The Egyptian Journal of Hospital Medicine (October 2022) Vol. 89 (2), Page 6438- 6444

Antibiofilm Activity of Fructophilic Lactic Acid Bacteria filtrate against *Klebsiella Pneumoniae* Virulence Gene Expression

[1*]Hussam Mahmood Hasan, [1]Ghada Mohammed Salih, and [2]Hameed M. Jasim
[1]Department of Biology, College of Science, University of Baghdad, Iraq; [2]Department of Molecular and Medical Biotechnology, College of Biotechnology, Nahrain University, Iraq
Email: hussam.alammar@sc.uobaghdad.edu.iq, **Mobile**: +9647704537478

ABSTRACT

Background: Fructophilic lactic acid bacteria (FLAB) are a group of LAB with unique growth features and are regarded as potential bioactive compound manufacturers. FLAB is discovered in D- fructose rich niches that favors D-fructose as a growth substrate triumphs over D-glucose. On D-glucose, they require electron acceptors to proliferate. The organisms have similar metabolic processes for carbohydrates. *Fructobacillus spp*. are *Lactobacillus kunkeei*, are members of this unique group.

Methodology: Inhibitory effect of filtrate from four FLAB isolates from honeybee was detected against the growth of pathogenic *Klebsiella pneumoniae* isolates. Biofilm formation, and the expression of *fim*H, *mrk*D, and *NDM*-1, and *OXA*-1 virulence genes were also tested.

Results: It was found that FLAB has antibacterial and antibiofilm effect. The incubation of *K. pneumoniae* with FLAB suspension at different three-time intervals (3, 6, and 9 hrs.) decreased expression of *fim*H, *mrk*D, *NDM*-1, and *OXA*-1 virulence genes compared with the expression of the same genes in the absence of FLAB.

Conclusion: all FLAB filtrate isolates tested, *K. pneumoniae fim*H, *mrk*D, *NDM*-1, and *OXA*-1 virulence gene expression was down-regulated, when compared to the control culture.

Keywords: Fructophilic lactic acid bacteria, *Klebsiella pneumoniae*, Virulence genes, Gene expression, Antibacterial activity

INTRODUCTION

Fructophilic lactic acid bacteria (FLAB) are cultivated in D-fructose-rich environments, including flowers, fruits, fermented fruits, and insects' gastrointestinal tracts [(1-3)]. They demonstrate relatively little growth on D-glucose as a growth substrate, but they actively metabolize D-fructose. Their growth on D-glucose is clearly improved by external electron acceptors, and O_2, pyruvate, and D-fructose are employed as electron acceptors.

Metabolically, FLAB belongs to a class of heterofermentative lactic acid bacteria (LAB), however end-product profiles from D-glucose metabolism clearly distinguish FLAB from other heterofermentative LAB. The main byproducts of the heterofermentative pathway of LAB are lactate, ethanol, and CO_2. When using FLAB, acetate replaces ethanol [(2,4)]. The fructophilic bacteria in honeybee guts have a positive effect on their hosts' health and length of life and are intriguing candidates for probiotics. These honeybee symbionts are abundant in fresh honey and play a crucial part in the production of honey by bees. Fresh honey is the best alternative for future wound healing because of its combination of osmolality and these bacteria's antibacterial and therapeutic characteristics [(5)]. The importance of FLAB as probiotics to enhance honeybee health has drawn considerable attention. These bacteria live in the digestive tracts of honeybees, shielding them from harmful infections and helping to maintain the good health of beehive colonies. FLAB indicates that bees' production of honey plays a significant influence. They support honey's medicinal and antibacterial qualities. Honey is a promising candidate to be used as a further aid in the therapy of wounds against various human and animal illnesses because it contains bioactive, antimicrobial compounds manufactured by FLAB and other LAB [(6)].

Two types of antigens are typically expressed on the cell surfaces of members of the *Klebsiella* genus. The lipopolysaccharide (LPS), which comes in nine different types, contains the first antigen, known as O. The second is K antigen, a polysaccharide capsular with over 77 different variants. Both support sero-grouping and contribute to pathogenicity[(7)]. According to the diseases they caused, the genus *Klebsiella* can be classified into three species: *K. pneumoniae*, *K. ozaenae*, and *K. rhinoscleromatis*. The *Klebsiella* species' most well-known and significant rod is *K. pneumoniae* and it accounts for 75-86% of all *Klebsiella* species[(8)]. *K. pneumoniae* is responsible for initiating enteritis, pneumonia, meningitis, and urinary tract infections that are recognized as pathogenic to humans. *K. pneumoniae* are also a common opportunistic pathogen in humans chiefly in immunocompromised and/or postoperative patients. In addition, *K. pneumoniae* is one of the most important pathogens that can product extended-spectrum β-lactamases, leading to its resistance to utmost antibiotic [(6)].

K. pneumoniae has a variety of strategies to thrive and protect itself from the host's immune due to its many virulence factors. The formation of hypercapsule in HV strains is one of the four main classes of virulence factors that have been thoroughly documented in *K. pneumoniae*. Other virulence factors include lipopolysaccharide (LPS), siderophores, and fimbriae,

Received:30/6/2022
Accepted:6/9/2022

sometimes known as pili, which simplify the microbe's ability to extent and evade body immune system, and cause infection of human hosts [9]. Therefore, this study aimed to learn the antibacterial influence of FLAB filtrate against *K. pneumoniae* and the expression of certain virulence genes; *fim*H, *mrk*D, and *NDM*-1, and *OXA*-1.

MATERIALS AND METHOD

Isolation of fructophilic lactic acid bacteria

Prior to honeybee segmentation, bees were disinfected to eliminate the exterior microbes with 94% alcohol solution, then immersed and rinsed in sterile distilled water [8]. Under a laminar flow hood, the entire nectar stomach was dissected aseptically with sterile forceps. Each bee's nectar stomach was taken out of the rest of the gut and put into sterile plane tubes with 10 ml of MRS broth and 2% fructose and 0.1% cysteine added to it. Homogenization was accomplished using a clean wooden applicator [10].

Identification fructophilic of lactic acid bacteria (FLAB)

From the homogenized mixture, 200µl was spread on a specific media using L-shaped glass spreader. MRS agar and broth were used for isolation and identification of FLAB. The MRS plates were incubated anaerobically in anaerobic jar at 28-30°C for 24–48 hrs [11]. The colonies were selected from each plate and moved to a new MRS plate for final purification. The colonies were well isolated and had the normal characteristics of being tiny, pure white, and having whole borders. Bacterial isolates were identified according to their morphological, cultural, physiological and biochemical features [11].

Antibacterial activity of FLAB bacterial filtrate

Fructophilic lactic acid bacteria filtrate was prepared according to **Seibert *et al***. [11], by cultivation FLAB in MRS broth containing 0.1% amino acid (cysteine) and 2% hexose sugar (fructose) anaerobically at 28-30°C during 24–48 hrs, then cultures were centrifuged at 6000 rpm for 5 min, and filtered throughout 0.22 µm Millipore filters. Minimal inhibitory concentration (MIC) of FLAB probiotic was determined by preparing serial dilutions (1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128).

The antibacterial activity of FLAB probiotic against *K. pneumoniae* isolates was done according to well diffusion method [13].

Antibiofilm activity of FLAB probiotic against *K. pneumoniae*

Antibiofilm activity of FLAB filtrate against *K. pneumoniae* isolates was performed using microtiter plate method [11]. After incubation of bacterial culture with FLAB filtrate, growth of *K. pneumoniae* isolates was measured at 460 nm, after staining with 1% crystal-violet, and dissolving the biofilm with an ethanol:acetone (70:30) mixture.

Expression of *K. pneumoniae* virulence genes

Effect of FLAB filtrate on the expression of *fim*H, *mrk*D, *NDM*-1, and *OXA*-1 was investigated **according to Carey *et al***. [14]. The most virulent isolate of *K. pneumoniae* obtained from previous study[15] was selected for this purpose as it was resistant to an extensive range of antibiotics, and the efficient biofilm producer **.**This isolate was incubated with FLAB filtrate at different incubation periods (3, 6, and 9 hrs.).

RNA extraction and cDNA synthesis

K. pneumoniae's total RNA was isolated in accordance with the company's guidelines for Enterobacteriaceae (RNeasy RNA isolation mini kit). Spectrophotometric measurements of RNA quantity (A260) and quality (A260/280 ratio) were made in molecular mark water and 10 mM Tris-HCl (pH 7.5), respectively. With the help of the reverse transcriptase kit, cDNA was created. Briefly stated, 2.5 µg of RNA was reverse transcribed with 1 µg/µl of each primer listed in table (1), 10 mM each dNTP, 4 µl 5× First Strand Buffer, 2 µl of 0.1 M DTT, 40U RNasin,(Biomolecular system, Australia).

Table (1): DNA nucleotide primers used for *K. pneumoniae* gene expression

Gene	Primer sequence (5'→3')	T_m (°C)	Amplicon Size (bp)	Reference
*fim*H	F:CGACCTCTCCACGCAGATTT R:CACGGTGCCTGAAAAACTCG	62	118	This primers were designed by Nabu foundation/Baghdad-Iraq
*mrk*D	F:CGGTGATGCTGGAACATGGT R:CCTCTAGCGAATAGTTGGTG	60	240	
NDM-1	F:ACAAGATGGGCGGTATGGAC R:GAAAGTCAGGCTGTGTTGCG	61.8	119	
OXA-1	F:TCTCCCAGTTAAAAACTCAGCCA R:TTGCTGTGAATCCTGCACCA	61.3	110	
16S rRNA	F:GGCAGGCTGGAGTCTTGTAG R:CCCAACATCTCACGACACGA	62.9	125	

Antibacterial activity of FLAB filtrate against *K. pneumoniae* isolates

Minimal inhibitory concentration (MIC) of 4 isolates of FLAB against *K. pneumoniae*, was determined. Results showed that among these isolates, FLAB No.3 isolate had the greatest antibacterial activity (15-18 mm inhibition zone) against thirty isolates of *K. pneumoniae*.

Ability of *K. pneumoniae* in biofilm formation

The ability of *K. pneumoniae* isolates in biofilm formation was examined. Result showed that *K. pneumoniae* isolates were variable in biofilm formation, in spite of all isolates had the ability to produce biofilm. Two isolates (6.67%) were classified as weak biofilm formers, 16 isolates (53.3%) as moderate biofilm formers, 12 isolates (40%) as strong biofilm formers with significant differences at (P≤0.01), as illustrated in Figure (1). All these isolates possessed *fimH* gene, and most of them (86%) possessed *mrkD* gene.

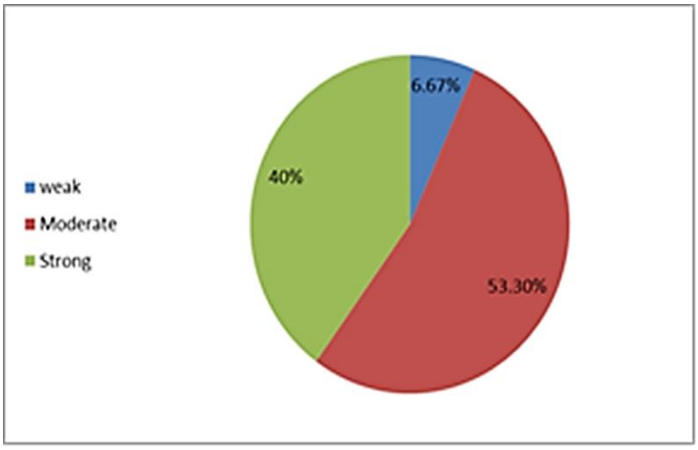

Figure (1): Ability of *K. pneumoniae* isolates in biofilm formation

Antibiofilm activity of FLAB filtrate

The antibiofilm activity of FLAB probiotic against *K. pneumoniae* isolates was tested. Results illustrated in figure (2) (showed that there is a different route for biofilm formation, where six isolates (20%) are weak in biofilm formation, 15 isolates (50%) are moderate, 9 isolates (30%) as still strong in biofilm formation without significant differences at (P>0.05). It was a clear that the ability of biofilm formation was affected slightly after treatment with FLAB suspension, where the percentage of strong biofilm former isolates were lowered from 40% before treatment to 30% after treatment with FLAB probiotic, moderate biofilm former isolates also was lowered from 53.3% before treatment to 50% after treatment with FLAB suspension, while weak biofilm former isolates increased from 6.67% to 20% after treatment with FLAB suspension.

Real-time PCR

A 25 (µl) net volume including 12.5 (µl) of Power SYBR® Green PCR Master Mix, 500 nM of each primer, 0.25U of reverse transcriptase, and nuclease-free water was used to real-time PCR amplification using the cDNA 1 (µl) as the template. Conditions for real-time PCR comprised: one cycle at (50 ºC) for (2 min) to activate UNG, one cycle at (95 ºC) for (10 min) and 40 cycles at (95 º for 15 s; 63 ºC for 1 min; 72 ºC for 30 s), followed by melt curve analysis, which involved heating the products to (95 ºC for 1 min), followed by cooling to (55 ºC) and slowly heating to (95 ºC) while monitoring fluorescence. At the conclusion of each cycle, fluorescent data were gathered using a quantitative PCR system. Using real-time PCR with a comparative critical threshold (Ct), relative mRNA levels were calculated. In other wells, treated and untreated controls were magnified. When comparing treated and untreated *K. pneumoniae*, the input quantity of RNA and the degree of expression were normalized using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The influence of FLAB on the expression levels of *K. pneumoniae fimH*, *mrkD*, *NDM-1*, and *OXA-1* was examined in the current investigation. Using comparative critical threshold (Ct) real-time PCR, mRNA levels were calculated, GAPDH an endogenous control, was used to normalize the data, and the levels of gene expression in the treated and untreated groups were compared.

Ethical approval

This study was approved from The Research Ethics Committee, College of Science Baghdad University. This work has been carried out in accordance with "Guide for the care and use of Laboratory Animals" for the use and welfare of experimental animals, published by the US National Institutes of Health (NIH publication No. 85–23, 1996).

The Statistical Analysis

System-SAS (2012) application was used to determine how various study parameters were impacted by various circumstances. The T-test was utilized to significantly compare between means. The Chi-square test was used to compare percentages. P<0.05 was considered significant.

RESULTS

Isolation of fructophilic lactic acid bacteria (FLAB). Fourteen isolates have been insulated from the guts of 40 honeybees. These isolates were identified as smooth with creamy white color on MRS agar. Results of biochemical tests showed that these isolates are catalase positive and hydrolyzing calcium carbonate on MRS plates.

6 and 9 hrs. of *K. pneumoniae* incubation with sub-inhibitory concentration of FLAB suspension compared with *NDM*-1 expression in the absence of FLAB suspension with significant differences at (P≤0.01), while 3 hrs of incubation didn't exhibit any change in gene expression.

On the other hand, results illustrated in figure (4) that expression of *OXA-1* decreased after 3 and 9 hrs. of *K. pneumoniae* incubation with FLAB suspension with significant differences at (P≤0.01), while 6 hrs of incubation didn't exhibit any change in *OXA-1* gene expression.

Furthermore, *fim*H gene expression, results indicated in figure (5) that the expression of this gene was downregulated after incubation of *K. pneumoniae* with FLAB suspension at all times intervals with significant differences at (P≤0.01). Finally, expression of *mrk*D was downregulated after incubation with sub-inhibitory concentration of *K. pneumoniae* with FLAB suspension at 6- and 9-hours intervals compared with *mrk*D expression in the untreated *K. pneumoniae* with significant differences at (P≤0.01), as shown in figure (6). Moreover, for all genes, incubation of *K. pneumoniae* with sub-inhibitory concentration of FLAB suspension exhibited highest deregulation of the studied genes among other incubation time and it was considered the most suitable for decreasing gene expression of the examined genes, and all these results were obtained with significant differences at (P≤0.01).

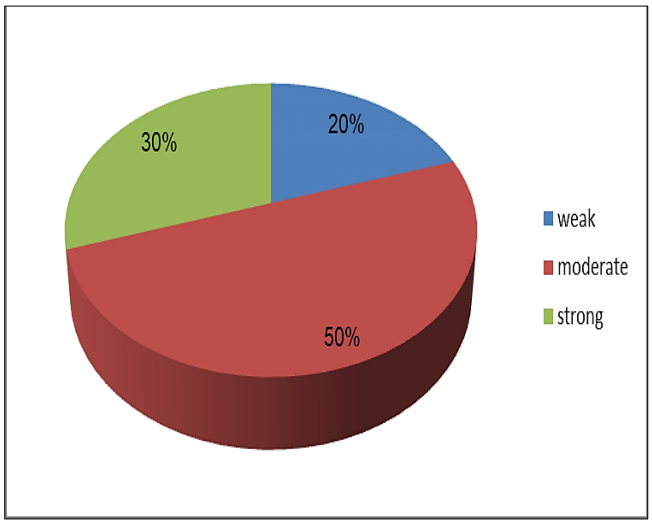

Figure (2): Showing percentage production of biofilm from *K. pneumoniae* after treatment with FLAB suspension

Effect of FLAB filtrate **on the expression of *K. pneumoniae* virulence genes**

Results showed that incubation of *K. pneumoniae* with sub-inhibitory concentration of FLAB suspension at different time intervals decreased expression of *fim*H, *mrk*D, *NDM*-1, and *OXA*-1 compared with the expression of these genes in the same isolates not incubated bacterium. Results illustrated in figure (3) showed that gene expression of *NDM*-1 decreased after

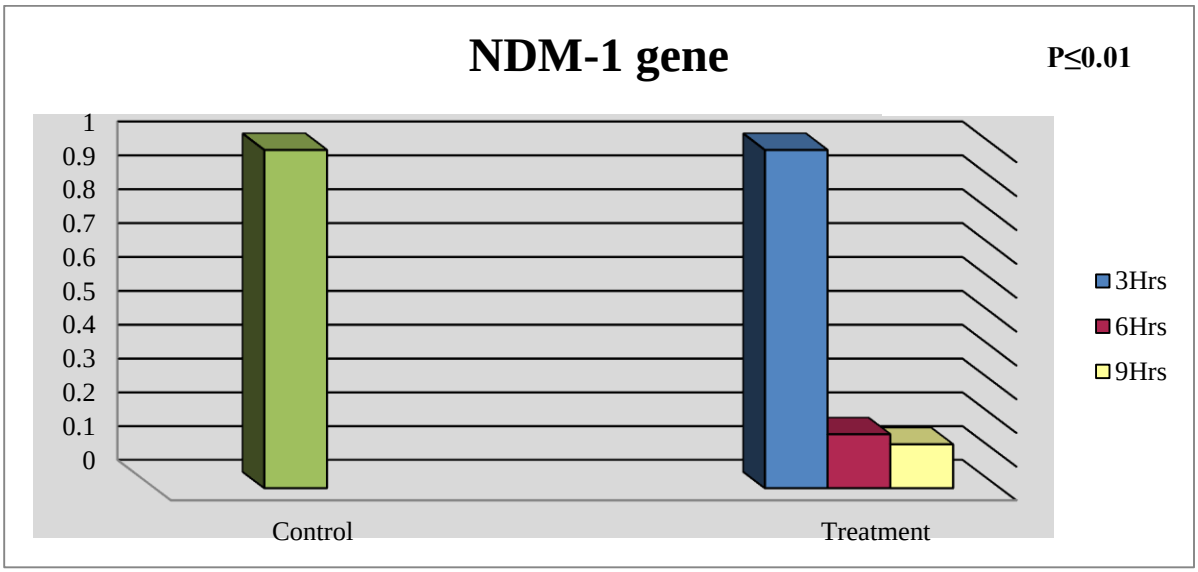

Figure (3): Fold expression of *K. pneumoniae NDM*-1 after incubation with FLAB probiotic for different time intervals using (comparative real-time PCR). The level of NDM-1 expression in (treated and untreated *K. pneumoniae*) was compared after the input amounts of RNA were normalized using the (housekeeping gene GAPDH)

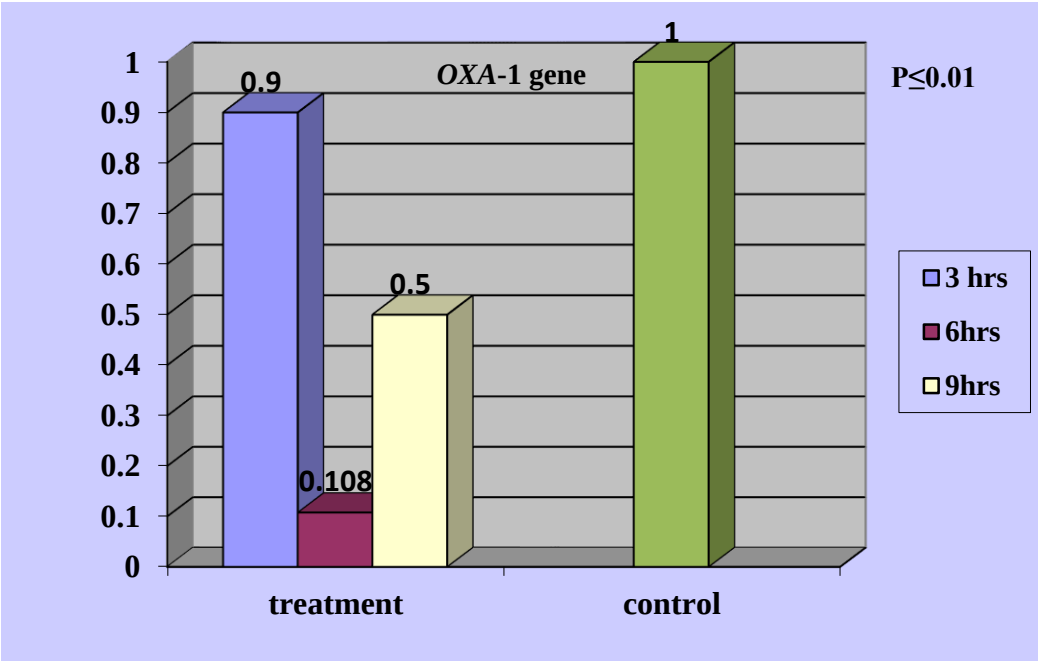

Figure (4): Fold expression of *K. pneumoniae OXA*-1 after incubation with FLAB for different time intervals by (using comparative real-time PCR).

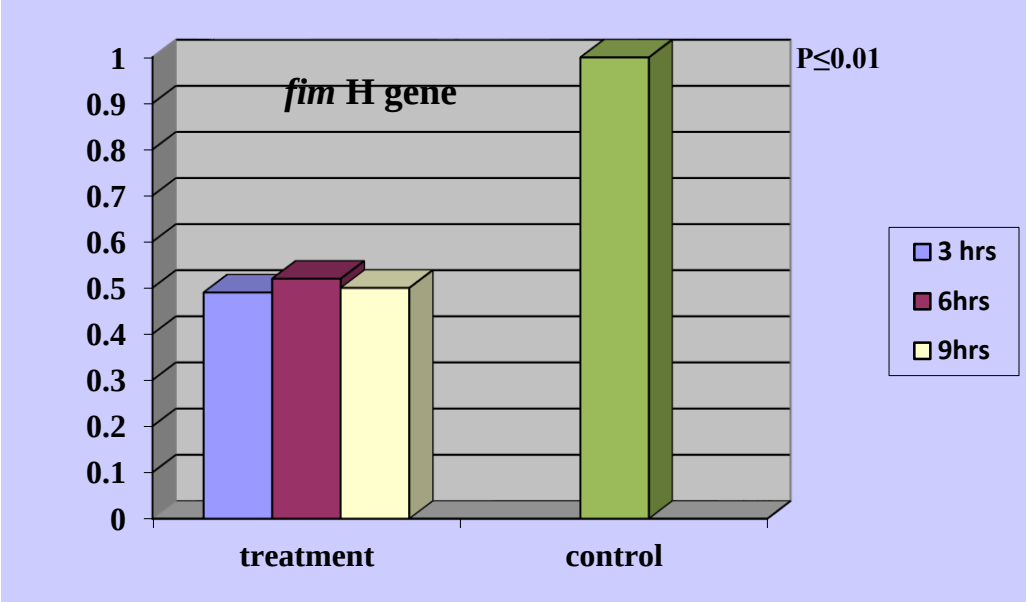

Figure (5): Fold expression of *K. pneumoniae fim*H after incubation with FLAB for different time intervals, (using comparative real-time PCR)

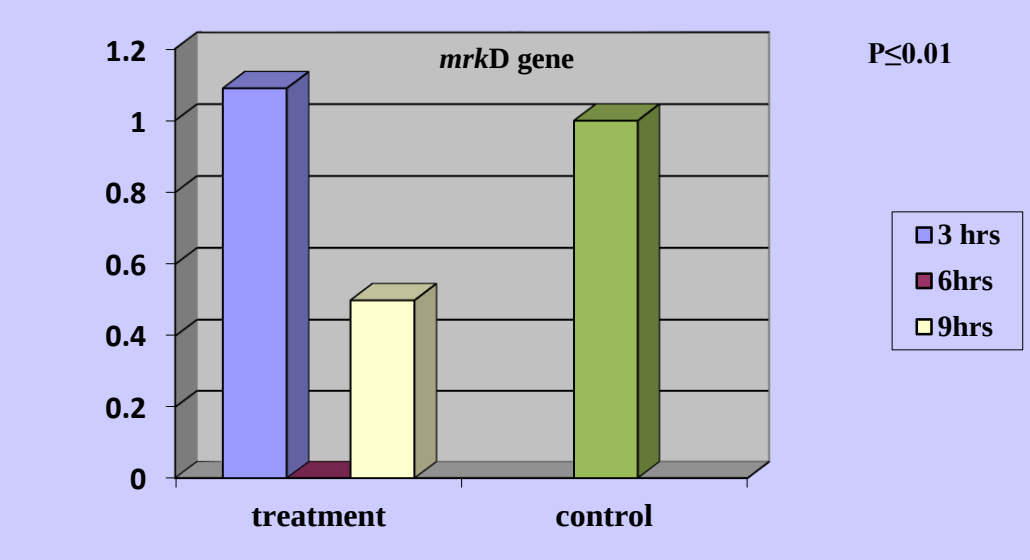

Figure (6): Fold expression of *K. pneumoniae mrkD* after incubation with FLAB for different time intervals (using comparative real-time PCR)

DISCUSSION

The effect of FLAB filtrate may be due to production of large quantities of probiotic metabolites such as acetate and lactate. These substances are killing and compete pathogenic isolates [(12)]. Some strains of FLAB are considered as probiotics for bees [(16)], while other strains originating from honey bees exhibits useful effects on health of humans, which increase the levels of secretory immunoglobulin A (IgA) [(17)].

Ballén *et al*. [(18)] identified 35 isolates of *K. pneumoniae* as weak biofilm formers (22.55%), 42 as moderate biofilm formers (33.07%), and 25 as strong biofilm formers (19.69%), and 25 isolates (19.69%) were considered as non-biofilm former. While **Ali *et al*.** [(19)] showed up one isolate (2.56%) that classified as moderate biofilm former, 21 isolates (53.85%) as weak biofilm former, and 17 isolates (43.59%) as strong biofilm formers. In another study, it was found that from 165 clinical isolates, 159 isolates had biofilm phenotype: 33 isolates (20%) had strong biofilm, 31 isolates (18.8%) had moderate biofilm, and 95 isolates (57.6%) produced weak biofilm, while 6 isolates (3.6%) had no biofilm [(19)].

The *fim*H and *mrk* D genes are responsible for production of type 1 and type 3 fimbriae respectively, which had a chief role in biofilm formation, Arota ***et al*.** [(20)] .

The results of antibiofilm were confirmed the effects of FLAB suspension on biofilm formation ability of the clinical isolates of *K. pneumoniae*, which may be due to the effect of FLAB suspension on the expression of *fim*H and *mrk* D genes that are responsible for type 1 and type 3 fimbriae formation respectively, that is as a result of lowering the clinical isolates' ability to form biofilm, Arota ***et al*.** [(20)] .

According to a number of research, probiotic bacteria may decrease the growth of *K. pneumoniae* [(21)]. However, it is unclear how FLAB suspension affects the expression of the virulence genes in *K. pneumoniae*.

Sugiyama *et al*. showed that FLAB extract has effect on nicotinamide mononucleotide (NMN), that is considered as an intermediate in nicotinamide adenine dinucleotide (NAD) biosynthesis. An essential cofactor in redox reactions in biological organizations is NAD [(21)].

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