Antibiofilm effect of *Lactobacillus pentosus* and *Lactobacillus plantarum* cell-free supernatants against some bacterial pathogens

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Lactobacillus **has a well known positive effect on human health. Therefore, this study aimed at applying the antimicrobial and antibiofilm effects of** ***L. pentosus*****,** ***L. plantarum*** **and** ***L. pentosus*** **HG against 2 common pathogens,** ***Bacillus cereus*** **(food born pathogen) and** ***Pseudomonas aeruginosa*** **(plant pathogen). All lactic acid bacterial strains were identified by partial sequencing of the 16S rRNA gene and exerted tolerance to stress conditions such as bile salts, gastric juice and NaCl. The cell- free** ***Lactobacillus*** **supernatant CFLS showed a good antimicrobial activity against both pathogens. The MIC90 of** ***L. pentosus*** **and** ***L. plantarum*** **were 30 µl and more than 40 µl, respectively. On the other hand, the MIC90 of** ***L. pentosus*** **HG was 30 µl in case of** ***P. aeruginosa*** **and more than 40 µl in case of** ***B. cereus*****. Both pathogens displayed remarkable reduction in biofilm formation in presence of 20 µl or more of CFLS. The antibiofilm dose of** ***L. plantarum*** **or** ***L. pentosus*** **HG was 20 µl against** ***B. cereus*****. Besides, 20 µl of** ***L. plantarum*** **supernatant is the antibiofilm dose against both pathogens. In conclusion, the three** ***Lactobacillus*** **strains isolated from traditional fermented milk are strongly recommended as probiotics as well as biocontrole agents against** ***B. cereus*** **and** ***P. aeruginosa*** **by inhibition of their ability to form biofilm.**

Keywords: *Lactobacillus*; pathogens; antibiofilm; bile salt; gastric juice.

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Introduction

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer health benefit on the host [1]. Food based probiotics have assumed great significance in recent years as different food products can harbor native and beneficial *lactobacilli* and thus can be used for both nutritional and therapeutic purposes [2]. *Lactobacilli* are characterized by their ability to inhibit the growth of bacteria throughout the production of antimicrobial materials such as bacteriocins and biosurfactants, thus preventing the formation of biofilms [3, 4]. Biofilms, a surface-associated bacterial community, are complex and ordered bacterial societies that can grow in connection with different biological and inert surfaces [5].

Microbial cells in biofilms are held together by extracellular matrix containing exo-polysaccharids, proteins, and even nucleic acids [6]. This biofilm can protect microorganisms from several stress conditions and disinfectants. Also, it supplements the injured micro-

organisms with micro and macro nutrients necessary to recovery and growth [7]. Many bacteria such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli* can form biofilms in body tissues, leading to many infections [8, 9]. Moreover, biofilms of *Bacillus spp*. and *Pseudomonas spp*. are recognized as a serious problem and important contaminants in many food industries [10]. These micro-organisms are primary colonizers of surfaces and provide a biofilm backbone to which other microorganisms can adhere [11, 12]. Drug resistance of such pathogens increases in biofilms comparing with what is normally seen with planktonic cells [13]. Besides, micro-organisms rapidly acquire the antibiotic resistance such as coliforms and *S. aureus* [14]. Therefore, drug resistant bacteria are a major problem in antibiotic therapy and alternative remedies have to be applied for the treatment of many infectious diseases. One of these safe remedies is the use of lactic acid bacteria which produce specific natural antibiotics [15].

Therefore, the aim of the current work is to determine the anti-biofilm efficacy of cell-free supernatant of three different, recently isolated, *Lactobacillus* isolates against two important bacterial pathogens. Moreover, bile and NaCl tolerance, and gastric juice resistance of these recent isolates were also investigated.

Materials and method

Bacterial strains

Three bacterial pathogens obtained from stock cultures maintained in the microbial culture collection, Department of Food Science, Faculty of Agriculture, Ain Shams University, Cairo, Egypt and used in the present study. These strains are *Bacillus cereus* DSMZ 345 and *Pseudomonas aeruginosa* ATCC 10145.

Isolation of *Lactobacillus*

Lactobacillus strains were initially isolated from traditional fermented milk samples. One gram sample was homogenized in 9 ml of 0.1% peptone water, serially diluted and plated on MRS (de Man, Rogosa, Sharpe) agar (Difco, USA) and grown at 30°C for 2-3 days under anaerobic conditions (Anaerobic Jar, Sigma-Aldrich; with anaerobic atmosphere generation bag, product No. 68061). Colonies that developed were preliminary characterized by some physiological and biochemical tests according to the criteria of Bergey's Manual of Systematic Bacteriology [16]. The studied characteristics were morphology of colony, cell shape, Gram reaction, catalase and oxidase activity, sporulation, and cell motility.

Partial sequencing of 16S rRNA gene

Genomic DNA of *Lactobacillus* isolates was extracted and purified with a genomic DNA isolation kit (Core Bio System, USA). The 16S rRNA gene was amplified from chromosomal DNA of *Lactobacillus* strains using the universal bacterial primers 518F/800R [17]. The reaction mix was composed of Template DNA, 2 µl BigDye-Mix, 1 µl Specific primer (10 µmol/l), and HPLC water to a final volume of 10 µl. The amount of template DNA applied was dependent on the concentration of target sequences to obtain about 10 ng DNA in the final mix. The PCR program was as follows; initial denaturation at 96°C for 2 min (1 cycle), denaturation at 96°C for 10 sec, annealing at 45°C for 5 sec , extension at 60°C for 4 min (30 cycles), and then cooling at 4°C. The PCR product was purified as recommended by the manufacturer and then sequenced. PCR fragments were analyzed by cycle sequencing, using the BigDye terminator cycle sequencing kit (Applied Biosystems, UK). These fragments were sequenced in both directions. The sequences were compared with those in the GenBank database by using BLAST [18]. The 16S rDNA sequences were finally deposited in the GenBank and the accession numbers were obtained.

Bile Tolerance

Bile tolerances of isolated *Lactobacillus sp.* were determined using the protocol described by Graciela and Maria [19]. Briefly, MRS broth was

prepared by supplementation of MRS broth with 0.5, 1.0, 1.5, and 3.0 g bile Oxgall (Difco Laboratories) to obtain a final concentration of 0.05, 0.1, 0.15, and 0.3 %, respectively. Cells grown anaerobically in MRS broth at 30°C for 28 hours were harvested by centrifugation at 5,000 g for 10 min. Pellet was washed twice with 0.1 M phosphate buffer (pH 7.0), and resuspend to the original volume with the buffer by vortexing. MRS broth was inoculated (0.5%) with the bacterial suspension, and anaerobically incubated at 30°C. The optical density at 595 nm wavelength (OD_{595}) against the blank (uninoculated broth) was measured every hour for the first 8 hours and after 24 hours of incubation.

Gastric juice resistance

Gastric juice resistances of isolated *Lactobacillus* from yoghurts were assayed using the protocol described by Graciela and Maria [19]. Briefly, artificial gastric juice (AGJ): NaCl (2 g), pepsine (3.2 g), adjusted at a final pH 3±0.5 with HCl, and the total volume was adjusted to 1 L with distilled water. The AGJ was sterilized by filtration using 0.22 µm filter membrane. The bacterial culture suspensions of G01, G02 and G03 strains were prepared by incubation anaerobically at 30°C for 48 hours. The artificial gastric juice pH 3±0.5 was inoculated (2%) with the bacterial suspension and incubated anaerobically at 30°C. Samples were taken at 0, 2, 4, 6, and 8 h and after 24 h for determining cell viability. Proper dilutions from 10-fold serial dilutions (in 0.1% peptone water) were plated in MRS agar. The plates were incubated at 30°C for 24 h under anaerobic conditions. The resulting colonies were counted and expressed as colony-forming units (CFU) per milliliter (CFU/ml).

NaCl tolerance

For the determination of NaCl tolerance of isolated *Lactobacillus*, 11 test tubes containing MRS broth were adjusted with different concentrations of NaCl (0-10%). After sterilization, each test tube was inoculated with 1% (v/v) fresh culture of *Lactobacillus* and incubated anaerobically at 30°C for 48 h. After that, growth was monitored by measuring OD_{595}. The normal growth in the absence of NaCl as a control was indicated as triple positive sign (+++), reduced growth as double or single positive sign (++ or +), and no growth as negative sign (-).

Antimicrobial activity of *Lactobacillus* cell-free supernatant

Cell-free supernatants of *Lactobacillus* isolates were prepared according to Sadoswska and his coworkers [20]. Overnight cultures in MRS broth incubated at 37°C were centrifuged (3,000 g, 15 min, 20°C) and filter-sterilized (0.22 µm Minisart; Sartorius, Germany). The stock of BLIS was stored at 4°C. Antimicrobial activity of free-cell supernatant of *Lactobacillus plantarum* strains was determined as minimum inhibitory concentration (MIC90) using a micro-dilution technique in 96-well plates. Different amounts of free-cell supernatant (10, 20, 30, 40 µl) were added to each well. 10 µl activated culture of each tested strain; *Bacillus cereus* and *Pseudomonas aeruginosa* (about 106 CFU/ml) were added. The total volume in each well was adjusted to 250 µl using tryptic soy broth (TSB). Control wells contained culture medium and the tested strain without adding free-cell supernatant. After 24 hours of incubation at 37°C, bacterial growth inhibition was determined by monitoring OD_{595}. The MIC90 was defined as the lowest concentration of free-cell supernatant inhibiting >90% of bacterial growth. All tests were carried out in triplicate (n=3) and the results were averaged.

Antibiofilm effect of *Lactobacillus* cell-free supernatants

Inhibitory effect of cell-free supernatant of *Lactobacillus* isolates against biofilm formation by *Bacillus cereus* and *Pseudomonas aeruginosa* was determined *in vitro* using the commonly used 96 wells polystyrene plate method. 10 µl activated culture of each tested strain; *Bacillus cereus* and *Pseudomonas aeruginosa* (about 106 CFU/ml) were added. Sub-MIC90 of CFLS (20 µl) was added per each well. The total

volume in each well was adjusted to 250 µl by using tryptic soy broth (TSB). Control wells contained culture medium and the tested strain without adding free-cell supernatant. After incubation at 37°C for 24 hours, content of the plates were poured off and the wells were washed three times with 300 µl of phosphate-buffered saline (PBS, pH 7.2). The remaining adhered bacteria were fixed with 250 µl of methanol per well. After 15 min, plates were emptied and air dried. The plates were stained with 250 µl per well of 1% crystal violet used for Gram staining for 5 min. The excess of stain were rinsed off by placing the plates under running tap water. After drying the plates, absorbance at 620 nm (A_{620} nm) was measured by using ELISA reader. The same experiment was repeated in the presence of NaCl (4%), bile salt (0.3%), and AGJ (pH 3 ±0.5). All tests were carried out in triplicate (n=3) and the results were averaged.

Based on A_{620} nm produced by bacterial films, strains were classified into four categories according to the classification of Christensen and his coworkers [21] which modified by Stepanovic *et al*. [22]. Briefly, the cut-off absorbance (Ac) was the mean absorbance of the negative control. Strains were classified as follows: A = Ac = no biofilm producer (0); Ac < A ≤ (2 x Ac) = weak biofilm producer (+); (2 x Ac) < A ≤ (4 x Ac) = moderate biofilm producer (++); (4 x Ac) < A = strong biofilm producer (+++). All tests were carried out in triplicate (n=3) and the results were averaged. The antibiofilm dose was defined here as the lowest CFLS concentration that caused prevention of biofilm formation after 24 h by each tested bacterium (to be in the category of no biofilm producer).

Statistical analysis

The data obtained from three replicates were analyzed by a one-way ANOVA (SAS 8.2, Cary, NC, USA). In all cases, the level of statistical significance was of $P < 0.05$.

Results and discussion

Partial sequencing of 16S rRNA gene

Three *Lactobacillus* strains were isolated and selected according to the genus characteristics. All strains were Gram-positive, catalase-negative, oxidase-negative, facultative anaerobic, endospore forming, and non-motile rod-shaped cells. The identification of *Lactobacillus* isolates was confirmed by determining the partial sequencing of the 16S rRNA gene. The obtained sequences were deposited to the DDBJ (DNA Data Bank of Japan). Results of nucleotide BLAST and the accession numbers were listed in table 1.

Table 1. Accession numbers of the new isolates

Bacteria	% Identity	Accession No.
L. pentosus	99	LC027962
L. plantarum	97	LC027963
L. pentosus strain HG	99	LC027964

Effect of bile salt

Bile tolerance is an important criterion in the selection of microbial strains for probiotic uses [23]. It enables the probiotic strain to survive, grow and exert its action in the small intestine [24]. In the present study, bile tolerance of the *Lactobacillus* species under test was tested by using serial dilutions, 0.05, 0.1, 0.15, and 0.3%, of bile oxgall (Figure 1). All isolated strains can tolerate the bile concentrations with different degrees. At the end of log phase (within approx 8 h), the OD_{595} of the studied strains decreased at the higher bile salt concentrations. *L. pntosus* and *L. plantarum* reached almost OD_{595} of 2.2 and 2.1, respectively upon using 0.3% bile. On the other hand, *L. pentosus* HG reached OD_{595} of only 1.8 when the same bile concentration was used. Suskovic *et al*. [23] stated that *L. acidophilus* M92 showed a satisfactory degree of tolerance against bile oxgall and concluded that this tolerance is a valuable parameter for selection of probiotic strains. Moreover, Mirlohi and his coworkers [25] declared that a native *L. plantarum* is considered as a potential

probiotic, regarding its bile resistance. According to our results, it can be concluded that the recently isolated *L. pentosus*, *L. pentosus* HG and *L. plantarum* could be considered as promising probiotics regarding their bile salt resistance.

Gastric juice tolerance

Gastric juice resistance of the isolated *Lactobacillus* strains was investigated overtime intervals, from 0 to 24 h (Figure 2). Generally, number of cells decreased with time in the presence of the artificial gastric juice (AGJ). The maximum reduction can be noticed after 24 h. Within 8 h, the isolates could withstand the stress giving around 1.65×10^4, 1.5×10^4, and 1.35×10^4 CFU for *L. pentosus*, *L. plantarum*, and *L. pentosus* HG, respectively. However, the isolates still resist the AGJ after 24 h and the CFU exceeded 1×10^4 for all of them. Similar results were obtained by Wang *et al*. [26]. They found that *L. casei* Zhang, which was isolated from soymilk and bovine milk, is a good tolerant to gastric and intestinal juice and maintain high viability (>10 CFU/g) during storage. Bautisa-Gallego *et al*. [27] found that 107 olives isolates were identified as *L. plantarum*, *L. pentosus*, and *L. paraplantarum*. Most of them showed lower survival to gastric than to pancreatic digestion.

NaCl tolerance

All of the tested *Lactobacillus* species tolerated 7% of NaCl (Table 2) and *L. pentosus* resisted till 8% of the salt. The reduction in bacterial growth is expected with increasing salt concentrations. This is may be due to the production of heat shock proteins as a response to salt stress [28]. Besides, Montano and his coworkers [29] declared that at pH 6.4, *L. plantarum* H4 showed growth at 6% salt. On the other hand, they reported that *L. plantarum* 221 exhibited no growth using 6% of NaCl. This indicates the intraspecies variability in response to stress conditions.

In general, *Lactobacillus* isolates under study showed good probiotic properties regarding tolerance to bile salts, AGJ, and NaCl. Therefore they have a potential for application in the development of probiotic food products.

Table 2. Growth of *Lactobacillus* isolates at different NaCl concentrations (0-10%)

NaCl %	*Lactobacillus* isolates		
	L. pentosus	*L. plantarum*	*L. pentosus* HG
0	+++	+++	+++
1	+++	+++	+++
2	+++	+++	+++
3	+++	++	+++
4	++	++	+++
5	++	++	++
6	++	++	++
7	+	+	+
8	+	-	-
9	-	-	-
10	-	-	-

Lactobacillus antimicrobial effect

The cell-free *Lactobacillus* supernatant (CFLS) showed significant antimicrobial activity against *Bacillus cereus* [30] (a food born pathogen) and *Pseudomonas aeruginosa* [31] (a plant and human pathogen) and its antimicrobial activity increases with higher volumes (Table 3). The lowest concentration of CFLS inhibiting > 90% of pathogen growth (MIC90) was 30 µl for *L. pentosus* against both studied pathogens. However, *L. plantarum* displayed about 80% inhibition in the growth of studied pathogens when 40 µl of CFLS were tested. On the other hand, the MIC90 of *L. pentosus* HG was 30 µl in case of *P. aeruginosa*, while only 85% of *B. cereus* cells were inhibited using 40 µl of CFLS. In conclusion, *L. pentosus* supernatant has the best antimicrobial effect against the tested pathogens. Francois *et al*. [32] also observed the antimicrobial activity of *L. plantarum* against *Klebsiella pneumonia*, *Pseudomonas sp*., and *Enterococcusfaecalis*. Moreover, Rao *et al*. [33] declared that different strains of *L. pentosus* and *L. plantarum* have a good antimicrobial activity against *B. subtilis*, *P. aeruginosa*, *S. aureus*, and others.

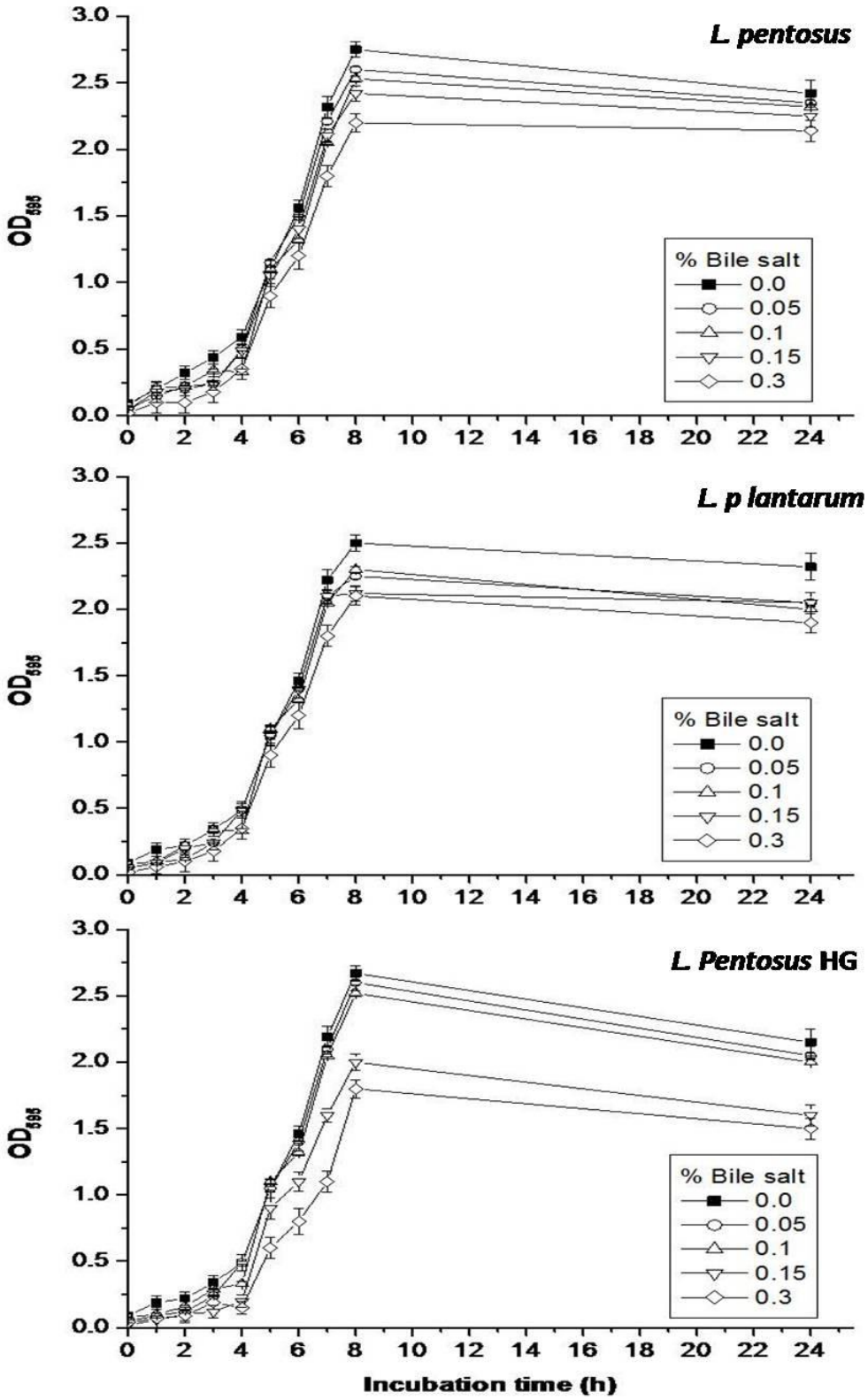

Figure 1. The effect of bile salt on growth of the three *Lactobacillus* isolates.

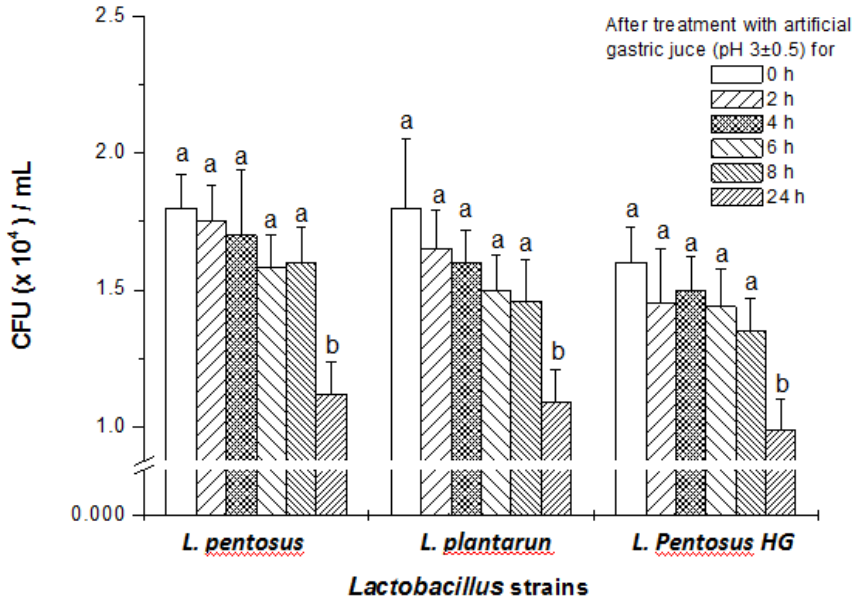

Figure 2. Tolerance of *Lactobacillus* isolates to treatment with artificial gastric juice (pH 3 ± 0.5). Columns with the same letter within each group are insignificantly different (*P > 0.05*).

Table 3. The antimicrobial activity of *Lactobacillus* cell-free supernatants against the bacterial pathogens

Volumes of *Lactobacillus* cell-free supernatants		**Pathogen growth at OD_{595}**	
Strain	**Amount (µL)**	***Bacillus cereus***	***Pseudomonas aeruginosa***
	0	0.423 ±0.025	0.394 ±0.021
L. pentosus	10	0.131 ±0.013	0.237 ±0.011
	20	0.067 ±0.012	0.178 ±0.021
	30	0.038 ±0.008	0.032 ±0.013
	40	0.023 ±0.007	0.028 ±0.0
L. plantarum	10	0.202 ±0.013	0.198 ±0.015
	20	0.113 ±0.021	0.157 ±0.014
	30	0.041 ±0.011	0.033 ±0.012
	40	0.039 ±0.081	0.033 ±0.077
L. pentosus HG	10	0.274 ±0.010	0.289 ±0.031
	20	0.213 ±0.013	0.222 ±0.023
	30	0.040 ±0.009	0.028 ±0.015
	40	0.037 ±0.004	0.023 ±0.011

Lactobacillus antibiofilm activity

After 24 h of incubation with different volumes of cell-free *Lactobacillus* supernatants (CFLS), the antibiofilm activity was recorded (Table 4). In absence of *Lactobacillus* supernatant, the pathogenic bacteria, *B. cereus* and *P. aeruginosa*, were characterized as strong and moderate biofilm producer, respectively. Both pathogens displayed remarkable reduction in biofilm formation in presence of 20 µl or more of CFLS. For instance, *B. cereus* lost its ability to form biofilm and became no biofilm producer (0) when exposed to at least 20 µl of *L. plantarum* or *L. pentosus* HG supernatants.

Therefore, this volume can be considered as antibiofilm dose against *B. cereus*. Moreover, 20 µl of *L. plantarum* supernatant is the antibiofilm dose against both pathogens, *B. cereus* and *P. aeruginosa* (Table 4). By comparing MIC90 of CFLS against the pathogens (Table 3) with the antibiofilm activity of *Lactobacillus spp*. (Table 4), it can be generally noticed that the results in both tables are in harmony and parallel to each other. For example, increasing CFLS volume leads to reduction in the pathogen growth, OD_{595}, (Table 3) and the pathogen ability to form the biofilm as well (Table 4). However, exceptions can also be found. The OD_{595} of *B. cereus* and *P. aeruginosa* in the presence of 20 µl of *L. plantarum* supernatant were 0.113 and 0.157, respectively (Table 3), although no biofilm was produced by both pathogens in correspondence (Table 4). This finding indicated that, the CFLS used in the present study had ability to inhibit biofilm formation at sub MIC90 levels. This means that the obtained antibiofilm effect referred to inhibiting adhesion of pathogenic strain to microtiter plate surface, but not to inhibiting their growth. Another declaration could be referred to the concept of quorum sensing (QS). It is well known that QS is an important regulatory mechanism of biofilm life style in a variety of bacterial species [34]. It involves the accumulation of signaling molecules in the surrounding environment which enables a single cell to sense the density of the number of bacteria and therefore the whole bacterial population can make a coordinated response [35]. Accordingly, many groups of researchers had focused to find out the most appropriate quorum sensing inhibitors (QSI) that could be used for inhibition of pathogenecity and virulence of multidrug resistant *P. aeruginosa* strains [36]. They finally found that the metabolites produced by probiotic strains exhibit inhibitory activity on different virulence properties of pathogenic bacteria [37, 38]. The antimicrobial activities of *Lactobacillus spp*. are well known against many pathogens [39, 40]. They secrete organic acids (acetic and lactic acids), bacteriocins, and other by-products that accumulate in the probiotic supernatant of *Lactobacillus spp*. [41]. Therefore the antibiofilm effect of CFLS prepared in the present study may be referred to its ability to inhibit QS. In parallel with our findings, Rao *et al*. [33] have declared that cell free supernatant of both *L. plantarum* and *L. pentosus* strains showed good antibiofilm activity against *P. aeruginosa* and *Klebsiella pneumonia*. Besides, they stated that these probiotics have good antimicrobial activity against some important pathogens such as *Escherichia coli*, *B. subtilis*, *P. aeruginosa*, and others. In addition, Das *et al*. [2] found that *L. plantarum* cell free supernatant inhibited the growth of *Salmonella enterica* Serovar Enteritidis without affecting the growth of other Gram-positive lactic acid bacteria. In addition, the cell free supernatant showed a significant reduction in the biofilm forming ability of *Salmonella enterica*.

Table 4. The antibiofilm activity of *Lactobacillus* cell-free supernatants against the bacterial pathogens

	Biofilm formation	
Bacterial supernatant (µl)	***Bacillus cereus***	***Pseudomonas aeruginosa***
L. pentosus		
0	+++	++
10	++	++
20	+	+
30	+	+
L. plantarum		
10	++	+
20	0	0
30	0	0
L. pentosus strain HG		
10	++	++
20	0	+
30	0	+

Lactobacillus spp. were classified as no biofilm producer (0), weak biofilm producer (+), moderate biofilm producer (++), or strong biofilm producer (+++); n=3

Conclusion

Lactobacillus strains isolated in the present study displayed potential probiotic properties, *i.e.* tolerance to the artificial gastric juice, bile salts, and NaCl. These strains had significant

antimicrobial effect against *B. cereus* and *P. aeruginosa*. The present study demonstrated (*in vitro*) the antibiofilm effect of CFLS against both pathogenic strains. Since the adhesion of bacterial pathogens to their host cells is well known as an important virulence factor in the pathogenicity, the CFLS could be suggested to control the infections caused by *B. cereus* (food-born pathogen) and *P. aeruginosa* (plant pathogen). However, further *in vivo* investigations are recommended to ensure this hypothesis.

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