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The Effect of *Bifidobacterium Breve* and *Lactobacillus Salivarius* on the Isoepoxydon Dehydrogenase (Idh) Gene Expression Involved in Patulin Biosynthesis Produced by *Penicillium Expansum*

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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, isoepoxydon dehydrogenase (idh) gene involved in the biosynthesis of patulin were measured using real time-PCR technique. The result of this study found that the supplementation with B. breve and L. salivarius down-regulate the relative expression of isoepoxydon dehydrogenase (idh) gene . 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 the these bacteria.*

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

1. Introduction

The majority of important toxigenic 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 occur 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 produce the mycotoxin patulin, especially in apple products .

Patulin(C6H7O4) is alow molecular weight ,produced by fungi belonging to several genera including *Penicillium*, Aspergillus and Byssochlamys. PAT has been found as a natural contamination 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]. Studied 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).

2. Material and Methods

2.1. Chemicals

Patulin (98%) was obtained from Sigma Aldrich (Poole, UK), suspended in methanol at 5 mg ml_1 and stored at -20 °C.

2.2. P. expansum and Lactic Acid Bacteria (LAB) Culture

P. expansum (297959) was obtained from CABI (UK) and sub cultured on potato dextrose agar (PDA) at 25 °C (Oxoid, UK). When grown Lablemco-Tryptone Broth (LTB) over 10 d. *P. expansum* would typically produce 58.4± 1.0 μg ml^{-1} of patulin and with 0.01 % manganese supplementation this would raise to 347.5± 9.3 μg ml^{-1} .

The 2 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 (107 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 (107) and incubated for 4 days at 25°C prior to addition of 1 ml LAB (107) and incubated for a LAB suspension(106) and incubated for 48h at 37°C before 1 ml of spore suspension(106) was added ; the incubation was then continued for 8 days at 25°C. Group A control treatment consisted of 1 ml spores only (106) 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 the sample volume of ethyl acetate, drying over anhydrous Na2SO4, evaporating to dryness with a rotary evaporator at 48°C , redissolving in 2 ml chloroform, evaporating under N2, and serially diluting in 1% CAN for HPLC analysis [13]

Gene Expression Analysis Using Quantitative Reverse Transcription Polymerase Chain Reaction (QRT-PCR) For *P. Expansum*

1 . RNA Extraction and Reverse Transcription

RNA was extracted using Gene Elute HP plant RNA extraction kit using 100mg of fungal mycelium according to manufacturer's protocol. All consumables and reagents used were free of contaminating DNAase and RNAase. Fungal mycelium grown in co-cultivation was homogenised using liquid nitrogen to make a fungal powder. 100 mg of fungal powder placed in Eppendorf tube and 500μl of lysis solution added and the mixture incubated for 10 minute at 56°C water bath until mixture becomes viscous. Then, tubes centrifuged at 13000rpm for 10 min, clear supernatant transferred to RNAase minicolumn filtrations tube and centrifuged for 1 min. equal volume of 70% ethanol was added to the supernatant and mixed by repeated pipetting and transfered

to binding column, centrifuged 1min at 13000rpm. To avoid genomic DNA contamination, one-column DNAase-I was used to remove all DNA in the samples. 80 µl of DNAase and DNAase buffer was added to the samples for 15 min for DNA digestion, followed by washing with washing solution 1 and 2 and the RNA was eluted using 50µl elute buffer. RNA quantity and purity measured using nanodrop spectrophotometer (Fig 3) . RNA samples stored in the freezer at -80°C until using. Reverse transcriptase method used to make up *complementary DNA (cDNA)* using Moloney Murine Leukemia Virus (M-MLV) using random nonamer primers. 1 µg of each sample used to generate cDNA. All conditions performed using GeneAmp PCR System 9700 machine. RNA denatured at 70°C for 10 min in the presence of dNTPs (dATP, dCTP, dGTP, TTP) and random nanomers. Reactions were cooled on ice for 5 minutes and then 1 unit of MMLV-reverse transcriptase was added to each reaction followed by incubation for 10 min at 21°C, 37°C for 50 min and 94°C for 5 min. RTs were stored at 4° C until used. (14).

2. Real Time –PCR and Gel Electrophoresis

Primers were designed using NCBI Information website, and primers designed according to the mRNA sequence of each gene published in the same website , Primer for genes summarized in table 3 , while β-tubulin had used as housing keeping gene. Reverse transcription reaction had run using Taq DNA polymerase and Master mixture of RT reaction is described in table 4 in final volume of 25 µl under several conditions included 94°C for 2 minutes, followed by 40 cycles at 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. PCR product then verified by 1% (w/v) TAE agarose gel electrophoresis using 1 kb ladder , and the product conformed by its size after loading with loading buffer (Orange dye).The agarose dissolved in appropriate volume of TAE buffer (Tris-acetate-EDTA) and heated in a microwave for 2 minutes and then cooled to 50°C. 1µl of ethidium bromide (10mg/ml) was then added to the gel to enable visualization of DNA under UV light. Gel was run at 60-100V according to the size of the product. Bands were checked for presence and size using UV gel documentation system (15).

3. Quantitative Real-time Polymerase Chain Reaction (QRT--PCR)

Quantitative PCR experiments were carried out to compare semi-quantitative analysis of the gene expression in the experiment relative to the reference (control) group. Quantitative RT-PCR was performed using a Step One PCR system and using the DNA-binding dye SYBR green for detection of PCR products. All samples were run three times according to software protocol to confirm the results. Reactions containing the following: 2µl of cDNA samples added to a final reaction volume of 25µl which contained Taq DNA polymerase, SYBR green, PCR buffer, reference dye and specific sense and antisense primers. The thermal protocol for PCR reactions were:3 min incubation at 95 °C, followed by 40 reaction cycles: 15 s at 95°C, 30 s at 60°C, 20 s at 72°C where the fluorescent amplification signal was read. Melting curves for PCR products were adjusted between 60-90°C. The data were analysed based on the differences between the reference (control group) and the treatment groups using a comparative Ct analyses, using the following equation (16).

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

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

Amount of target (RQ) = $2^{-(\Delta\Delta Ct)}$, Where Ct is threshold cycle.

3. Results and Discussion

Patulin production is controlled by network of transcriptional factors including idh. Therefore, to evaluate the effect of probiotic bacteria on *P. expansum* production, the expression idh gene involved in patulin biosynthesis was assessed using real time quantitative PCR technique. QPCR results (Fig. 1– Fig. 2) 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. In contrast, the high peak of idh expression was noticed by *P. expansum* group treated with *Lb. salivarius* after 4 days. These findings indicated that both *B. breve* and *Lb. salivarius* bacteria were able to reduce patulin availability through effect on idh mRNA expression result in defective in the signaling network produced patulin toxin. Moreover, it is clear that patulin bioavailability was affected by *B. breve* and *Lb. salivarius* bacteria treatment in early stages of fungi growing. However, the reason to increase idh in the group treated with *Lb. salivarius* after 4 days is unknown, but may belong to the effect on other genes participate in patulin synthesis than modulating idh. Many factors govern production of secondary metabolites from multi-gene pathogens. (6) showed that the expression of genes of the secondary metabolism is strictly controlled by nutrients ,inducers, products, metals and growth rate, and in most cases, regulation is at transcription level. This assumption was also supported by (14).

The second deposited gene, isoepoxydon dehydrogenase (idh),is the seventh enzyme of the patulin biosynthetic pathway. It catalyzes a reversible oxidation reaction converting isoepoxydon to phllostine in fungi capable of synthesizing patulin and has an absolute specificity for $NADP^+$ (17 ; 18). (15) showed that the expression of idh gene ,6 msas gene and cytochrome P450 was to be higher under patulin –permissive conditions, indicating for the first time that regulation of patulin biosynthetic in *P. expansum* is mediated at the level of gene transcription.(19) founded that the supplementation with *B. breve* 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. 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.

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 . (20) 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 (14) 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.

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TABLE 1 : Primers used for PCR reactions for the target genes. The length of bases for the genes ranged between 90 and 170 bases.

Primer / Gene	Sequence 5'-3'	Product size	Accession No.
β-tub	Forward: 5' ATG GTA CCT CCG ACC TCC AGC -3' Reverse: 5'- CGG CAC GGG GAA CGT ACT TGT -3'	150	AF003248.1
Idh	Forward: 5'- TGT GCC CGG ACT GTC ACC AAT-3' Reverse: 5'- CGT CAA TTC GTC CGA CTC GCT-3'	160	DQ084388.1

TABLE II. Preparation Of Reverse Transcription Master Mix.

Chemical name	Volume
Sample (cDNA)	2 µl
10 mM dNTPs	1
MgCl2	1.5
PCR buffer (10x)	2.5
Taq DNA polymerase	1
Forward primer	1
Reverse primer	1
M.H_2O	15µl
Total volume	25µl

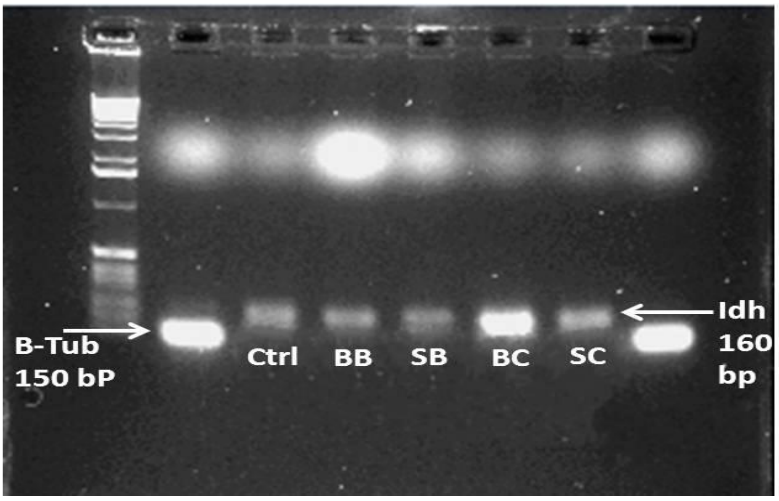

Fig. 1: RT-PCR amplification products obtained from *P. expansum* (patulin producer) with primers targeting genes involved in patulin biosynthesis (idh)(B:bacteria and mould growth at the same time,BB: *B.breve* and mould ; C:mould growth first for 4 days, SC: *Lb. salivarius* growth first).

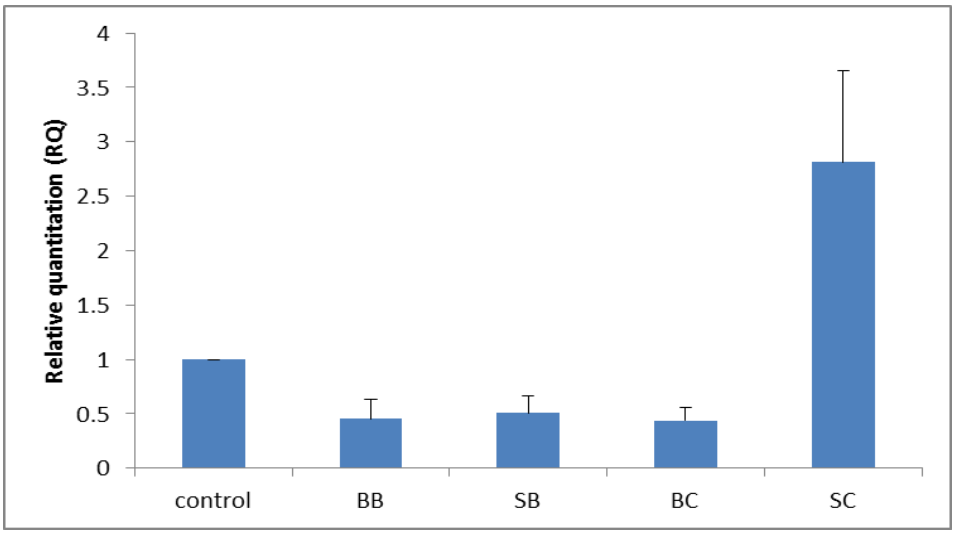

Fig. 2: Relative expression idh , genes in the toxigenic strain *P.expansum* grown for six days on LTB supplemented with *B. breve* and *L. salivarius.* Data were analysed using the 2−ΔΔCt method, and normalised for differences in the amount of total RNA added to each reaction using the β-Tub housekeeping gene.