Antibacterial Activity of Bacteriocin Produced by *Lactobacillus plantarum* Isolated from Horse Milk

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ABSTRACT

Lactic acid bacteria (LAB) effects are related to producing anti-microbial agents as bacteriocin. Our target is to isolate LAB from different horse milk samples and investigates its antimicrobial activities against many pathogenic bacteria. LAB was isolated from horse milk from Al-Taif city in Saudi Arabia on MRS medium. By agar well diffusion method, twelve LAB isolates demonstrated that the antibacterial activities against Gram-positive bacteria *S. aureus* ATCCBAA977, *ST. pneumonia* ATCC49619, and *E. coli* ATCC35218, *P. aeruginosa* ATCC27853. According to the 16S rDNA sequence, the highest activity LAB isolate was presented in a gene bank such as *Lactobacillus plantarum* with an identity of 99% under accessory LBP 135401.1. The results showed that the incubation at 35°C for 24h at pH 6.5 were the best conditions for producing the largest quantity of bacteriocin.

Key Words: *LAB, Antimicrobial, Bacteriocins, Pathogenic bacteria*

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INTRODUCTION

Bacteriocins are proteinaceous products produce by many Gram bacteria either positive or negative and have antimicrobial activities [1-4]. Bacteriocins are usually not called antibiotics to avoid anxiety about curative antibiotics could be unlawful allergic effects on humans health [5].

Bacteriocin is protein agents digest through digestive proteases and peptides make from ribosomes, which could improve its properties to enhance its activities [6]. Bacteriocins also can inhibit foodborne pathogens [7, 8]. Bacteriocins products by LAB are advantageous on survival rates of bacteria growth rates in the ecological niche and could exploit by the food industry for example to controlling undesirable bacteria [9]. *Lactobacilli* has gain attention related to its bacteriocins products [10].

LAB is nonpathogenic, nonsporulating, rod or coccus-shaped, and Gram-positive and is one of the commensal microorganisms found in gastrointestin, urogenit tracts, and milk maternal [11]. LAB was classified as bacteria majority (probiotics) [12, 13]. The LAB group is currently categorized in Firmicutes [14]. Various antimicrobials produce by probiotics, bacteria are effective molecules that subject to intense studies [15].

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MATERIALS AND METHODS

Samples

For isolating LAB, 10 samples of Hores milk were used in this research (nearly 150 ml) of Horse milk were collecting from different regions at Al-Taif city in Saudi Arabia.

LAB isolation

Dilution pour plate method uses to obtain LAB isolates from the milk samples (10-4). 0.1 ml of dilution transfer over solid MRS plates. Bacterial colonies were obtained after 24h (incubating / 35°C). Selected colonies were purified and cultured in slants and preserved at 4°C for other studies. Morphological properties of colonies (color, shape, and size) were examined, and morphological properties by using microscopic.

Test bacteria

For ATCC bacteria were used as test organisms: *S. aureus* ATCCBAA977*ST, pneumonia* ATCC49619, *E. coli* ATCC35218, *P. aeruginosa* ATCC27853. The test bacteria were obtained from IMC in Jeddah, Saudi Arabia.

Screening for production bacteriocin from LAB isolates by agar well diffusion assay

For the study of bacteriocin products and antibacterial activity in the cell-free filtrate (CFF). The cultural filtrate of the bacterial isolate experiments performed in 250 ml Erlenmeyer flasks had 25 MRS ml broth medium and inoculated by slant from preculture of selected isolate (*Lactobacillus plantarum*) at 35°C. post 24 h of incubation, CFF collecting throughout centrifugation at 5000 rpm / 20 min /4°C. Bacteriocin activities evaluation using agar-well diffusion [16]. 0.1ml of inoculum suspension pours and spread by sterile swap. Wells made by sterile cork borer and fill by supernatant (100 µl). Left plates for 30 min in refrigerator. Plates incubated at 35°C /24 h.

Molecular characterization of selected LAB isolates

Genomic DNA isolation by Gene JET Genomic DNA Purification Kit-Thermo, Fisher Scientific. An amplicon of 1500 bp fragment represented full-length 16S rRNA gene was amplified using highly conserved universal primers [17]. Primers designed for PCR amplification:

Universal primers forward: (27F) 5'AGAGTTTGATCCTGGCTCAG-3'

and reverse: (1492R) 5'TACGGYTACCTTGTTACGACTT-3'

DNA sequences were phylogenetically analyzed and compared with those of GenBank to check for close evolutionary relatives using the BLAST algorithm and RDP database.

Influences of growth conditions for bacteriocin producing by Lactobacillus plantarum H7 isolate

100 µl CFF from cultures place in 5-mm diameter wells and cut in agar plate seed with indicator bacteria. Plates were incubating at 35°C. Post 24 h diameter of inhibition of indicator measure triplicate time for each treatment.

Incubation temperatures effects

Culture broth of *Lactobacillus plantarum* H7 isolate was incubated for 24 h. at different temperatures (25, 30, 35, 40, and 45 ºC).

Incubation periods of effects

The influence of incubation periods was studied by incubating the culture broth of *Lactobacillus plantarum* H7 isolate at 35ºC, for different incubation periods (24, 48, and 72 h), the antibacterial activities measure.

PH effects

Different pH (4.5, 5.5, 6.5, 7.5, and 8.5) were used and incubated at 35°C. post 24 h. of incubation bacterial growth and antibacterial activities measured

Statistical analysis

Analyses data were used by SPSS, version 16. Significance of differences among samples determined by t-test. P>0.05.

RESULTS AND DISCUSSION

LAB isolating

Ten samples of fresh horse milk from several places in Jeddah city, in Saudi Arabia. Using MRS agar medium, twelve bacteria isolates were obtained from horse milk. The cultural characteristics of the bacterial isolates were obtained from different milk samples. All the cultural characteristics of the bacterial colonies were smooth surface, colony shape round, flat, and color characteristics white and morphological properties showed gram-positive isolate.

Screening and estimation of antimicrobial activity of LAB isolates by agar well diffusion assay against indicator bacteria

All the obtained LAB isolate screen for produce antibacterial against four different ATCC pathogenic bacteria by agar well diffusion assay. The anti-microbial activities of those were estimating throughout mean inhibition zone diameters **(Figures 1 and 2)**. The mean diameters of inhibition zones were ranged from (15.5-26mm) after 24h of incubation The highest antibacterial activity was by isolates H7 against *E. coli* ATCC35218. The inhibition zone was (26 mm).

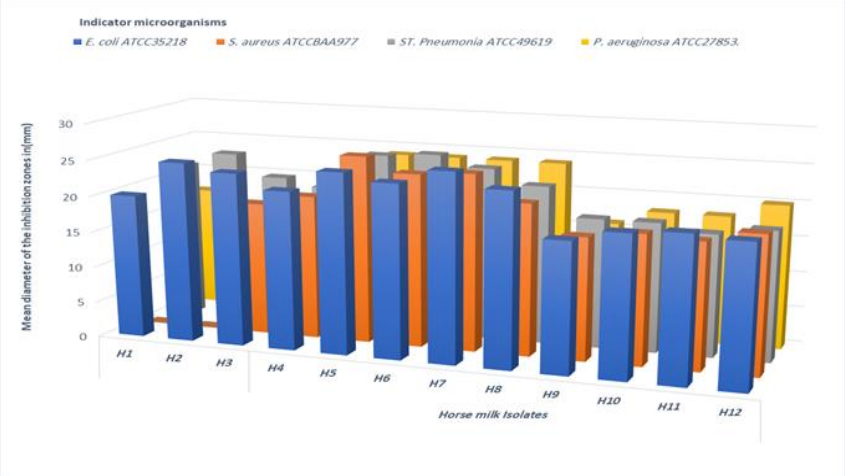
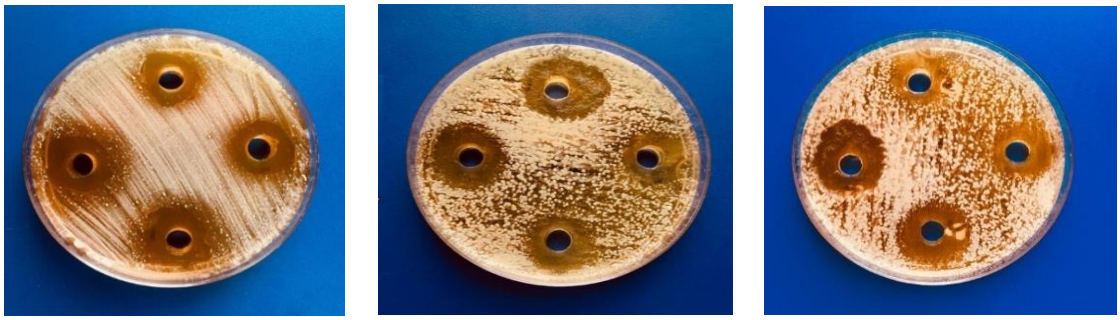


Figure 1. Screening and estimation of antimicrobial activities of LAB



Horse milk isolate **against** *S. aureus* ATCCBAA977

Horse milk isolate **Against** *St. Pneumonia* ATCC49619

Horse milk isolate **against** *E. coli* ATCC35218

Figure 2. Production of bacteriocin(s) by LAB

Molecular characterization

Based on the trimmed and merged 16S rDNA sequences comparison analysis for selected isolates by of nearest species sequences retrieve by NCBI BLAST where taxonomic affiliation with isolated strain. DNA sequences analyzed using nucleotides blast alignment tools of gene bank and showed isolated and identified as *Lactobacillus Plantarum*, with similarity percentages 99 %. The partial 16S rDNA sequence of the selected isolate was submitted into the Bacterial or Archaeal 16S ribosomal RNA sequences database under the accession numbers: LR135401.1 for strain H7 isolated from Horse milk **(Table 1)**.

Table 1. Identity percentage of 16S rDNA of H7

Bacterial isolates	Name and Accession of No. of the most related strain in NCBI GenBank.		Identity	Coverage	Suggested Name and Accession No. of the isolates obtained in the work.	
H7_27F	LR135401.1	*Lactobacillus plantarum*	99%	99%	LR135401.1	*Lactobacillus plantarum*

Cultural conditions influence bacteriocin productions by Lactobacillus plantarum H7 isolate

The various culture conditions were tested to study the optimal conditions on bacteriocin production by the selected isolate.

Incubation temperature effects on bacteriocin(s) productions by Lactobacillus plantarum H7 isolate

Results of this test in **Figure 3** show that the highest antibacterial activity against all indicator bacteria when isolate cultures incubating on 35ºC, the range of the inhibition zone varied from 23-25mm [18-22]. The highest antibacterial activity was 25mm against *E. coli* ATCC35218 and 24mm against *S. aureus* ATCCBAA977 and *ST. Pneumonia* ATCC49619 respectively.

Incubation period Effects on bacteriocin(s) productions by Lactobacillus plantarum H7 isolate

Results in **Figure 4** show that the highest antibacterial activity against all indicator bacteria when the selected isolate was incubating (24 h). The range of the inhibition zone varied from 24-26mm. The highest antibacterial activity 26mm against *E. coli* ATCC35218. Increasing the

incubation periods decreased the antibacterial activity of the selected isolate.

pH effects on bacteriocin productions by Lactobacillus plantarum H7 isolate

The highest antibacterial activity by *Lactobacillus plantarum* H7 which was grown at pH6.5 against all indicator bacteria after 24h of incubation at 35º and lower the pH value the production of the antibacterial activity decreased. The highest antibacterial activity was 26mm against *E. coli* ATCC35218 **(Figure 5)**.

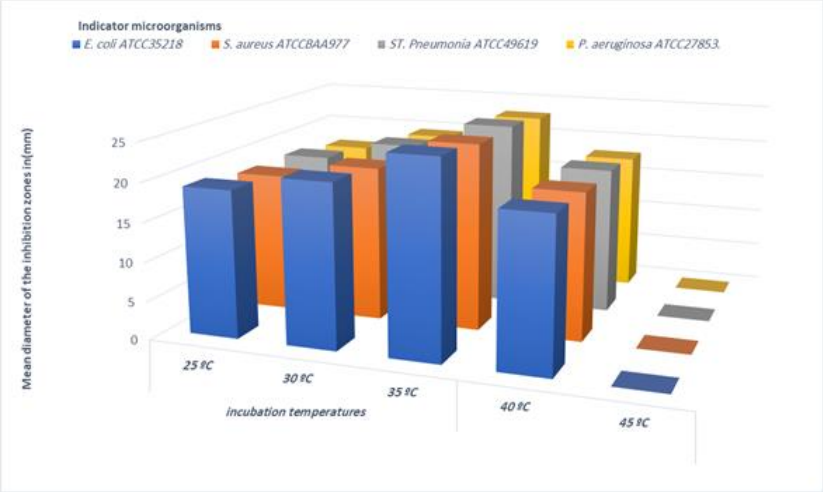


Figure 3. Incubation temperature effect on bacteriocin(s) productions by *Lactobacillus plantarum* H7

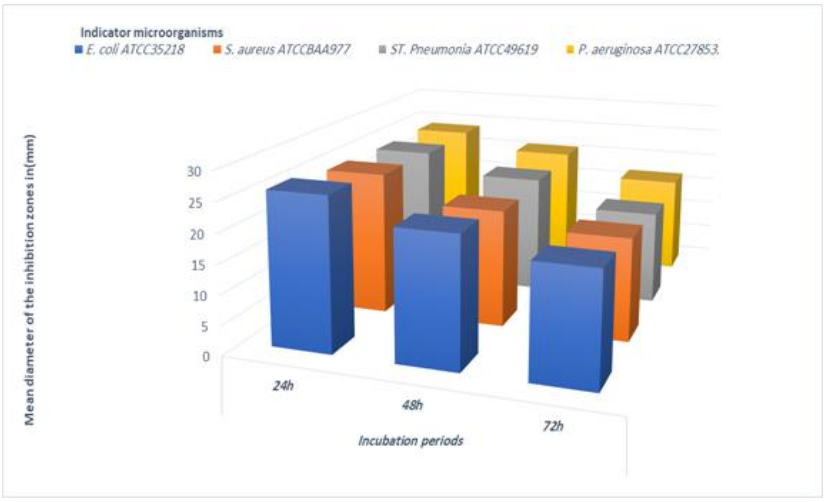


Figure 4. Effect of incubation period on the production of bacteriocin(s) by *Lactobacillus plantarum* H7 isolates

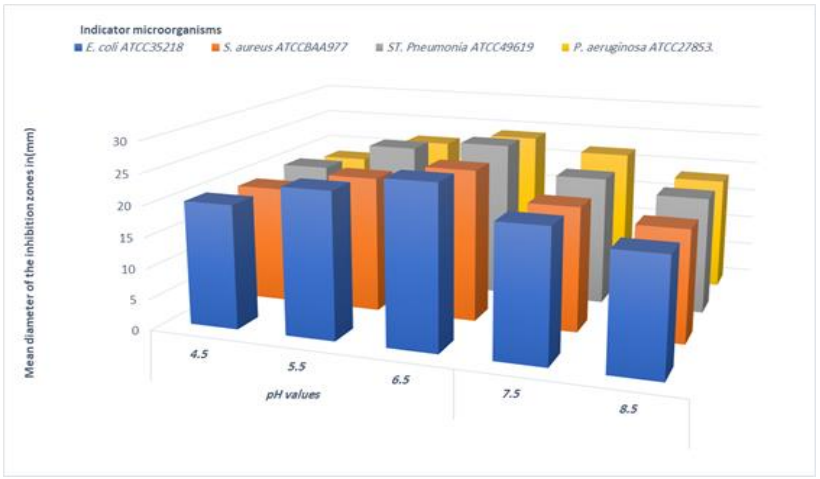


Figure 5. pH Effects on bacteriocin(s) productions by *Lactobacillus plantarum* H7 isolates

Many wild strains of LAB are prospective bacteriocins producers [23] and probiotics [24]. Six horse milk samples from Al-Taif city, kingdom Saudi Arabia, were used to isolates LAB bacterial isolates using MRS agar [25]. Our data approved LAB dominated samples microbial flora: twelve LAB strains were isolated from Horse milk. MRS is the best media for isolating LAB [26]. MRS medium is the best culture media for LAB isolating capable of producing bacteriocin [27]. Agar diffusion assay quantifies the abilities of antibiotics to inhibit the growth of bacteri [28].

In our present work, the indicator Gram-positive bacteria were, *S. aureus* ATCCBAA977, *ST. pneumonia* ATCC49619. While Gram-negative bacteria were *E. coli* ATCC35218 and *P. aeruginosa* ATCC27853. Most of the isolated lactic acid bacterial strains were had a good inhibitory toward Gram-positive and Gram-negative pathogenic indicator strains. Several investigations focus on LAB-secreted antibacterial products that can inhibit unwanted poultry disease pathogens such as E. coli [29, 30], Staphylococcus [31], a stronger antimicrobial property against positive bacteria such as S. aureus [32, 33].

We found the inhibition zones diameter varied between 23 to 25 mm. Schillinger and Lucke (1989) [34] found inhibition scored as positive if the width of clear zone around colonies of producer strain 0.5 mm or more. Our obtained results showed that LAB isolates were inhibited the growth of the Gram-positive and Gram-negative indicator bacteria, which might be related to bacteriocins productions and peptides with bactericidal activities against those strains of related species [35]. Also Danial *et al*. (2016) [36] found that the inhibition zone was screened by agar well diffusion method of forty isolates of lactic acid bacterial strains was ranged from 24.00 to 18.33 mm against *E. coli* ATCC and *S. aureus* ATCC25923 respectively.

Molecular identification of LAB, using DNA sequencing of 16S, and according to Nocker *et al*. (2004), [37] amplification 16S sequences of bacteria can produce 750bp amplicon. The DNA sequences analyzed using Blast alignment tools of GenBank and showed that one isolate was identified as *Lactobacillus plantarum* H7 with 99%. 16S rRNA gene, molecular marker, was ubiquitous to members of this domain [38].

The suitability of environmental conditions (incubation period, temperature, pH, and different media) on the productivity of the production of bacteriocin was investigated. Temperature is also required for growth and bacteriocin production, the highest antimicrobial activity at the optimum growth temperature of 35^{O}C followed by 30^{O}C. Sarika *et al*. (2010) [39] reported that bacteriocin activity recorded maximum in MRS medium at 30ºC compared to the other incubation temperatures (20, 25, 35, and 40ºC). According to Kumar *et al.* (2012) [40], the temperature was effective for bacteriocin production by *L. casei* LA-1 ranging from 33.5 to 34.5°C. These results are incompatible with Barman *et al.* (2018) [41].

Danial *et al*. (2016) [36] recorded the best conditions for bacteriocin production by *Leuconostoc mesentroides* in static incubation culture at 35°C for 24 hr. and pH 6.2. The isolate showed optimal inhibition activity against the tested bacteria after 24 and 48h of incubation. The antagonistic activity of isolates was decreased after 72 h of incubation. In general, the three isolates demonstrated optimal inhibition activity against the tested bacteria after 24 and 72 h of incubation [42]. Also Kp *et al*. (2016) [43] found protein concentration higher after 48 h of incubation than post 24 and 72 h from incubating.

The highest inhibition zone was recorded at pH 6.5 at 35^{O}C after 24h of incubation. The antibacterial agents were stable within variable pH ranges from 4.5 to 8.5. The inhibition effects were more obvious in the range of pH values (5.5 to 7.5). Also Kumar *et al*. (2012) [40] reported that pH (6.8 to 7.2) has an effect on bacteriocin productions by *L. casei* LA-1.

CONCLUSION

Bacteriocins have been used as biological preservatives for about 20 years. Many studies focused on inhibiting food spoilage and human pathogenic bacteria growth by using bacteriocins instead of chemical preservatives and antibiotics, which produced by many living organisms, especially many animals raw milk. In this study, we were able to produce and purify the bacteriocins produced by the lactic acid bacteria isolated from horse milk and improve the conditions such as temperatures, acidity, incubation period and the use of residues that affect the production.

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