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The effect of *Lactobacillus reuteri* cell free supernatant on growth and biofilm formation of *Paenibacillus larvae*

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Abstract

Background: *Paenibacillus larvae* is the etiological agent of American foulbrood (AFB) disease, the most lethal disease in honeybee (*Apis mellifera*) larvae. **Aims:** The aim of the present work was to study the antimicrobial effect of cell free supernatant (CFS) of probiotics on an Iranian isolate's biofilm formation. **Methods:** A local strain was identified by *16S rRNA* sequencing. The antibacterial effect of some probiotics was evaluated through drop plate method, minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC) and time-kill assay. The biofilm formation ability of *P. larvae* and the inhibition of biofilm formation by CFS were studied by microplate and scanning electron microscopy (SEM). The nature of the secondary metabolites in CFS was examined by microscale optical density assay (MODA). **Results:** Alignment of the results of *P. larvae* KB10 (GenBank accession number MH000685.1) *16S rRNA* with the database revealed more than 97% identity with *P. larvae.* The most antibacterial effect was observed in the CFS of *Lactobacillus reuteri* ATCC23272 with 12.75 ± 3.2 mm for zone of inhibition (ZOI) at 1000 µL/ml for MIC and MBC. Time-kill assay revealed that CFS eliminated 1.5×10^8 CFU/ml *P. larvae* KB10 at 2 h of exposure. Microtitre plate and SEM results revealed that CFS (at sub-MIC concentration) was able to inhibit biofilm formation by *P. larvae*. The results of MODA assay showed that antimicrobial activity were related to the production of organic acids. **Conclusion:** Cell free supernatant from *L. reuteri* ATCC 23272 had inhibitory effects on *P. larvae* KB10 growth and biofilm production due to its acidic nature. The obtained results can be used for antibiotic substitution in AFB control and treatment.

Key words: Anti-bacterial agents, Biofilm, *Lactobacillus reuteri*, *Paenibacillus larvae*, Virulence factors

Introduction

American foulbrood (AFB) disease, a devastating disease caused by *Paenibacillus larvae* spores, affects honeybee (*Apis mellifera*) larvae (Hamdi *et al*., 2013). Antibiotics treatment of AFB has risk of the residues in honey and also increases antibiotics resistance level in the pathogen (Alippi *et al*., 2007).

Bacteria in microbial populations exist in floating state and inside the biofilm. A biofilm is a growing microbial population on surfaces within a biopolymer matrix consisting of microbial organic materials. Regulation of gene expressions within the biofilm by phenotypic variation, food shortage and antimicrobial agents cause the bacteria to adapt to existing conditions (Garrett *et al*., 2008; Wei and Ma, 2013). It is essential to find a new therapeutic approach for the treatment of biofilm-related infections without using antibiotics. Previous research shows the prohibition of pathogen biofilms with the use of probiotic products (Fang *et al*., 2018).

Probiotics adopt different strategies such as producing organic acids (Mudroňová *et al*., 2011), antimicrobial compounds (Cleusix *et al*., 2007) and colonization (Dhanani and Bagchi, 2013) to inhibit or kill pathogens. Usually, probiotics do not have side effects on the host, cause environmental hazards or induce resistance. Many articles are published about the effects of probiotics on the *in vitro* prevention of *P. larvae* growth (Jaouani *et al*., 2014). *Lactobacillus* spp. produce several secondary metabolites which have inhibitory effects on biofilm formations of pathogens (Vahedi Shahandashti *et al*., 2016; Zamani *et al*., 2017). The possible effects of *Lactibacillus* spp. secondary metabolites on the growth and biofilm formation of *P. larvae* has not been studied earlier. The aim of this research was to evaluate the effect of *Lactobacillus reuteri* ATCC23272 cell free supernatant (CFS) on the biofilm formation, growth and viability of the *P. larvae* local isolate. Presumptive identification of effective secondary metabolites with inhibitory properties in the crude CFS was also undertaken.

Materials and Methods

Isolation and identification of *P. larvae*

Paenibacillus larvae were isolated from comb pieces of Isfahan apiaries according to Dingman and Stahly

(1983) on Muller Hinton yeast pyruvate glucose phosphate agar (MYPGP-agar) and Columbia sheep blood (CSA) agar by 7 days incubation at 37°C in a CO_2 gas pack containing jar (Cornman *et al*., 2013). Gram-positive, catalase negative, spore-forming bacilli were further examined for biochemical characteristics (Ayoub *et al*., 2013). Extracted DNA (Pouya Gene Azma kit) was amplified using the universal primers (Sinaclon) 27F (5´-AGA GTT TGA TGM TGG CTC AG-3´) and 1492R (5´-GGT TAC CTT GTT ACG ACT T-3´) (Mao *et al*., 2012). Polymerase chain reactions (PCR) were performed in a final volume of 50 µL (1 µM of each oligonucleotide primer, 200 µM dNTPs, 1 mM $MgCl_2$, 0.05 U/µL DNA *Taq* polymerase, 19 µL deionized water, 5 µL from 6× buffer) (all from Sinaclon) and 2 µL DNA (295.9 ng/µL). Two tubes without DNA were used as negative control. Polymerase chain reaction was performed as follows; 2 min denaturing step at 94°C followed by 30 cycles consisting of DNA denaturation at 94°C, 50 s, primer annealing at 59.1°C, 30 s; DNA elongation at 72°C, 1 min; final extension at 72°C, 10 min). Polymerase chain reaction products were then mixed with SYBR-gold and loaded in 1% w/v agarose gel (Sinaclon) prepared in 1× TBE buffer (Tris base 0.446 M, Boric acid 0.445 M, 20 ml EDTA 0.5 M, pH = 8). After electrophoresis with 100 v DC for 1 h, bands were visualized with Gel Doc (VILBER E-Box VX5). Gene sequencing was later performed using automated DNA sequencing (Bioneer, Korea). Pair wise and multiple DNA sequence alignments were then carried out comparing *16S rRNA* gene sequences available by basic local alignment search tool (BLAST) search in the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) using Bioedit 7.2.5 software. The sequences were submitted to GenBank for accession numbers. The phylogenetic tree was constructed by the neighbor-joining (NJ) method using the Molecular Evolutionary Genetic Analysis 7.0 (Mega version 7.0).

Preparation and antimicrobial activity of CFS from different probiotics

Overnight cultures of *Lactobacillus acidophilus* ATCC4356, *L. plantarum* ATCC8014, *L. reuteri* ATCC23272, *L. fermentum* ATCC9338, *L. rhamnosus* ATCC7469 in de Man, Rogosa and Sharpe (MRS)-broth (Merck)(pH = 5.4) in 5% CO_2 at 37°C were centrifuged and filter sterilized (0.22 µm) (Millipore) to achieve CFS. *Paenibacillus larvae* in MYPGP-broth (OD_{595}=0.1) was lawn cultured on MYPGP-agar after which a drop of 20 µL CFS was put on the culture. The zone of inhibition (ZOI) on the droplets' place was evaluated after 48 h incubation at 37°C. Sterile MRS-broth was used as negative control. Broth microdilution method was carried out to determine minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of CFS. One hundred fifty µL of CFS two-fold serially diluted in MRS-broth was dispensed in wells of a 96 well polystyrene microtitre plate (Sorfa). Each well was inoculated with 150 µL of overnight culture of *P. larvae* in MYPGP-broth (OD_{595}=0.1) then diluted 1/100. Minimum inhibitory concentrations and MBC were defined as Balouiri (Balouiri *et al*., 2016) comparing positive (MRS-broth instead of CFS) and negative (sterile MYPGP-broth) controls after 24 h at 37°C (Balouiri *et al*., 2016). A time-kill test was performed according to Bajpai with CFS at MIC concentration compared to the positive control (bacterial culture in MYPGP-broth).

Biofilm formation ability

Microplate technique was used to assay biofilm forming according to Cai *et al*. (2013). Optical density (OD) was read at 595 nm by a microplate reader (Epoch BioTek). The cut-off (OD_C) was defined as the mean OD value of the negative control (sterile MYPGP-broth). Biofilms were classified as no ($OD \leq OD_C$), weak ($OD_C < OD \leq 2\times OD_C$), moderate ($2\times OD_C < OD \leq 4\times OD_C$) or strong ($4\times OD_C < OD$) (Gómez *et al*., 2016).

Inhibition of biofilm formation by CFS

The effect of CFS on biofilm formation was assessed by co-incubation of *P. larvae* in MYPGP-broth (OD_{595}=0.1) with the same volume of CFS at sub-MIC concentration (1/2 CFS prepared in MRS-broth). For positive control, MRS-broth was used instead of CFS (Wojnicz *et al*., 2013). The inhibition of biofilm formation was calculated according to Vahedi Shahandashti *et al*. (2016).

Effect of CFS removal on biofilm

After 24 and 48 h of biofilm formation, its removal by CFS was assessed according to Vahedi Shahandashti *et al*. (2016). In the positive control, phosphate buffered saline (PBS) was used instead of CFS.

Microscopic observation techniques

Microscopic observation of biofilms on glass slides was evaluated using MYPGP-broth culture samples with CFS, comparing biofilms formed in sterile MRS-broth (positive control) and MYPGP-broth (untreated biofilm), according to Vahedi Shahandashti *et al*. (2016) after 48 and 96 h at 37°C and studied using scanning electron microscopy (SEM) (Zeiss).

Effects of different treatments on CFS

The growth inhibitory effect of CFS was examined by microscale optical density assay (MODA) following different treatments of NaOH, acetic acid, trypsin, pepsin and catalase after 24 h at 37°C by absorbance at 595 nm according to Lash *et al*. (2005). The growth was evaluated as the difference in absorbance between the control and the samples (Vahedi Shahandashti *et al*., 2016).

Statistical analysis

Statistical analyses were conducted using SPSS 20. One-way analysis of variance (ANOVA) and independent samples t-tests were performed after assumptions of normality and variances of homogeneity

were checked. Significance levels were set at P<0.05.

Results

Isolation and identification of *P. larvae*

Isolates of *P. larvae* were identified on the basis of cultural and biochemical characteristics. Sequence alignment of a partial *16S rRNA* amplified gene revealed that the bacterial strain was *P. larvae* with 100% identity to *P. larvae* strains and 97.48% identity with standard strains of *P. larvae larvae* (DSM 25430) in the database (NCBI). The sequences were submitted to GenBank. Accession numbers are mentioned on phylogenetic tree in Fig. 1.

Antimicrobial activity of CFS

According to the results of five repetitions, CFS of *L. reuteri* ATCC23272 was able to inhibit the growth of *P. larvae* KB10, with a larger ZOI (12.75 ± 3.2 mm) comparing to other tested probiotics. No growth inhibition was seen at the the negative control drop. Broth microdilution method showed that CFS from *L. reuteri* ATCC23272 with a concentration of 1000 µL/ml (1/1 v/v CFS/MRS) inhibited the growth of *P. larvae* KB10 and destroyed 99.9% of the living cells. Therefore, this concentration was considered as MIC and MBC of CFS from *L. reuteri* ATCC23272 (Fig. 2A). A time-kill test of CFS revealed significant inhibitory effects of CFS on *P. larvae* KB10 by the complete inhibition of cell viabilities during the first 2 h of exposure. The inhibition effect was directly related to CFS as compared to the control tube containing MRS-broth (Fig. 2B).

CFS effects on biofilm formation by *P. larvae* KB10

Biofilm formation ability, investigated by the microplate method, indicated that *P. larvae* KB10 was able to form biofilm. The biofilms in 24 and 48 h were weak (OD=0.181) and moderate (OD=0.243), respectively; compared to the negative control (sterile MRS-broth) (OD=0.117) (Fig. 3A). Inhibition of biofilm formation by CFS (at sub-MIC concentration) showed a significant reduction of 50% and 37.29% in 24 and 48 h exposure to CFS, respectively (P<0.05), compared to the positive control (biofilm in exposure to MRS-broth) (Fig. 3B).

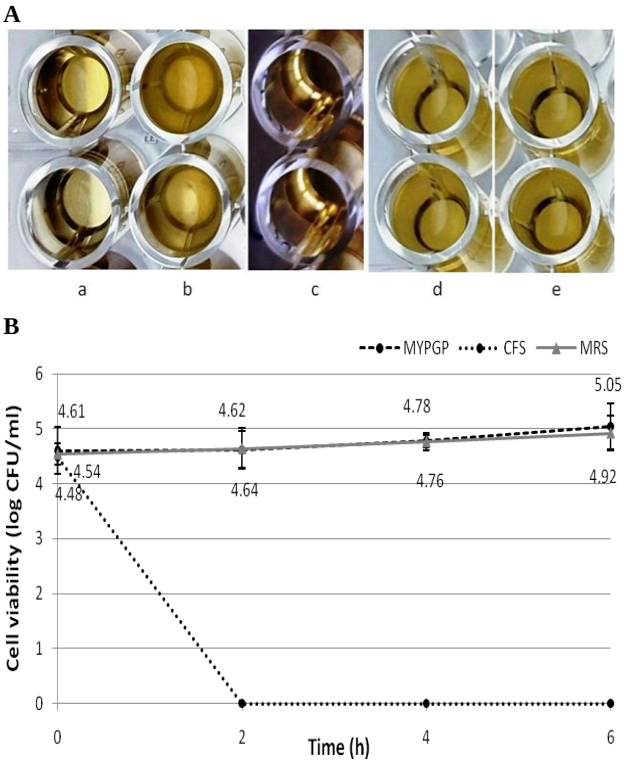


Fig. 2: Antimicrobial activity of cell free supernatant (CFS): (**A**) Microdilution plate of two fold dilutions of CFS in sterile de Man, Rogosa and Sharpe (MRS)-broth: 1000 µL/ml (a), 500 µL/ml (b), sterile Muller Hinton yeast pyruvate glucose phosphate agar (MYPGP) broth (negative control) (c), *P. larvae* KB10 in MYPGP broth (positive control) (d), and *P. larvae* KB10 in (MYPGP broth+MRS broth) (positive control) (e). (**B**) Time-kill test of CFS on *P. larvae* KB10. Data are the average of four replications ± SD

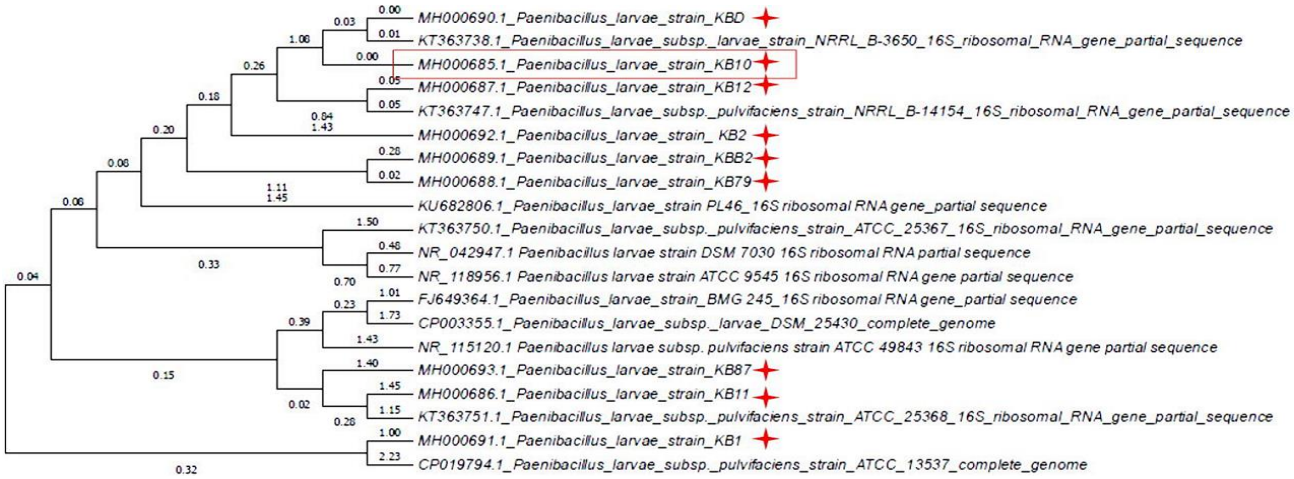


Fig. 1: Evolutionary relationships of taxa: the evolutionary history was inferred using the neighbor-joining method. The optimal tree with the sum of branch length = 24.22610255 is shown (next to the branches). The stars represent Iranian isolated strains. The strain in the box was studied in this research

The evaluation of CFS removal effects on formed biofilms showed no significant differences with the control (PBS treated biofilm) (P>0.05) (Fig. 3A). Microscopic observations confirmed biofilm formation in MYPGP-broth (Fig. 4A) and MYPGP/MRS-broth (Figs. 4B and C). Inhibition of biofilm formation in exposure to CFS was also confirmed in microscopic observations (Figs. 4D and E).

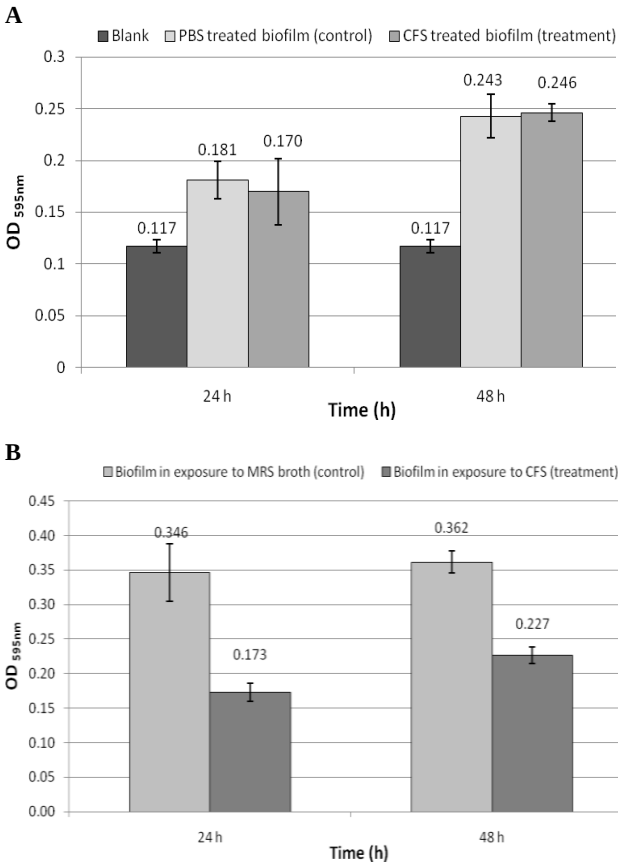
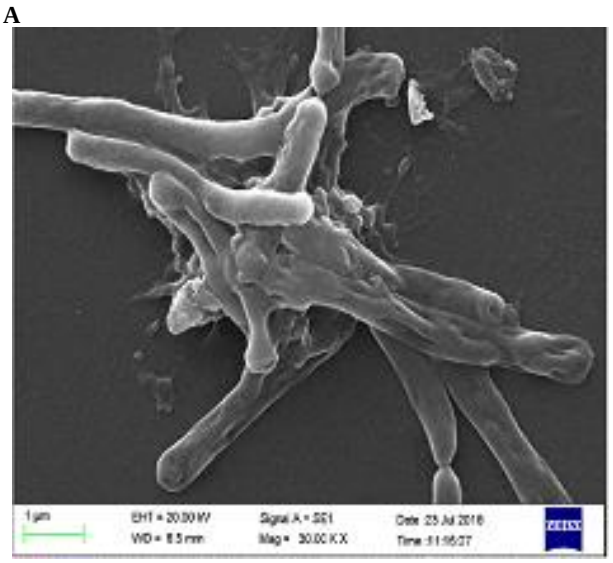
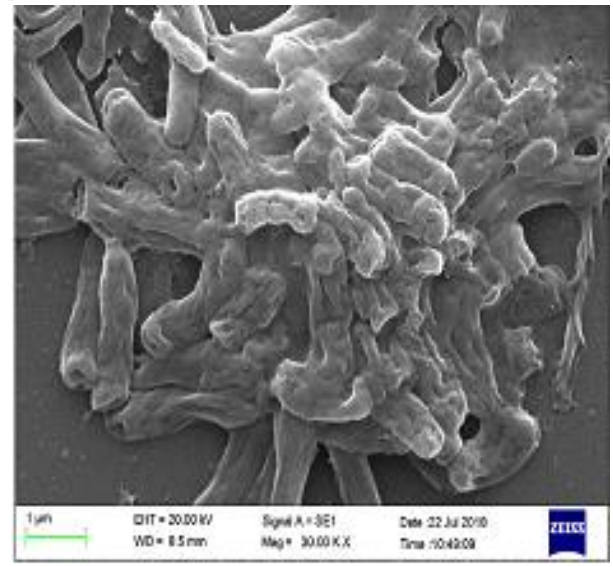
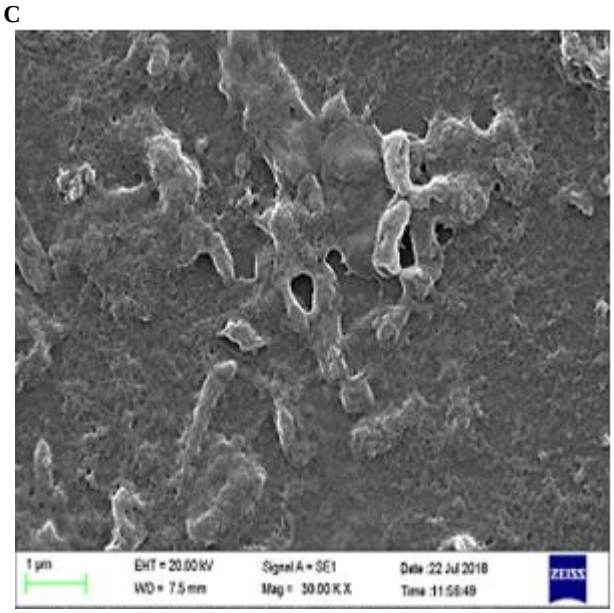
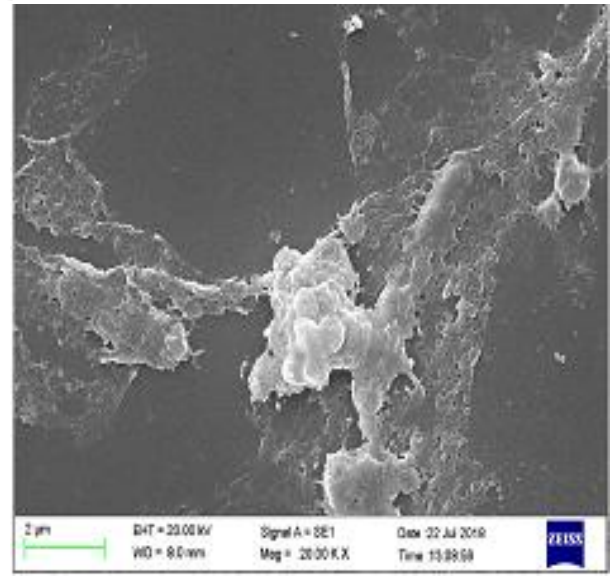


Fig. 3: Effects of cell free supernatant (CFS) of *L. reuteri* ATCC23272 at sub-MIC concentration (500 µL/ml) on: biofilm formation (**A**), and biofilm removal (**B**) of *P. larvae* KB10. Results are the average of three replications ± SD









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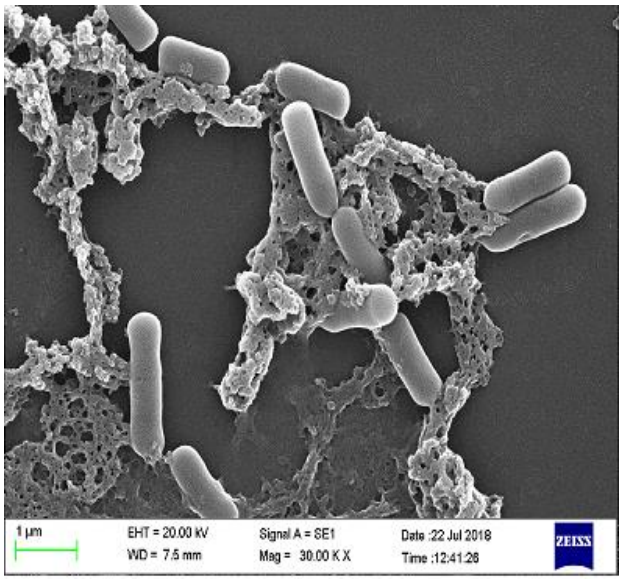

Fig. 4: Scanning electron microscopy images from biofilms of *P. larvae* KB10 on glass slides. (**A**) 24 h in MYPGP broth, (**B**) 48 h in a mix of MYPGP/MRS broth (1/1), (**C**) 48 h in MYPGP/CFS, (**D**) 96 h in MYPGP/MRS broth, and (**E**) 96 h in MYPGP/CFS (×30000)

Effects of different treatments on CFS anti-microbial activity

The results of MODA revealed that neutralizing CFS with NaOH caused an increase in the growth of *P. larvae* KB10 compared to untreated CFS ($P<0.05$). Bacteria growth reduced significantly in acid treated MRS-broth compared to untreated CFS as the control ($P<0.05$). Elimination of probable H_2O_2 (by catalase) and proteins (by pepsin and trypsin) in CFS did not change the growth rate ($P>0.05$). Boiled CFS showed a slight but not significant increase in growth compared to untreated CFS as control ($P<0.05$) (Fig. 5).

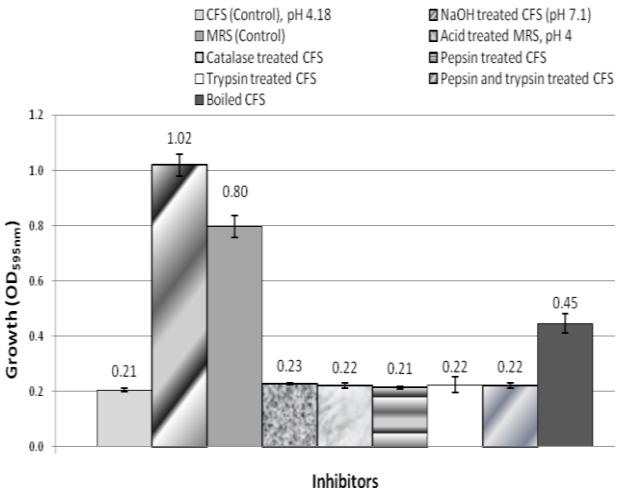

Fig. 5: Microscale optical density assay results of cell free supernatant (CFS) from *L. reuteri* ATCC23272 on the growth of 1/100 diluted of *P. larvae* KB10 (OD=0.1) following different treatments of NaOH, acetic acid, trypsin, pepsin catalase and boiling. Results are the average of five replications ± SD

Discussion

Sequence alignment of *P. larvae* KB10 showed 97.48% identity to the ERIC II genotype of the *P. larvae larvae* standard strain (DSM25430). The authors have shown the presence of a crystalline surface layer in this strain (unpublished data), given that this layer has been identified exclusively in the ERIC II genotype (Poppinga *et al.*, 2012). Determining the genotype group in this strain is necessary for the strain.

The quantified biofilm strength indicated an increase from 24 to 48 h incubation which was in accordance with biofilm formation dynamics (Cai *et al*., 2013). Scanning electron microscopy images of 24-h biofilm revealed a production of some extracellular scaffold-like matrices, which are the basis of adhesion in biofilm production and extension (Fig. 4A). Extracellular polymeric substance consists of exopolysaccharides, proteins, extracellular DNA and other compounds (Zamani *et al*., 2017). Bacteria in the 48-h biofilm bonded together and created a multi-layer structure (Fig. 4B). The created biofilm after 96-h was less three-dimensional (Fig. 4C) and had lower numbers of cells in the cementing mass compared to the 48-h biofilm (Fig. 4B); however, it still had an extracellular matrix. This is the detachment stage, in which the production of some secondary metabolites cause bacteria to disperse and leave a mass of extracellular material (Joo and Otto, 2012). This is the first report on the kinetics of biofilm formation in a local isolate of *P. larvae*.

Biofilm formation reduced when exposed to CFS compared to the control. The reduction of biofilm after 48 h was not significant compared to the reduction at 24 h of exposure (Fig. 3A) ($P>0.05$). This might be due to the strength (higher amount of extracellular polymeric substances) and lower bacterial metabolism in the 48 h biofilm comparing to the biofilm of 24 h.

The 48-h biofilm created in contact with CFS (Fig. 4D), encountered a decrease in attached numbers of bacteria and biofilm production. In the meanwhile, the 48-h *P. larvae* KB10 culture, exposed to MRS-broth (control) (Fig. 4B), caused the bacteria to gather, attach to the surface and form micro-colonies. Biofilm formation in contact with CFS ($OD_{595}=0.227$) reduced about 37.29% compared to the control ($OD_{595}=0.362$) (Fig. 3B). Similar findings regarding the effect of CFS on reducing biofilm production from other bacteria were reported by Zamani *et al*. (2017). Exposure to CFS (pH = 4.18) was more stressful for *P. larvae* KB10 than the control medium containing MRS-broth (pH = 5.4) (Fig. 4B). Therefore, the number of bacteria decreased and consequently, a very weak biofilm was produced. This significant effect may contribute to the acidity or other unknown compounds in CFS. In the present study, CFS at sub-MIC concentrations had no effect on *P. larvae* KB10 cell wall lysis. The biofilm in exposure to CFS after 96 h (Fig. 4E) differed from that formed when exposed to the control sterile MRS-broth. This difference consisted of fewer bacteria without extracellular materials on the surface and a soft and loose connection

to the matrix in the biofilm. The matrix appeared weak and porous and seems to be too unsuitable in amount or quality to bind to the substrate. *P. larvae* KB10 in CFS seems to have weak adhesion to glass surfaces. *Lactobacillus* sp. suppressive effects have been reported earlier by other researchers only with regards to the growth of *P. larvae* (Pehrson *et al*., 2015) Our research is, therefore, the first to represent the inhibitory effect of CFS on the biofilm formation of *P. larvae*. Although CFS ability in removing biofilm has been reported earlier (Vahedi Shahandashti *et al*., 2016) the results of our research showed that CFS had no significant removal effects on the formed biofilm (Fig. 3A). The reasons may be attributed to biofilm structure, CFS concentration and its mode of action on bacterial cells or penetration into the matrix barrier.

The time-kill assay showed that the 4.5 log CFU/ml viable cell count of *P. larvae* KB10 was completely eliminated after the first 2 h of contact with the CFS at MIC concentrations (Fig. 2B), which confirm the results of MBC. The results are acceptable comparing other similar investigations (Dasari *et al*., 2014).

According to the results obtained from the MODA test, it can be concluded that the inhibitory effect of CFS was related to acidity rather than H_2O_2 or protein compounds. Boiling CFS caused a slight pH increase (0.77 units) which caused a non-significant increase in the growth of *P. larvae* KB10 ($P>0.05$) (Fig. 5). It is reported that *L. reuteri* produces a number of heat-sensitive metabolites such as carbon dioxide (Morita *et al*., 2008) and ethanol (Chen *et al*., 2016). The evaporation of such materials in boiling temperatures might be the reason of increased *P. larvae* KB10 growth.

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Conflict of interest

The Faculty of Biological Sciences of Alzahra University supported this work. All authors disclose any financial and personal relationships with other people or organizations that might inappropriately influence or bias their work.

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