect of quercetin and umblelliferne on the transcript level of *Penicillium expansum* genes involved in patulin biosynthesis. Eur. J. Plant Pathol.,125:223-233.
16. Desjardins,A. E. & Proctor, R. H. (2007). Molecular biology of Fusarium mycotoxins .Journal of food Microbiology,119:47-50.
17- White, S. ; OCallaghan, J. & Dobson, A. (2006). Cloning and molecular characterization of *Penicillum expansum* up-regulated genes under conditions permissive for patulin biosynthesis. FEMS Microbiol. Lett., 255:17-26.
18- Puel, O. ; Tadrist, S. ; Delafarge, M. ; Oswold, I. & Lebrihi, A. (2007). The inability of *Byssochlamys fluva* to produce patulin is related to absence of 6-methylsalicylic acid synthase and isoepoxydon dehydrogenase genes. International Journal of Food Microbiology, 115:131-139.
19- Hawar, S. N.(2012). Activity of some probiotic bacteria against the mycotoxin patulin produced by *Penicillium expansum* isolated from apple fruits. Baghdad university, Athesis,pp:145.

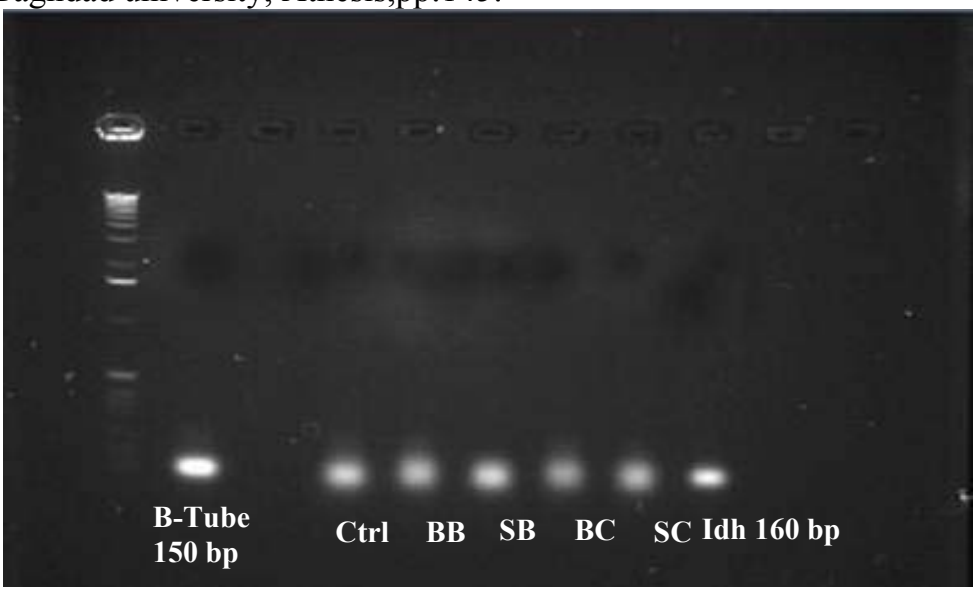

Figure N0. (1) : RT-PCR amplification products obtained from *P. expansum* (patulin producer) with primers targeting genes involved in patulin biosynthesis (6-mas)(B:bacteria and mould growth at the same time,BB: *B.breve* and mould ; C:mould growth first for 4 days, SC: *Lb. salivarius* growth first).

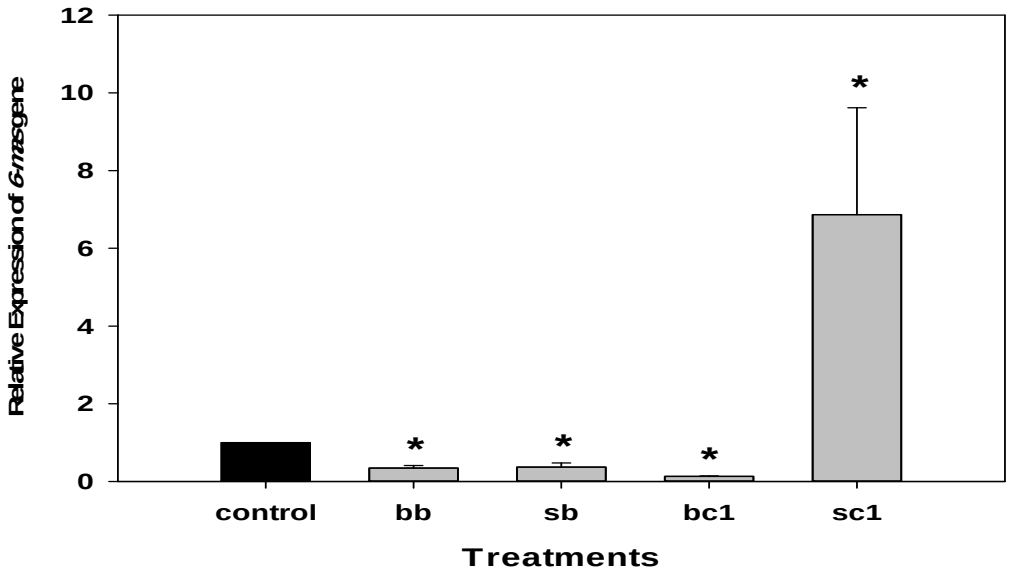

Figure No.(2): The effect of supplemented bacteria *B. breve* and *L. salivarius* on the relative expression of *6-mas* gene in the toxic strain *P.expansum*. Data expressed normalized to β- Tub; housekeeping gene, and data represent the mean + SEM. A P<0.05 versus control treated group.

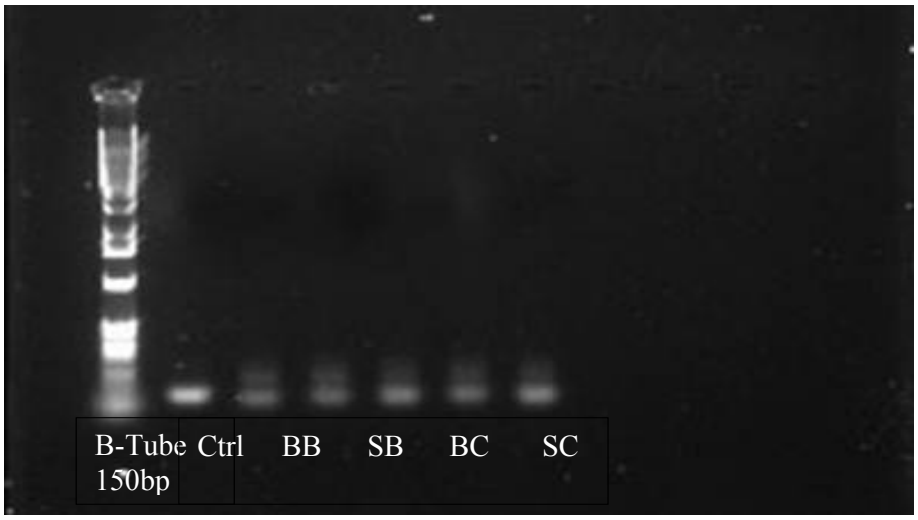

Figure N0 (3) : RT-PCR amplification products obtained from *P. expansum* (patulin producer) with primers targeting genes involved in patulin biosynthesis (P450)(B:bacteria and mould growth at the same time, BB: *B.breve* and mould ; C:mould growth first for 4 days, SC: *Lb. salivarius* growth first).

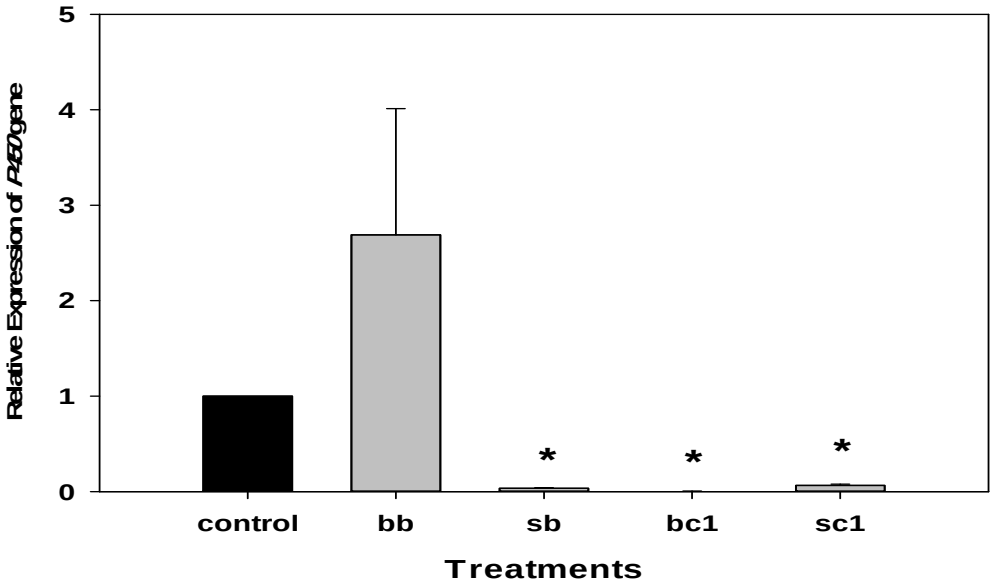

Figure N0. (4) : The effect of supplemented bacteria *B. breve* and *L. salivarius* on the relative expression of *p-450* gene in the toxic strain *P.expansum*. Data expressed normalized to β- Tub; housekeeping gene, and data represent the mean + SEM. A P<0.05 versus control treated group.

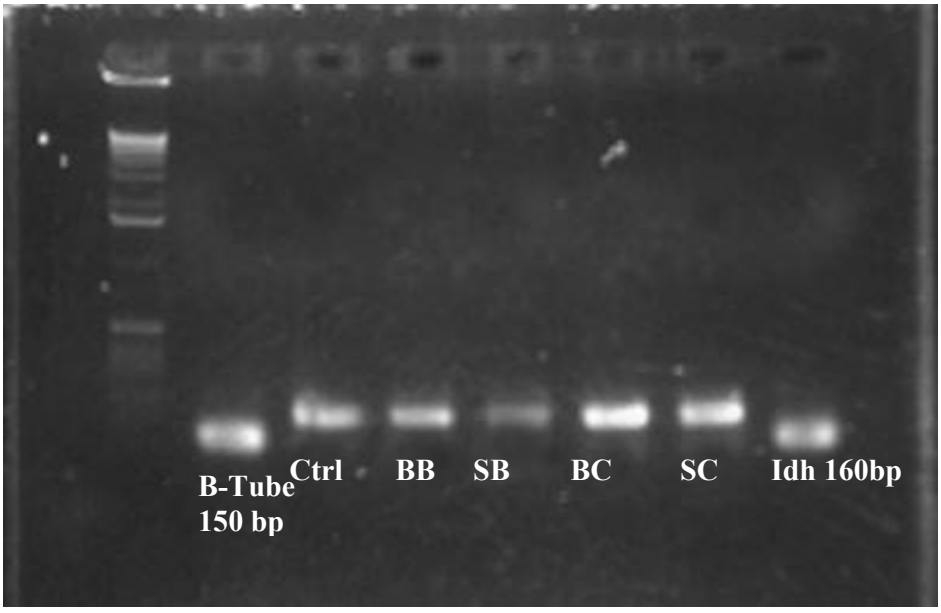

Figure N0. (5): RT-PCR amplification products obtained from *P. expansum* (patulin producer) with primers targeting genes involved in patulin biosynthesis (ABC)(B: bacteria and mould growth at the same time,BB: *B.breve* and mould ; C:mould growth first for 4 days, SC: *Lb. salivarius* growth first)

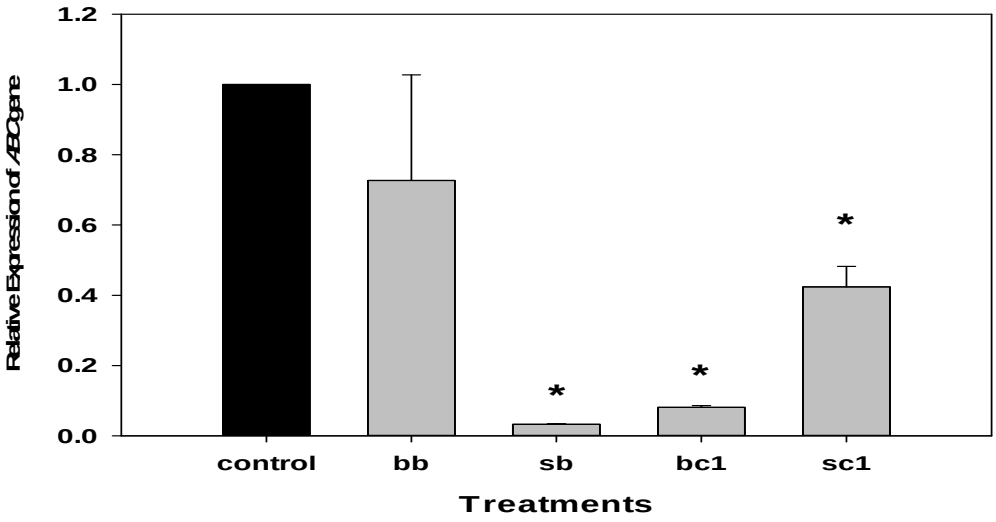

Figure No.(6) : The effect of supplemented bacteria *B. breve* and *L. salivarius* on the relative expression of *ABC* (ABC transporter mRNA, partial cds (gene coding an ATP-binding cassette (ABC) transporter gene) in the toxic strain *P.expansum*. Data expressed normalized to β- Tub; housekeeping gene, and data represent the mean + SEM. A P<0.05 versus control treated group.

تأثير بكتريا *Bifidobacterium breve* و *Lactobacillus salivarius* في التعبير الجيني للجينات المسؤولة عن البناء الحيوي لسم الباتيولين المنتج من قبل فطر *Penicillium expansum*

سميه نعيمه حوار
سحر سعدي غريب

قسم علوم الحياة\ كليه التربية للعلوم الصرفة (ابن الهيثم) \ جامعه بغداد

استلم البحث في :18/كانون الثاني / 2014 ، قبل البحث في 2/شباط / 2015

الخلاصة

ان فطر البنسليوم ينتج سم الباتيولين في التفاح الذي يكون ساما للانسان. لتحديد كيفية تأثير كل من بكتريا *Bifidobacterium breve* و *Lactobacillus salivarius* في انتاج سم الباتيولين من الناحية الجزيئية ، تم تقدير التعبير الجيني لثلاث جينات مسؤولة عن البناء الحيوي لسم الباتيولين بعد معاملة الفطر بالبكتريا المدروسة باستخدام تقنية سلسلة تفاعلات البلمرة PCR. بينت النتائج ان المعاملة ببعض السلالات من البكتريا المدروسة قد قللت من التعبير الجيني للجينات المدروسة ، جين methylsalicylic acid synthase (msas) ، ATP binding cassette transporter (ABC) و جين putative cytochrome P450 monooxygenases (*P450-1*). بالرغم من ذلك فان بعض السلالات لايوجد لها تاثير في التعبير الجيني للجينات المدروسة. ان نتائج الدراسة الحالية بينت ان البكتريا المدروسة قد خفضت من انتاج سم الباتيولين من خلال التاثير في الجينات المسؤولة عن البناء الحيوي لانتاج هذا السم.

الكلمات المفتاحيه: سم الباتيولين . بكتريا حامض اللاكتك . فطر البنسليوم . جين