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Research Article

Gene expression of *OmpA* and *EpsA* genes in clinical isolates of *Acinetobacter baumannii* treated with *Lactobacillus casei* and *Lactobacillus plantarum*

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Abstract: The study of gene expression was conducted by 8 *Acinetobacter baumannii* isolates. This study focused on effect of the *Lactobacillus casei* and *Lactobacillus plantarum* on Gene Expression of *OmpA* and *EpsA* Genes in *A. baumannii* isolates. The primers for *A.baumannii* targeting *OmpA* and *EpsA* genes are highly specific for expressing. Gene expression of *OmpA* and *EpsA* genes was quantified through Real- time PCR in *A.baumannii* isolates were before and after the treatment with the *Lactobacillus casei* and *Lactobacillus plantarum*, the isolates was selected that appeared highly Ct (cycle threshold) value. Ct value was determined as the point (cycle) at which the amplification plot crossed the threshold line. Eight isolates (AS-1, AS-2, AS-4, AS-5, AS-6, AS-7, AS-8 and AS-10) participated in their gene expression *OmpA* and *EpsA* genes. The qRT-PCR method has been used to examine the expression levels of the OmpA gene in *Acinetobacter baumannii* Isolates under the effect *Lactobacillus casei* and *Lactobacillus plantarum*. The results showed that the *Lactobacillus casei* and *Lactobacillus plantarum* increased the transcript levels of the tested gene in *A. baumannii* isolates with *Lactobacillus casei* and *Lactobacillus plantarum*. The results showed of (P1-P8) that *OmpA* gene gave a highly expression of different degrees by amplification of the gene. The Ct value of *Omp A* gene in the present study show the pattern of the amplification of the gene were (19.0, 23.49, 19.16, 19.14, 19.79, 20.03, 20.1 and 20.2) and folding (2.57, 0.23, 3.17, 2.59, 9.68, 5.73, 9.51 and 16.42). while (C1-C8) that OmpA gene expression recording Ct value (21.85, 21.37, 22.27, 21.83, 20.75, 20.25, 19.40 and18.59) and (1.03, 1.91,4.26,1.17,15.89,8.35,4.15 and 6.55) of folding compared to the control. The results showed of (P1-P8) that *eps A* gene gave a decrease expression of different degrees by amplification of the gene. The Ct value of *eps A* gene in the present study show the pattern of the amplification of the gene were (25.59, 25.41, 26.58, 26.09, 29.13, 30.48, 30.79 and 29.29) and folding (0.4, 1.1, 0.3,0.6,1.2,0.2,0.2 and 0.7). while (C1-C8) that *eps* A gene expression recording Ct value (27.37, 27.30, 30.54, 28.73, 30.56, 30.10, 30.34 and 28.66) and (0.4, 0.6, 0.2, 0.3, 1.4, 0.3, 0.1and 0.1) of folding compared to the control. Whereas gene expression of specific primer of rplB (Reference gene or House-keeping gene).

Keywords: *Lactobacillus,* gene expression**,** *OmpA, EpsA, Acinetobacter baumannii*.

INTRODUCTION

The *Acintobacter baumannii* growth appear non fermenter, small colonies 1-2 in diameter, white color, smooth surface, mucoid texture, non-hemolytic on blood agar, oxidase negative, catalase positive, citrate negative, aerobic, it is non-fastidious bacteria that easily grows in simple medium with single carbon and energy source. Also it can grow at a wide range of temperature degrees, grow optimally in 37˚C also can grow in 42-44˚C, and in different conditions of pH [1].

Acinetobacter spp. can cause infections in both hospital settings and in community. [2]. Infection caused by *Acintobacter baumannii* are formidable threats because of their ability to survive on inanimate objects and the remarkable emergence of Multi drug resistant (MDR) and pan-drug resistant (PDR) strains, these infections are difficult to treat owing to innate and acquired antimicrobial resistance [3].

Patients first become infected following colonization from the environment. Sources of contamination include surgical equipment, nasogastric tubes, and catheters. The length of stay at the intensive care units has repeatedly been associated with increased risk of colonization and infection. Colonization is usually asymptomatic but will increase the likelihood of subsequent infection, which may proceed when the host natural barriers are weakened by trauma, surgery or other invasive procedures [4].

Multiple bacterial virulence factors are required for pathogenesis of infections caused by *A.*

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baumannii; these include outer membrane proteins, gelatinase activity, biofilm production, capsular polysaccharides, bacterial phospholipases, penicillin-binding proteins, secreted outer membrane vesicles and siderophores [5]. As a class of important virulence factors in bacteria, outer membrane proteins (OMPs) have attracted much more attention. Among those OMPs of *A. baumannii*, *OmpA* is the most deeply studied virulence factor which plays key roles in regulating the adhesion, aggressiveness, and biofilm formation of *A. baumannii* and immune response of host. Other virulence factors such as exopolysacharide also have an important role in the invasion of these bacteria [6]. *Acinetobacter baumannii* produces a polysaccharide export outer membrane protein, called exopolysacharide or EPS, which is encoded by EpsA. EPS accumulates on the cell surface and provides protection to the cells against the harsh external environment [7]. Production of EPS is involved in the aggregation of bacteria which is associated with biofilm formation in many bacteria [8]. Lactic acid bacteria (LAB) are known as microorganisms that have probiotic properties Probiotics defined by the World Health Organization (WHO) , are live microorganisms that provide health benefits to hosts when ingested in adequate amounts [9, 10].

The mode of action by which a probiotic bacteria inhibit a pathogen can be facilitated by the production of antimicrobial substances such as bacteriocins and organic acids competition for nutrients, inhibition the adherence of pathogenic to epithelial and mucosal surface [11, 12] this study was aimed to examine the effect of probiotics on gene expression of *OmpA* and *EpsA* genes in *Acinetobacter baumannii* isolates.

MATERIALS AND METHODS

Table-1: Kits used in the RT-qPCR system

Kits	Company/ Origin
Chloroform	LiChrosolv, Germany
GoTaq® 1-Step RT-qPCR System, $MgCL_2$, Nuclease Free Water, Quantiflor RNA System.	Promega, USA
Isopropanol, 70% Ethanol	ROMIL pure chemistry, UK
Primers	Macrogen, Korea
TRIzol Reagent	Thermo Scientific, USA

Table-2: Primers used in RT-qPCR system

Primer name	Sequence	Annealing temperature
epsA-F	5`-AGCAAGTGGTTATCCAATCG-3`	
epsA-R	5`-ACCAGACTCACCCATTACAT-3`	
ompA-F	5`-CGCTTCTGCTGGTGCTGAAT-3`	50°C
ompA-R	5`-CGTGCAGTAGCGTTAGGGTA-3`	
rplB-F	5`GTAGAGCGTATTGAATACGATCCTAAC3`	
rplB-R	5`-CACCACCACCRTGYGGGTGATC-3`	

Sample Collection

This study was conducted during the period from December 2019 to April 2020, During the study period, eighty clinical burn wounds swabs samples were taken. Patients were interviewed and they answered several questions regarding personal information. Burn wounds samples from a total of 80 clinical different Wounds, samples were collected from Ghazi Al Hariri Hospital (40 samples) and Baghdad hospital (40 samples) in Baghdad / Iraq.

Culture media used for the detection of *Acintobacter baumannii* are Nutrient agar, Blood agar and MacConkey agar, Brain heart infusion aga and Chrmoagar media

Biochemical test used to detect *Acintobacter baumannii*:

Biochemical test

Biochemical test as Oxidase, Indole, Methyl red, Lactose fermentation, Citrate utilization, Catalase production and Urease test. were performed on the isolates to confirm their identification *Acintobacter baumannii*. All the tests were according to [13, 14], API (Analytical Profile Index), Identification Using Vitek 2 System.

Molecular Study

Quantitation of DNA

Quantus Fluorometer was used to detect the concentration of extracted DNA in order to detect the goodness of samples for downstream applications. For 1 µl of DNA, 199 µl of diluted Quanty Flour Dye was mixed. After 5 min. incubation at room temperature, DNA concentration values were detected.

Primer Preparation

Table-3: Preparation of the primer used in the present study

Primer Name	Vol. of nuclease free water (µl) water water (µl)	Concentration (pmol/µl) (pmol/µl)
epsA-F	300	100
epsA-R	300	100
ompA-F	300	100
ompA-R	300	100

These primers were supplied by Macrogen Company in a lyophilized form. Lyophilized primers were dissolved in a nuclease free water to give a final concentration of 100pmol/µl as a stock solution. A working solution of these primers was prepared by adding 10µl of primer stock solution (stored at freezer -20 °C) to 90µl of nuclease free water to obtain a working primer solution of 10pmol/µl.

Polymerase chain reaction to check the quality of DNA

Polymerase chain reaction master mix was prepared (with final volume 20 µl per one reaction) containing 10µM forward and reverse primers, 2 X of master mix, and 5µl of nuclease-free water was added until the volume reached to 17 µl. Then, 3 µl of DNA was added to mixture, as shown in Table-4. PCR tubes were transferred to the thermal cycler with the program used for amplification as shown in Table-5.

Table-4: Master Mix components of polymerase chain reaction

Master mix components	Stock	Unit	Final	Unit	V
Master Mix	2	X	1	X	10
Forward primer	10	µM	1	µM	1
Reverse primer	10	µM	1	µM	1
Nuclease Free Water					5
DNA		ng/µl		ng/µl	3
Total volume					20
Aliquot per single rxn	17µl of Master mix per tube and add 3µl of template				

Table-5: Polymerase chain reaction cycle

Steps	°C	m: s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	50	00:30	
Extension	72	00:30	
Final extension	72	07:00	1
Hold	10	10:00	

Agarose gel electrophoresis

After PCR amplification, agarose gel electrophoresis was adopted to confirm the presence of amplification. PCR was completely dependable on the extracted DNA criteria.

Preparation of agarose

- 100 ml of 1X TAE was taken in a beaker.
- 1 gm (for 1%) agarose was added to the buffer.
- The solution was heated to boiling (using Microwave) until all the gel particles were dissolved.
- 1µl of Ethidium Bromide (10mg/ml) was added to the agarose.
- The agarose was stirred in order to get mixed and to avoid bubbles.
- The solution was allowed to cool down at 50-60C°.

Casting of the horizontal agarose gel

The agarose solution was poured into the gel tray after both the edges were sealed with cellophane tapes and the agarose was allowed to solidify at room temperature for 30 minutes. The comb was carefully removed, and the gel was placed in the gel tray. The tray was filled with 1X TAE-electrophoresis buffer until the buffer reached 3-5 mm over the surface of the gel.

DNA loading

Polymerase chain reaction products were loaded directly. For PCR product, 10µl was directly loaded to well. Electrical power was turned on at 100v/mAmp for 75min. DNA moves from Cathode to plus Anode poles. The Ethidium bromide stained bands in gel were visualized using Gel imaging system.

RNA Purification

RNA was isolated from isolates according to the protocol of TRIzol™ Reagent as the following steps:

Sample Lysis

Cells grown in suspension:

For pellet calls, 1.4 ml of cells culture was precipitate by centrifugation for 2 min at 13000 rpm, supernatant then discarded and 0.6 mL of TRIzol™ Reagent was added to pellet. The lysate was homogenized by pipetting up and down several times.

For three phase's separation:

- For each tube, 0.2 mL of chloroform was added to the lysate, then the tube cap secured.
- All mixes were incubate for 2–3 minutes then centrifuge for 10 minutes at 12,000 rpm, the mixture was separate into a lower organic phase, interphase, and a colorless upper aqueous phase.
- The aqueous phase containing the RNA was transferred to a new tube.

For RNA precipitation:

- 0.5 mL of isopropanol was added to the aqueous phase and incubated for 10 minutes then centrifuge for 10 minutes at 12,000 rpm.
- Total RNA was precipitate formed a white gel-like pellet at the bottom of the tube.
- Supernatant then discarded.

For RNA washing

- For each tube, 0.5mL of 70% ethanol was added and vortex briefly then centrifuge for 5 minutes at 10000 rpm.
- Ethanol then aspirated and air-dried the pellet.

For RNA solubility:

- Pellet was rehydrated in 20-50 µl of nuclease free water then incubated in a water bath or heat block set at 55–60°C for 10–15 minutes.

Determine RNA, cDNA yield
Fluorescence method

Quantus Fluorometer was used to detect the concentration of extracted RNA in order to detect the goodness of samples for downstream applications. For 1 µl of RNA, 199 µl of diluted QuantyFlour Dye was mixed. After 5min incubation at room temperature in dark place, RNA concentration values were detected.

Table-6: Primer preparation and concentration

Primer name	Concentration (Pmol/µl)	Volume of nuclease free water (µl)
rplB- F	100	300
rplB- R	100	300

These primers were supplied by Macrogen Company in a lyophilized form. Lyophilized primers were dissolved in a nuclease free water to give a final concentration of 100pmol/µl as a stock solution. A working solution of these primers was prepared by adding 10µl of primer stock solution (stored at freezer -20 C) to 90µl of nuclease free water to obtain a working primer solution of 10pmol/µl.

Reaction Setup and Thermal Cycling Protocol
One Step RT-PCR

Polymerase chain reaction master mix was prepared, with total volume of 10 µl per one reaction for single sample, containing 5 µl of 2X of qPCR master mix, 0.25 µl of 1X of RT mix, then addition of 0.25 µl of $Mgcl_2$ as cofactor and 0.5 µl of 10 *p*mol forward and reverse primers, 2.5µl of Nuclease-Free Water was added. Then, 1 µl of RNA was added to mixture for each reaction, as shown in Table 7 & 8.

Table-7: Master mix components for polymerase chain reaction

Master mix components	Stock	Unit	Final	Unit	Volume
qPCR Master Mix	2	X	1	X	5
RT mix	50	X	1	X	0.25
MgCl2					0.25
Forward primer	10	µM	1	µM	0.5
Reverse primer	10	µM	1	µM	0.5
Nuclease Free					2.5
RNA		ng/µl		ng/µl	1
Total volume					10
Aliquot per single rxn	9µl of Master mix per tube and add 1µl of Template				

Table-8: Thermal cycling of reverse transcription polymerase chain reaction

Hold steps	Hold at 37°C for 15:00 min.
	Hold at 95°C for 5:00
Cycling 40 cycles	95°C for 15s
	50°C for 20s acquiring on Green
	72°C for 20s
Melt on Green	Melt from 72°C to 95°C at 0.3°C/s

Assessment of the extracted RNA

The concentration of the extracted total RNA was quantified by the use of Quantus fluorometer system following the manufacturer's instructions (Promega, USA). This assay was performed by adding 99 µl of Tris EDTA to 1 µl of extracted RNA in eppendorf tube and then 100 µl of Quanti Fluor RNA dye was added. The mixer was kept for 5 minutes at dark place. Then the quantity of RNA (ng/ml) was measured, the appropriate application was chosen (Nucleic acid, RNA).

The Taq Man minor groove binder (MGB) probes was contained:

1. A reporter dye (FAM™ dye) linked to the 5′end of the probe.
2. A minor groove binder (MGB) at the 3′ end of the probe. This modification increases the melting temperature (Tm) without increasing probe length, which allows the design of shorter probes.
3. A non-fluorescent quencher (NFQ) at the 3′end of the probe.

The 5′ nuclease assay process was occurred during PCR amplification. This process was occurred in each cycle and did not interfere with the exponential accumulation of product.

During PCR, the TaqMan MGB probe anneals specifically to a complementary sequence between the forward and reverse primer. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Förster- type energy transfer.

The DNA polymerase cleaves only probes that are hybridized to the target. Cleavage separates the reporter dye from the quencher dye; the separation of the reporter dye from the quencher dye results in increased fluorescence by the reporter. The increase in fluorescence signal occurs only if the target sequence is complementary to the probe and is amplified during PCR. Because of these requirements, nonspecific amplification is not detected.

Polymerization of the strand continues, but because the 3′end of the probe is blocked, there is no extension of the probe during PCR.

For reproducible and accurate results in RNA quantification, a normalization control was used in real-time PCR, where it was crucial to normalize the target RNA amount by the use of an appropriate reference RNA. This method is called a relative quantification. Factors that may result into inaccurate quantification are corrected through this normalization. These factors include differences in quantity of RNA input, probability of degradation of RNA, the availability of inhibitors in the samples of RNAs, as well as the variation in sample handling. Furthermore, normalization makes it possible to compare different samples directly.

Preparation of reverse transcription reaction for *OmpA* and *EpsA* genes:

1. Firstly, the kit components of Reverse transcription (RT) master mix were allowed to thaw on ice.
2. In a polypropylene tube, RT master mix was prepared by scaling the volumes listed below to the desired number of RT reactions as in Table-9.
3. Then, the components were gently mixed and centrifuged to bring solution to the bottom of the tube.
4. The RT master mix was placed on ice until preparation the microRNA reaction.
5. After that, the RT primer and RNA template were thawed on ice and before the use, by vortex the RT primer tube was mixed and then briefly centrifuged.
6. Each 10 µL RT reaction was consisted of 6.5 µL master mix, 2 µL RT primer, and 1.5 µL total RNA that added into a 0.2 ml polypropylene reaction tube. Then, gently mixed and centrifuged to bring the solution to the bottom of the tube.
7. After that, the tubes were loaded thermal cycler and the RT run was started according to following parameter values as in Table-10.

Table-9: Components of Master Mix of *OmpA* and *EpsA* genes for volume 10 µl Reaction

Component	**Master mix volume**
dNTPs (with dTTP) 100 mM	0.3µl
M.MLV Reverse transcriptase	0.15µl
Reverse Transcription Buffer 10x	2.0µl
RNase Inhibitor (20U/µl)	0.5µl
Nuclease-free water	3.55µl
Total	6.5µl

Table-10: Conditions for RT run for both genes

Step	**Time**	**Temperature**
Hold	30 minutes	16 °C
Hold	30 minutes	42 °C
Hold	5 minutes	85 °C
Hold	∞	4 °C

At the end of the incubation period, the product from RT reaction (cDNA) was stored in - 20°C to be proceeded with real-time PCR in the next day.

Preparation of quantitative PCR (qPCR) reaction for *OmpA* and *EpsA* genes

1. Firstly, thawing of the template cDNA (sample), Go Taq probe qPCR (master mix 2x), and primers for both genes on ice.
2. A reaction mix was prepared for a 10 µL per well reaction volume according to below as in Table-11. The template cDNA was dispensed into the individual tubes of the 0.2 ml optical 8-Tube strip.
3. The reaction was mixed thoroughly and gently, and then dispensed into the tubes of the optical 8- Tube strip containing template cDNA.
4. The optical 8-Tube strip was carefully and tightly sealed with the optical 8- Cap strip.
5. The sealed optical 8- Tube strip containing 10 µL reaction volumes were loaded into the real time PCR machine.
6. By the use of real-time PCR system software program, the comparative quantification experiment type was selected from the programming window. Then real-time PCR machine was programmed according to below as in Table-12, and the run was started.

Table-11: Components of reaction mix for *OmpA* and *EpsA* genes

Components	Volume per 10 µL reaction
Go Taq probe qPCR(MM 2x)	5.0 µL
OmpA primer	0.4 µL
EpsA primer	0.4 µL
Mgcl2	0.25 µL
Nuclease free water	2.45 µL
Product from RT reaction (cDNA)	1.5 µL

Table-12: PCR conditions for *OmpA* and *EpsA* genes

Step	°C	m: s	Cycle
RT. Enzyme Activation	37	15:00	
Initial Denaturation	95	05:00	1
Denaturation	95	00:15	
Annealing	50	00:20	40
Extension	72	00:20	

Analysis of data

Real time PCR machine analysis

Analysis of data was performed after the end of PCR reaction. Software associated with the Magnetic induction cycler quantitative PCR was used to find C_T values and to demonstrate the amplification plots as well as the dissociation curves for each reaction. The threshold settings were also the same across all runs of PCR included in the same analysis to permit comparison of results. Expression analysis was performed by the relative quantification.

Relative quantification (the $\Delta\Delta C_T$ method)

The $\Delta\Delta C_T$ method, also referred to as the Comparative C_T method, is a means of measuring relative quantification and was described by Livak and Schmittgen 2001. It determines the relative change in gene expression between a target gene under investigation and that of calibrator (control) gene. Most frequently, the untreated control is used as the calibrator.

The difference between the C_T of the target gene ($C_{T,\ target}$) and the C_T of the endogenous control ($C_{T,\ ec}$) is the ΔC_T of the sample:

$$"\Delta C_T = C_{T,\ target} - C_{T,\ ec}"$$

The term $\Delta\Delta C_T$ is calculated as the ΔC_T of the target gene in the treated sample minus the ΔC_T of the target in the untreated, calibrator sample:

$$\Delta\Delta C_T = \Delta C_{T,\ target\ in\ treated\ sample} - \Delta C_{T,\ target\ in\ calibrator\ sample}$$

The calibrator, since it is untreated, should have no change in its $\Delta\Delta C_T$ value during the course of the experiment. Its change, therefore, is equivalent to zero. Since 2^0 equal one, the calibrator gene's expression is unity.

When the $\Delta\Delta C_T$ method is used to measure gene expression, therefore, the results are expressed as a " fold" change in the expression level of the target gene normalized to the endogenous control and relative to the calibrator. It is given by the equation:

$$\text{Relative Fold Change} = 2^{-\Delta\Delta CT}$$

The amplification efficiency (E) for the sequence of interest was calculated by the PCR machine software.

RESULTS AND DISCUSSION

Effect of Probiotics (*Lactobacillus casei* and *Lactobacillus plantarum*) on Gene Expression of *OmpA* and *EpsA* Genes in *Acinetobacter baumannii* Isolates

From 70 samples inoculated on the Chromagar *Acinetobacter*, 10 isolate grew on the medium, *A. baumannii* isolates on the Chromagar were appeared as bright red colonies after 24h and incubation at 37° C, gene expression was conducted by 8 *A. baumannii* isolates.

Gene Expression Analysis of *OmpA* and *EpsA* Genes by Using Quantitative Reverse Transcriptase Real Time qRT PCR Technique

The study of gene expression was conducted by 8 *A. baumannii* isolates. this study focused on effect of the Lactobacillus casei and Lactobacillus plantarum on Gene Expression of *OmpA* and *EpsA* Genes in *A.*

baumannii isolates. The primers for *A.baumannii* targeting *OmpA* and *EpsA* genes are highly specific for expressing. Gene expression of *OmpA* and *EpsA* genes was quantified through Real- time PCR in *A.baumannii* isolates were before and after the treatment with the *Lactobacillus casei* and *Lactobacillus plantarum*, the isolates was selected that appeared highly Ct (cycle threshold) value. Ct value was determined as the point (cycle) at which the amplification plot crossed the threshold line. Eight isolates (AS-1, AS-2, AS-4, AS-5, AS-6, AS-7, AS-8 and AS-10) participated in their gene expression *OmpA* and *EpsA* genes

Total RNA Concentrations

Total RNA was successfully extracted from the isolates as mentioned in Table-13. The concentration of total RNA ranged from 13 to 42 ng / µl. Absorbance ratios were determined using a Nanodrop spectrophotometer.

Table-13: Total RNA concentrations (ng/ µl)

Sample	Conc.
P1	25
P2	21
P3	22
P4	23
P5	18
P6	15
P7	20
P8	13
C1	37
C2	39
C3	38
C4	36
C5	36
C6	34
C7	29
C8	42
CON.1	183
CON.2	155
CON.3	110
CON.4	123
CON.5	113
CON.6	157
CON.7	143
CON.8	107

Gene Expression of *OmpA* and *epsA* genes

Real time PCR Quantification of *OmpA* Gene Expression

The qRT-PCR method has been used to examine the expression levels of the OmpA gene in *Acinetobacter baumannii* Isolates under the effect *Lactobacillus casei* and *Lactobacillus plantarum*. The results showed that the *Lactobacillus casei* and *Lactobacillus plantarum* increased the transcript levels of the tested gene in *Acinetobacter baumannii* isolates with *Lactobacillus casei* and *Lactobacillus plantarum.* The results showed of (P1-P8) that *OmpA* gene gave a highly expression of different degrees by amplification of the gene. The Ct value of *Omp A* gene in the present study is shown in Table 14, 15 & 16 and Figures 1 & 2 that show the pattern of the amplification of the gene were (19.0, 23.49, 19.16, 19.14, 19.79, 20.03, 20.1 and 20.2) and folding (2.57 ,0.23, 3.17, 2.59, 9.68, 5.73, 9.51 and 16.42). while (C1-C8) that OmpA gene expression recording Ct value (21.85, 21.37, 22.27, 21.83, 20.75, 20.25, 19.40 and18.59) and (1.03, 1.91,4.26,1.17,15.89,8.35,4.15 and 6.55) of folding compared to the control.

Folding $=2^{-\Delta\Delta CT}$

$\Delta\Delta CT = \Delta CT$ Treated - ΔCT Control

ΔCT =CT gene - CT House Keeping gene

Table-14: Correlation between DCT and DDCT of *ompA* gene

Samples	rplB	*ompA*	DCT	DDCT	Folding
P1	24.96	19.00	-5.96	-1.36	2.57
P2	25.43	23.49	-1.94	2.12	0.23
P3	25.09	19.16	-5.94	-1.67	3.17
P4	25.30	19.14	-6.16	-1.37	2.59
P5	27.70	19.79	-7.91	-3.27	9.68
P6	27.24	20.03	-7.20	-2.52	5.73
P7	28.02	20.10	-7.92	-3.25	9.51
P8	28.07	20.20	-7.87	-4.04	16.42
C1	26.49	21.85	-4.65	-0.05	1.03
C2	26.36	21.37	-4.99	-0.93	1.91
C3	28.63	22.27	-6.36	-2.09	4.26
C4	26.84	21.83	-5.01	-0.22	1.17
C5	29.38	20.75	-8.63	-3.99	15.89
C6	28.00	20.25	-7.75	-3.06	8.35
C7	26.13	19.40	-6.73	-2.05	4.15
C8	25.14	18.59	-6.54	-2.71	6.55
CON.1	25.45	20.86	-4.60	0.00	1.00
CON.2	25.05	20.99	-4.06	0.00	1.00
CON.3	25.60	21.33	-4.27	0.00	1.00
CON.4	25.26	20.47	-4.79	0.00	1.00
CON.5	24.63	19.99	-4.64	0.00	1.00
CON.6	25.32	20.63	-4.68	0.00	1.00
CON.7	25.03	20.36	-4.67	0.00	1.00
CON.8	25.22	21.39	-3.83	0.00	1.00

P 1-8: *Lactobacillus plantarum; C 1-8: Lactobacillus casei*; CON: Control

Cycling: ompA	
Target	ompA → ompA
Normalisation	Dynamic
Ignore	First 1 cycles
Exclusion	Extensive with fluorescence cutoff of 5%
Threshold	3.477 starting at cycle 1

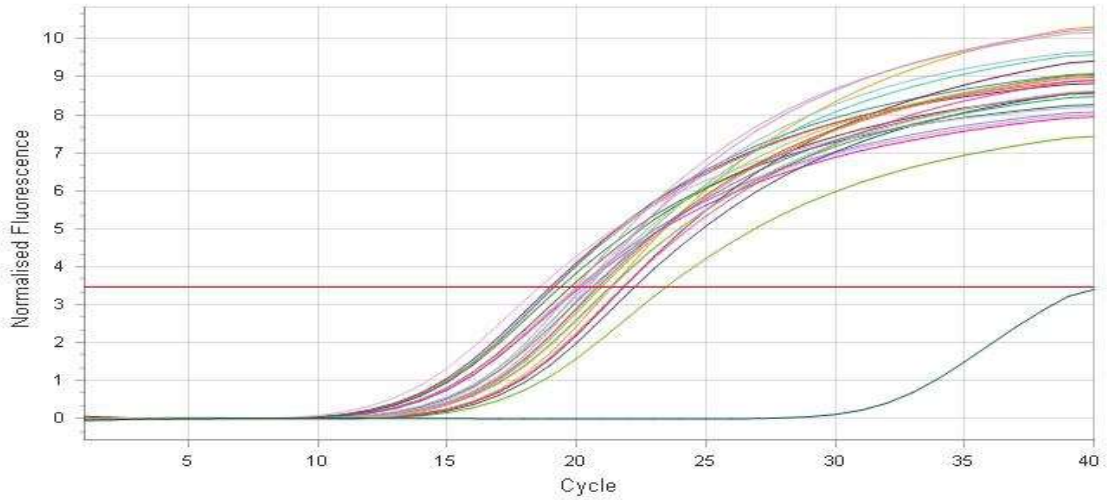

Fig-1: Amplification plots obtained by real time PCR machine for *ompA* gene cycling

Melt: ompA	
Target	ompA → ompA
Invert Data	No
Threshold	0.077 starting at 74.18°C

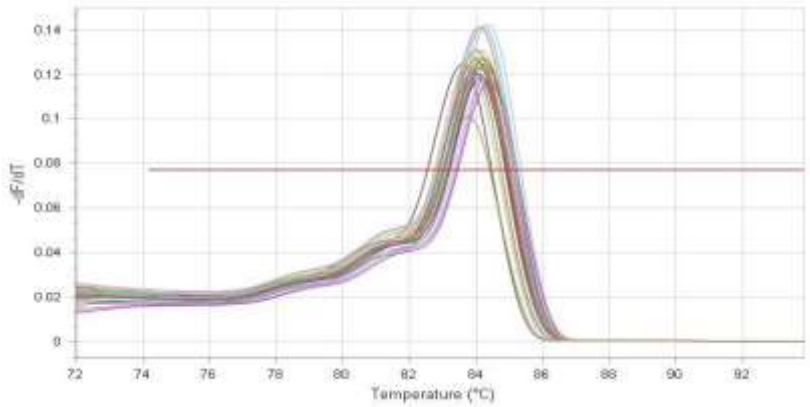

Fig-2: Amplification plots obtained by real time PCR machine for *omp A* gene melting temperature

Table-15: Correlation between efficiency and efficiency R2 in cycling

Well	Colour	Sample Name	Cq	Efficiency	Efficiency R^2
1		P1	19.00	0.67	0.99648
2		P2	23.49	0.60	0.99738
3		P3	19.16	0.67	0.99562
4		P4	19.14	0.69	0.99631
5		P5	19.79	0.65	0.99760
6		P6	20.03	0.64	0.99753
7		P7	20.10	0.65	0.99755
8		P8	20.20	0.64	0.99745
9		C1	21.85	0.64	0.99745
10		C2	21.37	0.70	0.99652
11		C3	22.27	0.64	0.99737
12		C4	21.83	0.66	0.99669
13		C5	20.75	0.74	0.99580
14		C6	20.25	0.73	0.99627
15		C7	19.40	0.65	0.99714

Well	Colour	Sample Name	Cq	Efficiency	Efficiency R^2
16		C8	18.59	0.66	0.99740
17		Cont.1	20.86	0.66	0.99645
18		Cont.2	20.99	0.67	0.99647
19		Cont.3	21.33	0.65	0.99722
20		Cont.4	20.47	0.71	0.99594
21		Cont.5	19.99	0.72	0.99739
22		Cont.6	20.63	0.64	0.99767
23		Cont.7	20.36	0.64	0.99758
24		Cont.8	21.39	0.64	0.99733
25		NC	-	0.68	0.99536

Table-16: Melting temperature obtained by real time PCR technique

Well	Colour	Sample Name	Tm (°C)
1		P1	83.57
2		P2	83.72
3		P3	83.96
4		P4	83.98
5		P5	84.20
6		P6	84.28
7		P7	84.36
8		P8	84.37
9		C1	84.01
10		C2	84.15

11		C3	84.12
12		C4	84.14
13		C5	84.19
14		C6	84.33
15		C7	84.23
16		C8	84.10
17		Cont.1	84.06
18		Cont.2	84.07
Well	**Colour**	**Sample Name**	**Tm (°C)**
19		Cont.3	84.07
20		Cont.4	84.10
21		Cont.5	84.07
22		Cont.6	83.88
23		Cont.7	83.81
24		Cont.8	83.79
25		NC	

The results showed of (P1-P8) that *eps A* gene gave a decrease expression of different degrees by amplification of the gene. The Ct value of *eps A* gene in the present study is shown in Table 17, 18 &19) and Figures-3, 4 & 5 that show the pattern of the amplification of the gene were (25.59, 25.41, 26.58, 26.09, 29.13, 30.48, 30.79 and 29.29) and folding (0.4, 1.1, 0.3,0.6,1.2,0.2,0.2 and 0.7). while (C1-C8) that *eps* A gene expression recording Ct value (27.37, 27.30, 30.54, 28.73, 30.56, 30.10, 30.34 and 28.66) and (0.4, 0.6, 0.2, 0.3, 1.4, 0.3, 0.1and 0.1) of folding compared to the control. Whereas gene expression of specific primer of rplB (Reference gene or House-keeping gene) showed as Table-20 & 21 and Figure-6.

Table-17: Correlation between DCT and DDCT of *epsA gene*

Samples	rplB	epsA	DCT	DDCT	Folding
P1	24.96	25.59	0.63	1.19	0.4
P2	25.43	25.41	-0.02	-0.16	1.1
P3	25.09	26.58	1.48	1.84	0.3
P4	25.30	26.09	0.80	0.76	0.6
P5	27.70	29.13	1.43	-0.20	1.2
P6	27.24	30.48	3.25	2.69	0.2
P7	28.02	30.79	2.76	2.18	0.2
P8	28.07	29.29	1.22	0.52	0.7
C1	26.49	27.37	0.87	1.43	0.4
C2	26.36	27.30	0.94	0.80	0.6
C3	28.63	30.54	1.90	2.26	0.2
C4	26.84	28.73	1.89	1.85	0.3
C5	29.38	30.56	1.18	-0.46	1.4
C6	28.00	30.10	2.10	1.54	0.3
C7	26.13	30.34	4.21	3.63	0.1
C8	25.14	28.66	3.52	2.82	0.1
CON.1	25.45	24.89	-0.56	0.00	1.0
CON.2	25.05	25.18	0.13	0.00	1.0
CON.3	25.60	25.24	-0.36	0.00	1.0
CON.4	25.26	25.30	0.04	0.00	1.0
CON.5	24.63	26.26	1.63	0.00	1.0
CON.6	25.32	25.87	0.56	0.00	1.0
CON.7	25.03	25.61	0.58	0.00	1.0
CON.8	25.22	25.92	0.69	0.00	1.0

Cycling: *epsA*	
Target	epsA → epsA
Normalisation	Dynamic
Ignore	First 1 cycles
Exclusion	Extensive with fluorescence cutoff of 5%
Threshold	1.410 (Automatic) starting at cycle 1

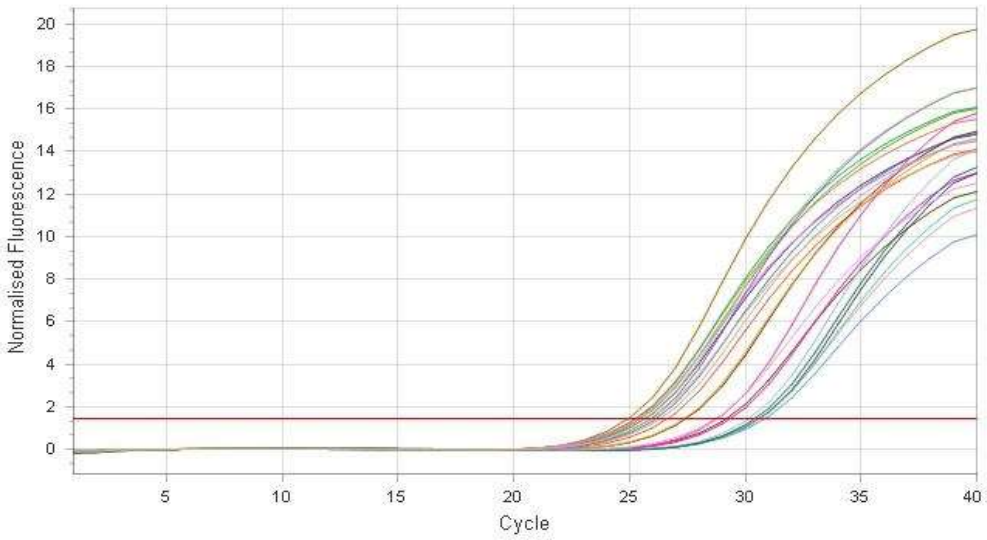

Fig-3: Amplification plots obtained by real time PCR machine for *eps A* gene cycling

Table-18: Correlation between efficiency and efficiency R2 in cycling

Well	Colour	Sample Name	Cq	Efficiency	Efficiency R^2
1		P1	25.59	0.73	0.99820
2		P2	25.41	0.74	0.99786
3		P3	26.58	0.73	0.99826
4		P4	26.09	0.75	0.99725
5		P5	29.13	0.72	0.99734
6		P6	30.48	0.72	0.99712
7		P7	30.79	0.69	0.99781
8		P8	29.29	0.73	0.99696
9		C1	27.37	0.74	0.99752
10		C2	27.30	0.74	0.99754
11		C3	30.54	0.74	0.99778
12		C4	28.73	0.81	0.99626
13		C5	30.56	0.73	0.99769
14		C6	30.10	0.80	0.99557
15		C7	30.34	0.78	0.99687
16		C8	28.66	0.71	0.99775
17		Cont.1	24.89	0.81	0.99619
18		Cont.2	25.18	0.73	0.99669
19		Cont.3	25.24	0.74	0.99744
20		Cont.4	25.30	0.70	0.99750

21		Cont.5	26.26	0.71	0.99738
22		Cont.6	25.87	0.79	0.99632
23		Cont.7	25.61	0.77	0.99783
24		Cont.8	25.92	0.70	0.99733

Melt: epsA	
Target	epsA → epsA
Invert Data	No
Threshold	0.100 starting at 74.18°C

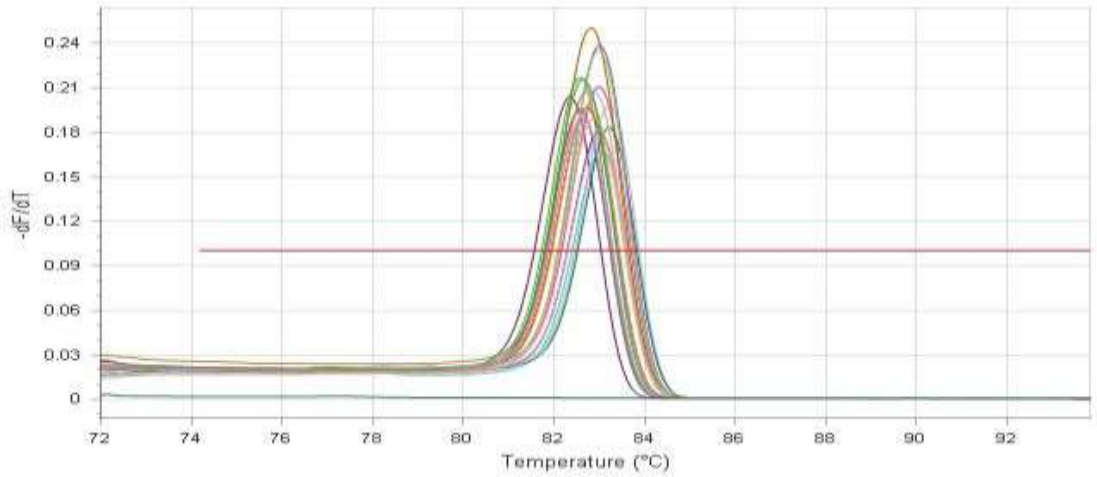

Fig-4: Amplification plots obtained by real time PCR machine for *eps* A gene melting temperature

Table-19: Melting temperature obtained by real time PCR technique

Well	Colour	Sample Name	Tm (°C)
1		P1	82.35
2		P2	82.57
3		P3	82.57
4		P4	82.57
5		P5	83.65
6		P6	83.77
7		P7	83.83
8		P8	83.81

9		C1	82.73
10		C2	82.74
11		C3	82.99
12		C4	82.99
13		C5	83.04
14		C6	83.22
15		C7	83.21
16		C8	83.00
17		Cont.1	82.82
18		Cont.2	82.72

Well	Colour	Sample Name	Tm (°C)
19		Cont.3	82.64
20		Cont.4	82.62
21		Cont.5	82.91
22		Cont.6	83.01
23		Cont.7	82.99
24		Cont.8	82.89
25		NC	

Cycling: rplB	
Target	rplB → rplB
Normalisation	Dynamic
Ignore	First 1 cycles
Exclusion	Extensive with fluorescence cutoff of 5%
Threshold	0.992 starting at cycle 1

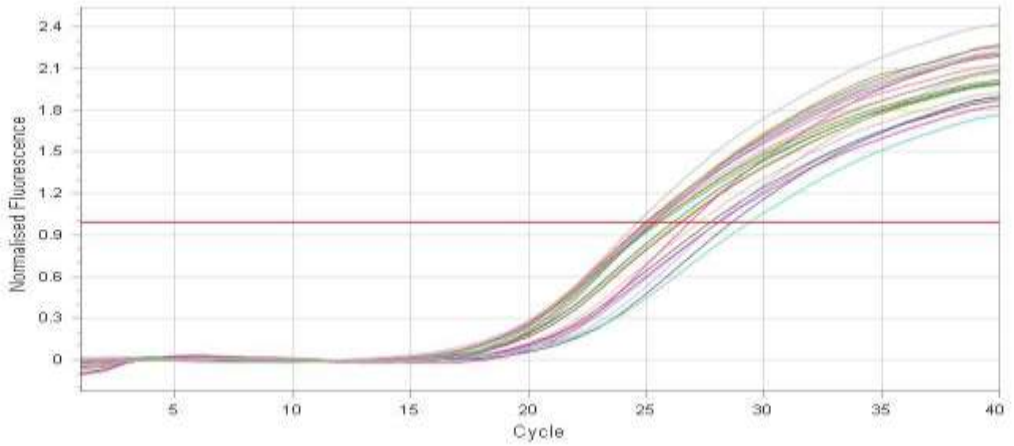

Fig-5: Amplification plots obtained by real time PCR machine for *rplB* gene cycling

Table-20: Correlation between efficiency and efficiency R2 in cycling

Well	Colour	Sample Name	Cq	Efficiency	Efficiency R^2
1		P1	24.96	0.87	0.99514
2		P2	25.43	0.58	0.99822
3		P3	25.09	0.80	0.99901
4		P4	25.30	0.79	0.99151
5		P5	27.70	0.61	0.99588
6		P6	27.24	0.61	0.99693
7		P7	28.02	0.56	0.99647
8		P8	28.07	0.57	0.99479
9		C1	26.49	0.60	0.99660
10		C2	26.36	0.59	0.99709
11		C3	28.63	0.58	0.99633
12		C4	26.84	0.78	0.99685
13		C5	29.38	0.46	0.99830
14		C6	28.00	0.56	0.99805
15		C7	26.13	0.61	0.99665

Well	Colour	Sample Name	Cq	Efficiency	Efficiency R^2
16		C8	25.14	0.48	0.99901
17		Cont.1	25.45	0.61	0.99680
18		Cont.2	25.05	0.60	0.99764
19		Cont.3	25.60	0.60	0.99719
20		Cont.4	25.26	0.61	0.99750
21		Cont.5	24.63	0.69	0.99535
22		Cont.6	25.32	0.85	0.99365
23		Cont.7	25.03	0.78	0.99372
24		Cont.8	25.22	0.80	0.99533
25		NC	-	0.66	0.99690

Melt: rplB	
Target	rplB → rplB
Invert Data	No
Threshold	0.079 starting at 74.19°C

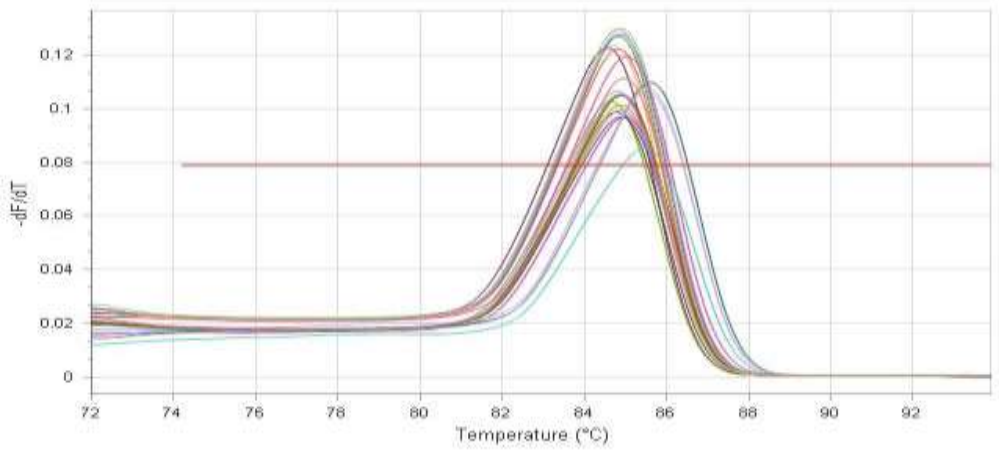

Fig-6: Amplification plots obtained by real time PCR machine for *rplB* gene melting temperature

Table-21: Melting temperature obtained by real time PCR technique

Well	Colour	Sample Name	Tm (°C)
1		P1	84.52
2		P2	84.61
3		P3	84.79
4		P4	84.79
5		P5	84.74
6		P6	84.82
7		P7	84.85
8		P8	84.94
9		C1	84.87
10		C2	84.86
11		C3	84.85
12		C4	85.00
13		C5	85.43
14		C6	85.48
15		C7	85.57
16		C8	85.50
17		Cont.1	84.88
18		Cont.2	84.93

Well	Colour	Sample Name	Tm (°C)
19		Cont.3	84.82
20		Cont.4	84.76
21		Cont.5	84.87
22		Cont.6	84.85
23		Cont.7	84.78
24		Cont.8	84.69
25		NC	

The infection symptoms caused by *Acintobacter baumannii* are closely associated with its virulence factor. But the virulence genes distribution in clinical *A. baumannii* was rarely reported, except for *bap*, *omp33-36* and traT [15]. Beneficial health effects of probiotic bacteria, is done by inhibition the growth of pathogenic microorganism isolates and their colonization in the gastrointestinal tract of humans [16].

The mode of action by which a probiotic bacteria inhibit a pathogen can be facilitated by the production of antimicrobial substances such as hydrogen - peroxide, bacteriocins and organic acids competition for nutrients, inhibition the adherence of pathogenic to the epithelial and mucosal surface [11, 12]. Saeed *et al.*, [17] study the effect of *Lactobacillus plantarum* on expression of biofilm genes of Streptococcus mutans, transcriptional levels of *GtfB* and *LuxS* genes were remarkably down regulated 9.43 -and 4.24- fold change, respectively, when treated with *lactobacillus plantarum* in comparison with the control group.Conclusions: *Lactobacillus plantarum* as probiotics can be used effectively to reduce the chance of dental caries by decreasing the effects of *S. mutans* and their gene expression to conserve good oral health Wasfi *et al.*, [18]. Using qPCR, examined the effect of *Lactobacillus* sp. spent supernatant on the expression levels of ten target genes (*gtfb, gtfc, gtfd, sacB, comC, comD, vick, vicR, aguD and atpD*) involved in glucan production, fructan production, quorum sensing and acid tolerance in *Streptococcus mutans*, the qPCR data were expressed as the fold change in expression levels of genes in *Streptococcus mutans* ATCC 25175 cells exposed to SCS of the four tested *Lactobacillus* sp. as compared to their levels in the untreated cells.

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