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Creating Optimal Conditions for Bacteriocin Production from *Lactiplantibacillus plantarum* Isolated from Traditionally Fermented Fruits and Vegetables

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Abstract

Lactic acid bacteria produce a large number of antimicrobial metabolites which are effective against pathogenic microorganisms. Amongst LAB's antimicrobial agents, bacteriocins have been found as potentially safe biopreservatives. In this research, LAB isolates from less studied non-dairy fermented products were screened for simultaneous production of bacteriocins. Among 253 dominant isolates from 48 types of fruit and vegetable fermented products, strains which found to be potent bacteriocin producers were selected. Further, DNA of the strains was evaluated by the 16S rRNA sequencing. Five isolates identified as *Lactiplantibacillus plantarum* (10A, V3, S6, Sa, and Ab), showed the highest inhibitory effect against the growth of pathogenic indicators *Listeria monocytogenes* PTCC 1165, *Escherichia coli* ATCC 25923, *Salmonella enterica* serovar typhimurium PTCC 1609 and *Staphylococcus aureus* ATCC 25922. Response surface methodology was used to optimize the culture conditions based on various carbon/nitrogen sources in different temperatures (30, 32, 35, 37 ºC) and incubation periods. This led to an increase in efficiency of bacteriocin production by 70%. The bacteriocin production curve was plotted within 58 hours showing that the maximum production and activity happened at 35 °C/48 h in culture containing peptone, yeast extract, tween 80 and glucose. Inhibitory effect of bacteriocins was significantly reduced when treated with proteolytic enzymes. Among the five isolated strains, 10A with an activity of 64000 Au/mL exhibited the highest inhibitory effect, even more than nisin. This study successfully recommends the selective potential of bacteriocin 10A as a candidate food biopreservative to control the growth of food pathogenic and spoiling bacteria.

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Keywords
Antibacterial activity
Food biopreservation
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Lactiplantibacillus plantarum

Introduction

Fermentation of various fruit and vegetable food stuffs is a widely practiced and ancient technology in Iran. For millennium diverse plant materials have been fermented by various bacteria, yeasts, and

fungi to make excellent healthy foods, and these techniques apparently will continue to grow, regarding their profound effects on improving food quality and food safety. Having typical features that depend on local and regional traditions, these fermented foods have attracted attention not only as traditional foods but also for their superior nutritive qualities that might promote prolonged life and thus experiencing a burst of popularity as functional healthy foods. The production of antimicrobials by lactic acid bacteria (LAB) as a method of eliminating pathogenic bacteria in fermented products has long been recognized. LAB are known for their ability to produce colorless and tasteless bacteriocins (Mahrous, Mohamed, Abd El-Mongy, El-Batal, & Hamza, 2013; Yi, Guo, Liu, Shao, & Lü, 2017).

Bacteriocins are a kind of bioactive peptides synthesized by ribosomal machinery of some bacteria, especially LABs, which have been considered as potential promising alternatives to antibiotics and current chemical preservatives. Bacteriocins extracted from lactobacilli can be used as natural antimicrobial metabolites in food and pharmaceutical products, due to their inhibitory properties, against a wide range of pathogeninc bacteria over various thermal, acidic, alkaline processes (Alizadeh Behbahani, Noshad, & Jooyandeh, 2021). These compounds are released extracellularly and have bactericidal or bacteriostatic effects against Gram-positive and Gram-negative indicators (Nes, Yoon, & Diep, 2007).

Bacteriocins could increase the permeability of target cell cytoplasmic membranes, release small cytoplasmic particles, make pores in cell walls, affect on depolarization of membrane potential, inactivation of lipid II and elimination of membrane potential, decrease intracellular ATP concentration, and eventually cause cell death (Simha, Sood, Kumariya, & Garsa, 2012). Pediocin, pelantarcin, nisin and Micocin® are FDA-approved bacteriocins (Radaic, de Jesus, & Kapila, 2020). Nisin is the most well-known and widely used bacteriocin in the food industry, which is used as an antimicrobial compound in many fermented dairy-based products such as cheese and milk (Hou *et al*., 2019). Although many LAB's with the ability to produce bacteriocin are found in different habitats, only a small number have been identified and isolated from fermented fruit and vegetable products. On the contrary, bacteriocin production has been repeatedly reported by various LABs from dairy products, the producing strains have been identified, and in some cases, their bacteriocin has been purified. Heretofore, there are few reports of their separation and purification from fermented vegetables and fruits and optimizing production conditions to achieve more significant quantities. The isolation and purification of bacteriocin from LABs in curly kale juice pickle (Szutowska & Gwiazdowska, 2021), fermented blueberry (Xiang *et al*., 2021), and wild Himalayan fig fruit pickle (Kumari, Sharma, & Kaundal, 2018), have been reported, and the results indicated high activity of purified bacteriocin against Gram-positive and Gram-negative indicators, as well as heat tolerance, and its stability to different pH values.

This paper introduces some bacteriocin-producing strains of LAB isolated from selected fermented fruit and vegetable of Iran. The objective of this study was to investigate the diversity of LAB communities and their potential antimicrobial activities in less studied traditionally fermented fruit and vegetable products, by analyzing various samples collected from different regions in Iran. In addition, optimizing culture conditions such as temperature, pH, incubation period, and substrate concentration to evaluate its effect on the number of bacteriocins was implemented. Moreover, this study was performed to determine the optimized growth conditions for LAB strains from specific non-dairy fermented

products to produce more bacteriocin with higher antimicrobial activities. This can be remarkably practical in food safety applications, leading to the development of desirable microflora and their antimicrobial compounds in food preservation.

Materials and methods

Bacterial strains and preparation of samples

Fourthy-eight samples of less studied fermented fruit and vegetable products, including fermented Abu Jahl watermelon (*Citrullus colocynthis*), common medlar, Crataegus azarolus, mixed fruit containing quince and green apple, mushroom-corn pickle, and fermented mango, were collected from the local markets in different regions of Iran and stored at room temperature until used. The bacteria used as indicator strains for evaluating bacteriocin screening as antimicrobial activities, were *Staphylococcus aureus* ATCC 25922, *Escherichia coli* ATCC 25923, *Listeria monocytogenes* PTCC 1165, and *Salmonella enterica* serovar typhimurium PTCC 1609 obtained from the Research Institute of Food Science and Technology (RIFST, Mashhad, Iran), Microbiology laboratory. Nisin has been used as a positive control in all tests

Isolation and identification of dominant strains

Various strains of LAB were isolated from fermented products according to the method described by (Ahn, Kim, & Kim, 2017). Lactobacillus spp. were isolated and identified based on phenotypic and physiological experiments using the methods described by (Hwanhlem, Ivanova, Biscola, Choiset, & Haertlé, 2017). The final backslope of MRS agar medium was determined by surface plate counting to identify the predominant LAB. Pure single colonies of LAB isolates were obtained from streak plates and subjected to Gram stain, catalase assay, and species-specific PCR. Next, the genomic DNA of the isolates was extracted with CTAB (cetyltrimethylammonium bromide) and subjected to PCR according to (Cotter, Stanton, Ross, & Hill, 2012). As a result of PCR, an 800 base pair (bp) fragment amplified, and the target sequence is derived from the variable region of 16S rDNA in LAB. The sequences of the forward and reverse specific primers were 27F: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R: 5′-GGTTACCTTGTTACGACTT-3′, respectively (Edalatian *et al*., 2012). After agarose gel electrophoresis, the PCR products were one way sequenced by the Gene Fanavaran Company (Tehran). Sequences were edited and aligned by ClustalW in BioEdit. Results were compared against genomic data available on NCBI (http://www.ncbi.nlm.nih.gov/genbank/) using the BLASTn procedure (Bartkiene *et al*., 2020).

Culture media and growth conditions

The LAB strains were cultured in de Man Rogosa Sharpe (MRS) broth medium (Scharlau-Spain). The modified culture medium was including a certain amount of peptone protease (10 g/L), yeast extract (4 g/L), glucose (20 g/L), dipotassium hydrogen phosphate (2 g/L), sodium acetate trihydrate (2 g/L), triammonium citrate (5 g/L), manganese sulfate tetrahydrate (0.20 g/L), and magnesium sulfate heptahydrate (2 g/L) (Brandl & Huynh, 2014). Various microbial culture media were made by changing the amounts of nitrogen (peptone, yeast extract) and carbon (glucose) sources in the formula. Strains were cultured in MRS broth for 18 h at 37 ºC then collected by centrifugation at 5000 *g* - for 6 min. The cell suspension was inoculated in MRS broth, that manually prepared by the above formulation supplemented with varying concentrations of yeast extract (0.06, 1.03 and 2% w/v), peptone (0.06, 1.03, 2% w/v), tween 80 (50, 100, 150 µL), glucose (0.06, 1.03, 2% w/v), and a mixed culture (MYGPT) inclusive yeast extract (1.03%)

+ tween 80 (100 µL) + glucose (1.03%) and peptone (1.03%). The culture was evaluated for bacteriocin production after 24 and 48 h (Yang *et al*., 2018).

The growth curve plotting

The selected isolates were inoculated into the final (MYGPT) MRS formula, that were incubated at the optimum temperature of bacteriocin production (35 °C). The growth curve was plotted against time by measuring the optical density at hourly intervals using a spectrophotometer (chromtech, Bad Camberg, Germany) at 620 nm for 72 h. In addition, to plot bacterial populations over time, MRS cultures were sampled every two hours, and live-cell counts were calculated using standard plate counting methods. pH analysis was also performed at 2 h intervals (Yang *et al*., 2018).

Design and determining the optimal conditions

Statistical analysis of bacterial growth medium optimization was performed with Response Surface Methodology (RSM) based on Box–Behnken design with three replications at the central point and the help of Design-Expert software (version 10, USA). Six central repetition points were used to estimate the test error and calculate the process repeatability. The multivariate model was defined for each of the evaluated responses to predict the effect of the variables. The next step consisted of graphically presenting the model relation and determining the optimal operating conditions. Finally, the optimal operating conditions were searched using the numerical optimization technique, with the created models to find the best conditions that join the desired optimization goals. Optimization models were implemented for production of bacteriocin in a modified medium having extra amount of carbon and nitrogen sources with maximum values for all dependent variables according to Table 1 (Suganthi & Mohanasrinivasan, 2015).

Table 1. Box–Behnken experimental design to determine the levels of effective parameters

Runs number	Yeast extract	Peptone	Glucose
1	1.03	1.03	0.06
2	0.06	0.06	1.03
3	1.03	1.03	1.03
4	1.03	1.03	2
5	1.03	0.06	0.06
6	1.03	2	1.03
7	1.03	0.06	2
8	0.06	1.03	0.06
9	0.06	2	1.03
10	1.03	2	2
11	2	1.03	1.03
12	2	2	1.03
13	2	0.06	1.03
14	2	1.03	2
15	0.06	1.03	2
16	2	1.03	0.06
17	1.03	0.06	1.03
18	2	1.03	1.03
19	0.06	1.03	1.03
20	1.03	2	0.06

Screening for bacteriocin production and partial purification

Production of bacteriocins was tested by spotting on MRS agar as described by Shokria *et al*. (2013). Isolates were incubated in MRS broth at 35 °C for 48 h, after centrifugation at 8000 *g* for 15 min, the supernatant was separated from the microbial cell to obtain a cell-free supernatant (CFS) by filtering through 0.22 µm filters. The pH of CFS, was adjusted to the range of 6.5-7 with 10 N NaOH in order to reduce the inhibitory activity of the organic acids, then Catalase enzyme (2 mg/mL) was used to neutralize the effect of H_2O_2 in the CFS Ammonium sulfate (75%) precipitation and chloroform/methanol extraction methods, reported by (Shokria, Zaghian, Fazeli, Mobasherizadeh, & Ataei, 2013), were applied for bacteriocin purification. After 24 h storage at 4 °C and centrifugation at 23000 *g* for one hour, potassium phosphate buffer (pH=7) was added to the resulting precipitate buffer in a ratio of 1:1 and methanol-chloroform solution a ratio of 2:1 then centrifuged at 23000 *g* for one hour. The resulting precipitate was extracted and dissolved twice in sterile

water(Shokria et al., 2013) (Shokria *et al*., 2013).

Detection of antibacterial activity at different temperatures

The selected strains that showed the highest antimicrobial activity against tested pathogenic strains were added to modified MRS broths (MYGPT) supplemented with different amounts of glucose, yeast extract, tween 80, and peptone at 30, 32, 35, and 37 °C under anaerobic conditions for 24 and 48 h. Cell-free supernatants of the *Lactobacillus* isolates were obtained by centrifugation at 8000 *g* for 10 min. The pH was adjusted to 6-6.5 with 10 N NaOH, and the samples were filtered through a Millex-GV microfilter with an orifice size of 0.22 mm (Millipore Sigma, US), After concentrating CFS in a freeze-dryer (R114-Operon/ Korea) for 48 h, the cell-free concentrated and neutralized supernatant was evaluated as crude bacteriocin in the non-proliferation zone by a well diffusion assay. Indicators (*S. aureus*, *E. coli*, and *S. typhimuriyum*) were cultured in Mueller-Hinton broth and *L. monocytogenes* was cultured in BHI broth medium. For the well-diffusion assay, 100 µL of the freeze-dried supernatant was transferred into the wells (For every 50 ml of the dried medium, 1000 µl of the sterile distilled water was added), while the sterile MRS medium was used as the control. For better diffusion, the petri dish was cooled in the refrigerator for 30 min, then incubated at 35 °C for 18 h. At the end of the incubation, the inhibition zones around the wells were measured in mm. *Lactobacillus ruteri* ATCC 1608 was used as a bacteriocin producer and control strain (Fagundes, de Sousa Santos, Francisco, Albano, & de Freire Bastos, 2017; Udhayashree, Senbagam, Senthilkumar, Nithya, & Gurusamy, 2012).

Evaluation of MIC and MBC

The minimum inhibitory concentration (MICs) of extracted bacteriocins, was determined by the microtiter dilution method in a 96-well microplate (Kalazist, Iran). After overnight incubation at 35 °C the minimum concentration of non-growing bacteria, was defined as MIC. The cultures from the MIC test were incubated overnight at 35 °C, then the concentration which, no colony, was defined as the minimum bactericidal concentration MBC (Lakshmanan, Kalaimurugan, Sivasankar, Arokiyaraj, & Venkatesan, 2020).

The effect of proteolytic enzymes on bacteriocin activity

The effects of proteolytic enzymes such as proteinase K, chymotrypsin, lysozyme, and pepsin on the antibacterial activity of bacteriocin were measured by a well diffusion assay on Mueller-Hinton agar. Bovine serum albumin was used as a control sample. The specific amounts of concentrated supernatants that were obtained from the previous step were treated with different enzymes (25 µl of a concentration of 20 mg/ml of enzyme), and then the prepared samples were incubated for two hours at 30 °C. Finally, the inhibitory activity was examined by the well diffusion method examined the (Schelegueda, Vallejo, Gliemmo, Marguet, & Campos, 2015).

Statistical analysis

The results of this study were statistically analyzed, based on a completely randomized design, in three replications with the factorial method, and using SAS software (version 9.1), for table analysis. Microsoft Office Excel was used to draw the charts (2013). The means were compared using the least significant difference test (Duncan) at the 95% level.

Results and discussion

Screening for LAB strains with antimicrobial activity

A total of 253 dominant strains were isolated from the 48 types of fruit and vegetable fermented pickles and salinities. Isolates were characterized as Gram-positive, oxidase-negative, indole-negative and catalase-negative bacteria (data are not

shown). Strains collected from fermented Abu Jahl watermelon or *Citrullus colocynthis* (V3), medlar pickle (Ab), *Crataegus azarolus* pickle (Sa), mixed fruit pickle containing quincedesign and green apple (10A), mushroom-corn pickle and fermented mango (S6), that showed the highest antimicrobial activity were identified as superior strains with the most significant growth inhibition zones on the pathogenic strain. Then the bacteriocins produced by each of the five characterized strains were elected as bacteriocins b10A, bV3, bS6, bSa, and bAb.

Strain identification

The selected strains (Fig. 1) were identified by analysis of the 16S rDNA gene, and the results are presented in Table 2. The identification of bacteriocinogenic isolates revealed that *Lactiplantibacillus plantarum* (formerly classified as *Lactobacillus plantarum*) was the predominant population in evaluated fruit and vegetable fermented products. Previous studies shown that *L. plantarum* was dominant among the lactic acid bacteria and bacteriocin-producing strains of this bacterium have been reported from many fermented foods. According to a study conducted by (Szutowska & Gwiazdowska, 2021), on changes in the population of LAB and the determination of probiotic potential during fermentation of curly kale juice, at the beginning of fermentation, the main microbiota was *Leuconostoc mesenteroides*, but during long fermentation, spontaneously replaced with Lactobacillus species, mainly *Lactobacillus plantarum, Lactobacillus sakei, and Lactobacillus coryniformis.* The result of the molecular test was consistent with the previous studies on fermented products (Hu, Zhao, Zhang, Yu, & Lu, 2013).

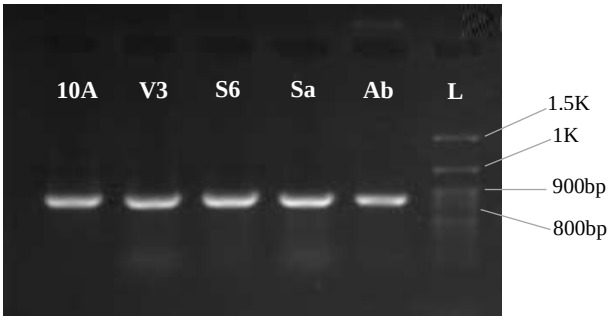

Fig. 1. Demonstration of PCR product (800 bp fragment) resulting from amplification of 16s rRNA gene in five selected isolates with a ladder on 1% agarose gel.

Table 2. 16S rDNA sequencing results of isolates selected from fermented fruit and vegetable products

Isolate code	Name of bacteria	% ID in NCBI	Accession number
V3	*Lactiplantibacillus plantarum strain* Heal 19	99.52%	CP055123.1
10A	*Lactiplantibacillus plantarum strain* 3360	99.42%	MT613643.1
S6	*Lactiplantibacillus plantarum strain* 7232	98.75%	MT645503.1
Sa	*Lactiplantibacillus plantarum strain* Lan4	99.38%	LT853604.1
Ab	*Lactiplantibacillus plantarum strain* lb51	98.99%	AY590774.1

Antimicrobial activity

the antimicrobial activity of five bacteriocins, b10A, bV3, bS6, bSa, and bAb was determined against the indicator microorganisms: *S. aureus*, *E. coli*, *S. typhimurium*, and *L. monocytogenes*. Obtained results show that the indicators were strongly inhibited by all bacteriocins (Table 3). According to the results, strain 10A has a significant difference in the level of 95% compared to other bacteriocins against all pathogens. Bacteriocins mostly show aggressive activity against Gram-positive bacteria and the activity against Gram-negative bacteria is an uncommon phenomenon (Behera, Ray, & Zdolec, 2018), which has been informed for the bacteriocins produced by *Pediococcus* sp. and *Lactobacillus bulgaricus* BB18 (Simova, Beshkova, & Dimitrov, 2009), that had close conformity with the present study.

Table 3. The inhibitory zone of cell free concentrated and neutralized supernatant (mm) incubated for 24/48 h in MRS broth against 10^8 cfu/mL pathogenic strains

Indicator microorganisms supernatants	*S. aureus*	*E. coli*	*S. typhimurium*	*S. aureus*	*E. coli*	*S. typhimurium*
	24 h			48 h		
b10A	13^{aA}	12^{aB}	12^{aB}	14^{aA}	13^{aB}	13^{aB}
bV3	11^{aA}	10^{aB}	12^{aB}	13^{aA}	13^{aB}	13^{aB}
bS6	12^{aA}	11^{aB}	11^{aB}	13^{aA}	13^{aB}	13^{aB}
bAb	10^{bA}	8^{bB}	10^{bB}	10^{bA}	11^{bB}	12^{bB}
bSa	9^{cA}	7^{cB}	8^{cB}	9^{cA}	10^{cB}	10^{cB}

b: bacteriocin; 10A: *L. plantarum* strain 3360; V3 *L. plantarum* strain Heal 19; S6: *L. plantarum* strain 7232; Sa: *L. plantarum* strain lb51*;* Ab: *L. plantarum* strain Lan4.
[a-c] Different superscript letters in the same column between samples denote signifcant differences ($P < 0.05$).
[A-B] Different superscript letters in the same row between samples denote signifcant differences ($P < 0.05$).

Optimization of production conditions

According to Box–Behnken experimental design and the results of Table 4, the MYGPT growth medium was used as the final composition, due to higher bacteriocin activity and larger growth-inhibitory region. Yeast extract provides not only a relatively more significant proportion of free amino acids and short peptides (two/ three amino acids) but also more growth factors than other protein hydrolysates. The maximum bacteriocin action was seen in the supplemented MRS broth with 1.03% peptone + 1.03% glucose + 1.03% yeast extract and 100 µL of tween 80. This culture was found better than other media for cell growing and bacteriocin production. An increase in glucose content had a significant effect on the inhibition zone ($p < 0.05$) but a high amount of glucose (2%) without peptone decreased the bacteriocin production. The addition of yeast extract and glucose in basal MRS broth increased the amount of bacteriocin production, specific amounts of yeast extract and peptone (Fig. 2a/2b) in the final formula had a significant effect on inhibition zone ($p < 0.01$). This finding is supported by such observations around bacteriocin production in broth culture medium with different concentrations of yeast extract and different amounts of tween-80 (Brandl & Huynh, 2014; Yang *et al*., 2018; Zhou *et al*., 2014). Optimal growth conditions for achieving more bacteriocin produced by *Lactobacillus fermentum* (Mahrous *et al*., 2013; Wiegand, Hilpert, & Hancock, 2008), had a good consistent with the present study.

Table 4. Comparison of bacteriocin activity from various modified growth media based on the size of inhibitory zones (mm) of pathogen

Culture media	Pathogen	
	S. aureus	*E. coli*
MG (0.06%)	8.166 ± 0.288^{eA}	8.000 ± 0.000^{eA}
MYG (0.06%)	8.333 ± 0.288^{eA}	8.000 ± 0.000^{eA}
MYGT (0.06%)	16.666 ± 0.577^{cA}	13.166 ± 0.288^{cB}
MYGPT (0.06%)	20.666 ± 0.577^{bA}	19.000 ± 0.000^{bB}
MYGPT*(1.03%)	26.833 ± 0.288^{aA}	22.500 ± 0.500^{aB}
MYGPT (2%)	13.833 ± 0.288^{dB}	12.166 ± 0.288^{dA}

MRS broth (M) with glucose (G), peptone (P), yeast extract (Y) and tween 80 (T) or combination of them like MYGPT: MRS broth enriched with a certain percentage of yeast extract, glucose, peptone and tween 80. Results expressed as mean values of triplicates ± standard deviation.
[a-e]Different superscript letters in the same column between samples denote signifcant differences ($P < 0.05$).
[A-B]Different superscript letters in the same row between samples denote signifcant differences ($P < 0.05$).

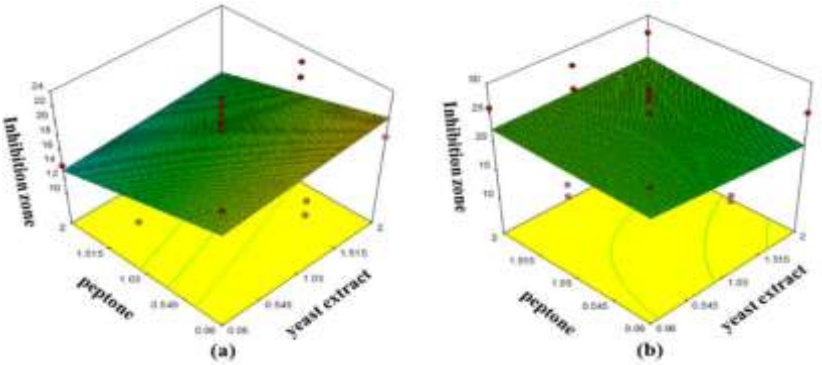

Fig. 2. Interaction of yeast extract and peptone on the diameter of inhibition zone against a: *E. coli* - b: *S. aureus.*

Evaluation of the fitted model for dependent variables of the inhibition zone of pathogens

According to Table 5, the statistically optimal model proposed by Design-Expert software for inhibition zone of *S. aureus* and *E. coli* was a 2F1 model (Equations 1 and 2). The high values of the explanation coefficient and the corrected explanation coefficient indicate the high suitability of the model for data fitting.

$$Y = +16.97 - 1.08X1 + 0.83X2 - 2.75X3 + 0.002X1.X2 + 0.75\,X1.X3 + 0.0003X2.X3 \quad (1)$$

Y: The diameter of the inhibition zone for *E. coli*, X1: yeast extract, X2: peptone, X3: glucose.

In this model, the non-fitting test was not significant and the values of coefficient of explanation and coefficient of variation were relatively appropriate and showed the accuracy of the degree of fit on the model. The interaction of yeast extract-glucose, glucose-peptone and yeast extract-peptone on the diameter of inhibition zone against the growth of *E. coli* shown in Fig. 3a/3b.

As the figures show, increasing the amount of peptone has a significant effect on increasing the size of the inhibition zone. The results of this experiment are closely consistent with the studies of Suganthi & Mohanasrinivasan (2015).

$$Y = +21.70 - 0.58\,X1 + 0.92\,X2 - 3.58\,X3 + 0.75\,X1.X2 + 0.75\,X1.X3 + 0.25X2.X3 \quad (2)$$

Y: the diameter of the inhibition zone for *S. aureus*, X1: yeast extract, X2: peptone, X3: glucose. In this model, the non-fitting test was not significant and the values of coefficient of explanation and coefficient of variation were relatively appropriate and showed the accuracy of the degree of fit on the model. According to Fig. 4a/4b, increasing equal amounts of carbon and nitrogen sources had a significant effect on increasing the inhibition zone of *S. aureus*. The results of this experiment are closely consistent with the studies of (Suganthi & Mohanasrinivasan, 2015).

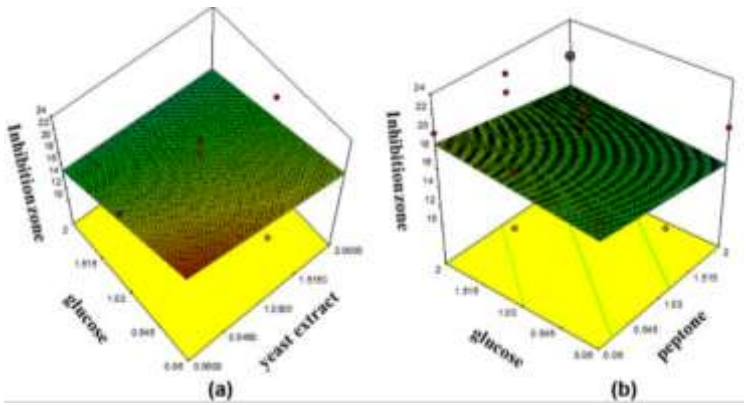

Fig. 3. Interaction of a: yeast extract-glucose and b: yeast extract-peptone on the diameter of inhibition zone against the growth of *E. coli.*

Table 5. The ANOVA analysis of multivariate models in the conventional inhibition zone of *E. coli* and *S. aureus*

Indicator	Source	Sum of squares	df	Mean square	*F* value	*P* value
E. coli	Model	115.42	6	19.24	1.38	0.2628
	A-yeast extract	14.8	1	14.08	1.01	0.3245
	B-peptone	8.33	1	8.33	0.60	0.4465
	C-glucose	90.75	1	90.75	6.53	0.0177
	AB	0.000	1	0.000	0.000	1.0000
	AC	2.25	1	2.25	0.16	0.6911
	BC	0.000	1	0.000	0.000	1.0000
	Residual	319.55	23			
	Lack of fit	284.22	12	7.37	7.37	0.0012
	Pure error	35.33	11			
	Cor total	434.97	29			
S. aureus	Model	173.00	6	28.83	1.12	0.3828
	A-yeast extract	4.08	1	4.08	0.16	0.6944
	B-peptone	10.08	1	10.08	0.39	0.5380
	C-glucose	154.08	1	154.08	5.97	0.0226
	AB	2.25	1	2.25	0.087	0.7704
	AC	2.25	1	2.25	0.087	0.7704
	BC	0.25	1	0.25	0.009692	0.9224
	Residual	593.30	23	25.80		
	Lack of fit	564.30	12	47.02	17.84	<0.0001
	Pure error	29.00	11	2.64		
	Cor total	434.97	29			

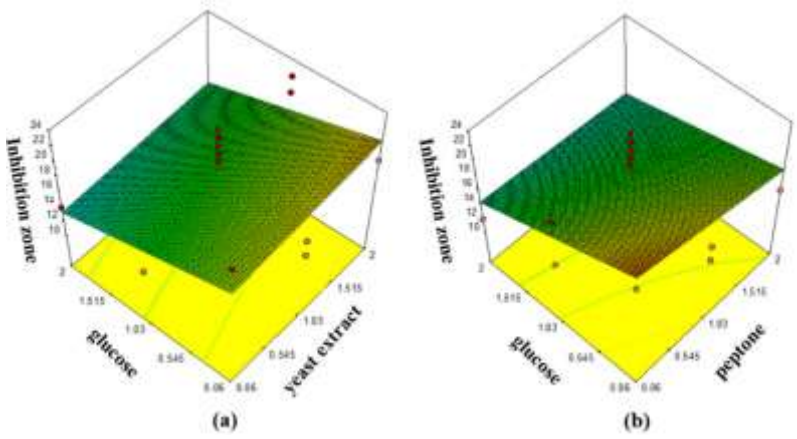

Fig. 4. Interaction of a: yeast extract-glucose and b: yeast extract-peptone on the diameter of inhibition zone against the growth of *S. aureus.*

Determination of MIC and MBC

According to the results of Table 6, a dilution of 480 ppm of bacteriocin from isolates 10A and Ab was considered as the minimum inhibition concentration (MIC). Between isolates 10A and Ab, in terms of the size of the inhibition zone, 10A was identified as the strongest bacteriocin against *S. aureus* and *E. coli*. The results of this experiment are closely consistent with the studies of (Ashari, Nissa, Nursiwi, Sari, & Utami, 2019). The high antimicrobial properties of the bacteriocins in this experiment, compared to an equal amount of commercial nisin, indicated their high potency and activity in inhibiting pathogens.

Table 6. Evaluation of MIC and MBC of bacteriocins extracted against *E. coli* and *S. aureus*

Indicator 10^6CFU/mL	(ppm) / Bacteriocin	240	480	650
Gram +/-	b10A	MIC	MBC	-
	bV3	-	MIC	MBC
	bS6	-	MIC	MBC
	bSa	-	MIC	MBC
	bAb	MIC	MBC	-
	nisin	-	MIC	MBC

Detection of best temperature for bacteriocin production

To evaluate the effect of incubation temperature on cell growth and antimicrobial activity, cultures of the *L. plantarum* strains were maintained at different temperatures (30, 32, 35, and 37 °C).The results showed that ambient growth temperature plays a vital role in increasing bacteriocin production. Regarding the effect of incubation temperature, isolates are incubated for 24 h and 48 h in mixed media at 30, 32, 35, and 37 °C (Table 7). The results showed that growth at 35 °C for 48 h (equivalent to the end of the stationary phase) is the appropriate time and temperature for achieving a high level of bacteriocin production. The antimicrobial activity of bacteriocin in the modified MRS broth at 35 °C compared to different temperatures was recorded at the maximum value. The research results by (Yang et al., 2018) were in close agreement with the present study.

Table 7. Bacteriocin activity and inhibition zone (mm) of strains incubated in enriched MRS broth at different temperature for 48 h

Indicator	T (°C)	10^3 CFU/mL				10^8 CFU/mL			
		S. aureus	*E. coli*	*S. typhimurium*	*L. monocytogenes*	*S. aureus*	*E. coli*	*S. typhimurium*	*L. monocytogenes*
b10A	30	18^{aA}	15^{aC}	18^{aB}	18^{aB}	13.5^{aA}	11.5^{aC}	12^{aB}	13^{aB}
bV3	30	16^{bA}	14^{bC}	22^{bB}	16^{bB}	12.5^{bA}	11^{bC}	12^{bB}	12.5^{bB}
bS6	30	15^{bA}	12^{bC}	19^{bB}	15^{bB}	11.5^{bA}	10^{bC}	11^{bB}	11.5^{bB}
bAb	30	16^{bA}	13.5^{bC}	17^{bB}	16^{bB}	12^{bA}	10^{bC}	11.5^{bB}	12^{bB}
bSa	30	14.5^{bcA}	13^{bcC}	14^{bcB}	15^{bcB}	12^{bcA}	12^{bcC}	11.5^{bcB}	12^{bcB}
nisin	30	14^{cA}	12.5^{cC}	13^{cB}	14^{cB}	10^{cA}	10^{cC}	10^{cB}	11^{cB}
b10A	32	20^{aA}	17^{aC}	18^{aB}	18^{aB}	15^{aA}	14^{aC}	14^{aB}	14^{aB}
bV3	32	18^{bcA}	16^{bcC}	17^{bcB}	17^{bcB}	13^{bcA}	11^{bcC}	12^{bcB}	14^{cB}
bS6	32	18^{bA}	15^{bC}	15^{bB}	15.5^{bB}	14^{bA}	12^{bC}	12^{bB}	13^{bB}
bAb	32	19^{bA}	15^{bC}	15^{bB}	16^{bB}	15^{bA}	12^{bC}	12^{bB}	12.5^{bB}
bSa	32	18^{bcA}	16^{bcC}	17^{bcB}	17^{bcB}	13^{bcA}	11^{bcC}	12^{bcB}	14^{bcB}
nisin	32	16^{cA}	15^{cC}	15^{cB}	15^{cB}	14^{cA}	12^{cC}	12^{cB}	14^{cB}
b10A	35	27^{aA}	22^{aC}	22^{aB}	24^{aB}	21^{aA}	17^{aC}	18^{aB}	20^{aB}
bV3	35	24^{bA}	21^{bC}	21^{bB}	22^{bB}	19^{bA}	16^{bC}	16.5^{bB}	18.5^{bB}
bS6	35	23^{bA}	20.5^{bC}	20^{bB}	21^{bB}	18^{bA}	15^{bC}	15^{bB}	18^{bB}
bAb	35	23^{bA}	17.5^{bC}	18^{bB}	19.5^{bB}	19.5^{bA}	15^{bC}	15^{bB}	17.5^{bB}
bSa	35	22^{bcA}	18^{bc}	18.5^{bcB}	21^{bcB}	17.5^{bcA}	14^{bcC}	15^{bcB}	17^{bcB}
nisin	35	20^{cA}	18^{cC}	18^{cB}	19^{cB}	17^{cA}	15^{cC}	16^{cB}	16^{cB}
b10A	37	18.5^{aA}	16^{aC}	17^{aB}	17^{aB}	18^{aA}	15.5^{aC}	14^{aB}	15^{aB}
bV3	37	17^{bA}	15^{bC}	17^{bB}	16^{bB}	16^{bA}	14^{bC}	14^{bB}	14^{bB}
bS6	37	16^{bA}	14.5^{bC}	15^{bB}	15^{bB}	15.5^{bA}	13.5^{bC}	14^{bB}	14^{bB}
bAb	37	16.5^{bA}	14^{bC}	15.5^{bB}	16^{bB}	15^{bA}	13^{bC}	12.5^{bB}	14^{bB}
bSa	37	14.5^{bcA}	13.5^{bcC}	14^{bcB}	15^{bcB}	14^{bcA}	13^{bcC}	13^{bcB}	12^{bcB}
nisin	37	16^{cA}	14.5^{cC}	15^{cB}	15^{cB}	15^{cA}	12^{cC}	13^{cB}	14^{cB}

b: bacteriocin; 10A: *L. plantarum* strain 3360; V3 *L. plantarum* strain Heal 19; S6: *L. plantarum* strain 7232; Sa: *L. plantarum* strain lb51*;* Ab: *L. plantarum* strain Lan4.

[a-c] Different superscript letters in the same column between samples denote significant differences ($P < 0.05$).

[A-C] Different superscript letters in the same row between samples denote significant differences ($P < 0.05$).

Growth curve and the best time for bacteriocin production

Cultures of the five strains of *L. plantarum* were evaluated for their growth, plus

production of lactic acid and bacteriocin during 72 h. From Fig. 5 we can deduce that extracellularly antimicrobial action was produced at the end of the logarithmic phase after about 19 h, then in the middle of the stationary phase 32 h production of lactic acid and bacteriocin was increased conceivably to eliminate the growth of rival strains. Additionally, bacteriocin production reached its maximum at the end of the stationary phase, about 48 h. Obtained results indicated that the secretion of antimicrobials is correlated with biomass production. Also, the inhibitory zone against Gram-negative and Gram-positive pathogens was examined as indicator strains. Inhibition against *S. aureus* (Fig. 5a), was higher than that of *E. coli* (Fig. 5b). Due to the similarity of the production process in all five strains and the avoidance of overlap in the Fig. 5a/5b was drawn for strain 10A as the producer of the highest amount of bacteriocin. According to the growth curve of the number of living organisms (Fig. 6 and Fig. 7), in the logarithmic phase, due to increased competition for food and space (Hosseininezhad & Yazdi, 2016), the pH of the environment decreased from 6.5 to 3.2 and the production of lactic acid and bacteriocin increased as a defense system to reduce competing microorganisms (Aroutcheva *et al.*, 2001). Findings agreement with the experimental results of Chin study (Chin, Shim, Kim, Yang, & Yoon, 2001).

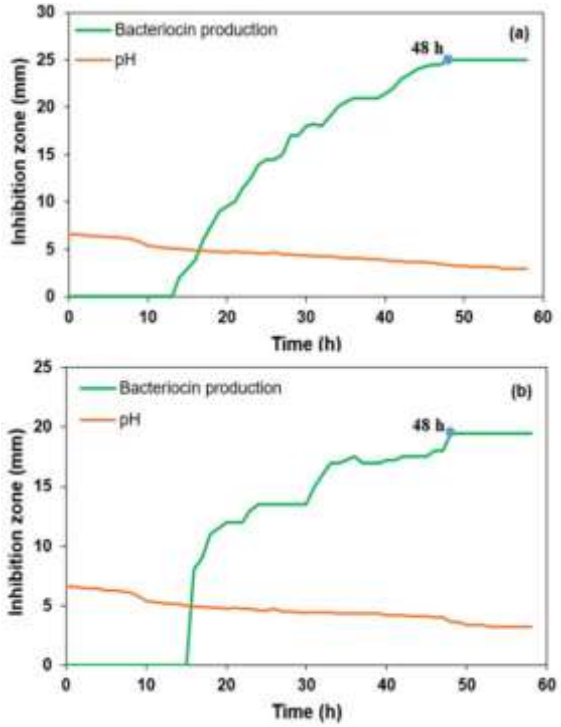

Fig. 5. (a) pH reduction and bacteriocin production curve for *S. aureus* at the different interval times of isolate growth. (b) pH reduction and bacteriocin production curve for *E. coli*.

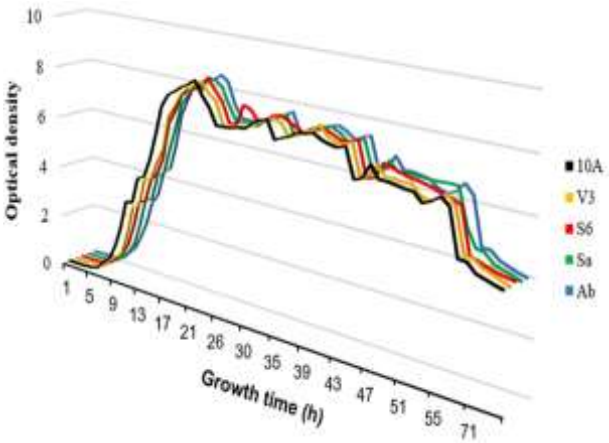

Fig. 6. Growth curve for isolated strains during 72 h at 600 nm.

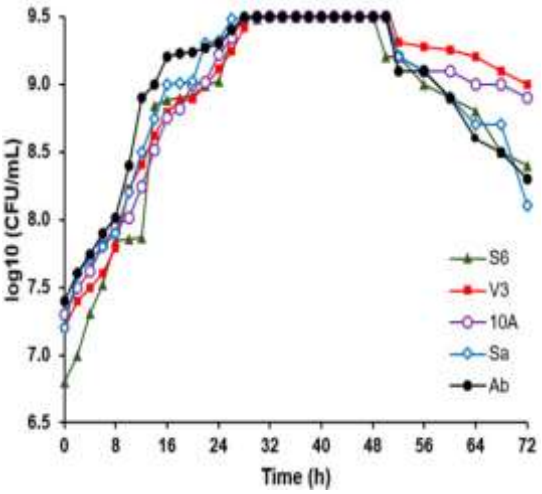

Fig. 7. The viable cell count of the 5 strains of *L. plantarum* isolated from fermented products during 52 h.

Detection of antibacterial activity

Crude bacteriocin from 24-hour cultures at the dose of 10^8 pathogen created a growth zone. According to Fig. 8a, The growth zone was not clear, and tiny colonies grew on the inhibitory zone, due to low volume bacteriocin production or low bacteriocin activity. Partially purified bacteriocin of the middle stationary phase (48-hour cultures) for the five LAB isolates showed large and clear inhibitory zones against the tested indicators in dilution of 10^8 CFU/mL (Fig. 8b). By increasing the culturing time, these areas were still stable even at higher dilutions, and obvious zones could be seen around the wells at a concentration of 10^8 indicators.

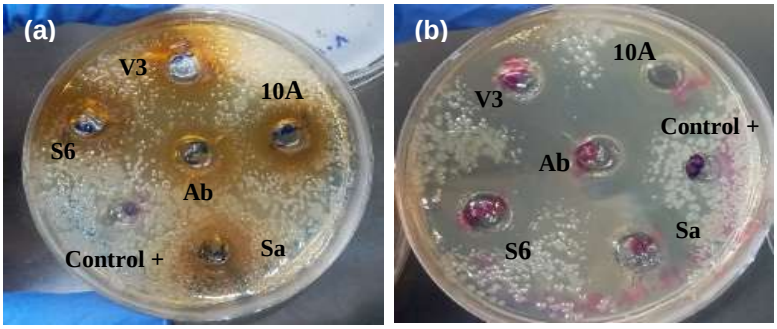

Fig. 8. Inhibition zone of a: Crude bacteriocin from 24 h cultures at the dose of 10^8 pathogen and b: partially purified bacteriocin from 48 h cultures.

Effect of proteolytic enzymes on bacteriocin activities

Treatment with the proteolytic enzymes (proteinase K, chymotrypsin, lysozyme, and pepsin) caused inactivation of the antimicrobial compounds, hence identified them as proteinaceous substances. Partially purified lyophilized bacteriocins were significantly reduced when treated with proteolytic enzymes, and in many cases, the inhibitory effect of bacteriocin was eliminated (Table 8). This test confirmed the protein nature of the inhibitory agent and showed that these compounds were easily digested by proteases. The results of this study were consistent with the results of (Hassan *et al*., 2020; Ma, Guo, Fu, & Jin, 2020) for bacteriocin inhibition with proteinase enzymes, showing the elimination of bacteriocin activity from *Pediococcus acidilactici* by using lysozyme.

Table 8. Effect of different enzymes on partial purified bacteriocins

Indicators bacteriocins	Enzymes 20 mg/mL	*S. aureus*	*E. coli*	*S. typhimurium*
b10A	lysozyme	-	-	-
	chymotrypsin	-	-	-
	proteinase K	-	-	-
	pepsin	-	-	-
bV3	lysozyme	-	-	-
	chymotrypsin	-	-	-
	proteinase K	-	-	-
	pepsin	-	-	-
bS6	lysozyme	-	-	-
	chymotrypsin	-	-	-
	proteinase K	-	-	-
	pepsin	-	-	-
bSa	lysozyme	-	-	-
	chymotrypsin	-	-	-
	proteinase K	-	-	-
	pepsin	-	-	-
bAb	lysozyme	-	-	-
	chymotrypsin	-	-	-
	proteinase K	-	-	-
	pepsin	-	-	-
Ctr	With out	+	+	+

b: bacteriocin; 10A: *L. plantarum* strain 3360; V3 *L. plantarum* strain Heal 19; S6: *L. plantarum* strain 7232; Sa: *L. plantarum* strain lb51*;* Ab: *L. plantarum* strain Lan4.

Conclusion

In this study, antimicrobial activities of LAB from less studied traditional fermented fruit and vegetable products collected from different regions in Iran, such as fermented colocynth, hawthorn, mixed fruits, and medlar pickle were investigated. Five strains of *L.plantarum* found as the dominant strain of LAB with the highest inhibitory effect against pathogenic bacteria. The present study demonstrated the production of bacteriocins (b10A, bV3, bS6, bSa, and bAb) by the dominant strains. Furthermore, it was shown that upon extraction, the amount of bacteriocin under environmental conditions such as temperature, growth time, and culture medium, with carbohydrates and nitrogen sources, was significantly different. Maximum production and bacteriocin activity was observed at 35 °C in 48h culture containing peptone, yeast extract, tween 80 and glucose. Loss of bacteriocin activity during culture at 30 °C may be associated with proteolysis, because LAB depending on the different strains they have, produce extracellular proteases at optimum temperatures of 25 to 55 °C. The resulting bacteriocins possess a broad spectrum of antimicrobial activity and the highest inhibition zones against pathogenic and food spoilage microorganisms. Pure and partial-purified bacteriocins can be used as biological preservatives in food industries. The use of biological preservatives, in addition to increasing the shelf life of food, reduces the use of chemical preservatives, heat, and other physical treatments, which in turn satisfies the need for consumers to use fresh and less processed foods. This study provides the basis for the antimicrobial activity of *L.plantarum* bacteriocin, which exhibits potential as a natural food preservative.

Conflicts of interest

The authors declare that there is no conflict of interest.

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ایجاد شرایط بهینه برای تولید باکتریوسین از *لاکتی پلانتی باسیلوس پلانتاروم* جدا شده از میوه و سبزی تخمیری بومی

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چکیده

باکتری‌های اسید لاکتیک (LAB) تعداد زیادی متابولیت ضدمیکروبی به‌نام باکتریوسین، تولید می‌کنند که به‌عنوان نگهدارنده‌های زیستی ایمن و بالقوه شناخته شده اند. در این پژوهش، جدایه‌های LAB از محصولات تخمیری غیر لبنی، برای بررسی تولید باکتریوسین غربالگری شدند. از بین ۲۵۳ سویه غالب جدا شده از ۴۸ نوع فراورده تخمیری بر پایه میوه و سبزی، پنج سویه (10A، V_3، S_6، Sa و Ab) بر مبنای تولید بیشترین مقدار باکتریوسین، با توالی‌یابی 16S rRNA به عنوان *لاکتی پلانتی باسیلوس پلانتاروم* شناسایی شدند. باکتریوسین‌های حاصل، بیشترین تأثیر بازدارندگی را در برابر رشد *لیستریا مونو سایتوژنز ۱۱۶۵* PTCC، *اشریشیا کلی ۲۵۹۲۳* ATCC، *سالمونلا تایفی موریوم* ۱۶۰۹ PTCC و *استافیلوکوکوس اورئوس ۲۵۹۲۲* ATCC نشان دادند. بهینه‌سازی شرایط کشت بر مبنای منابع مختلف کربن، نیتروژن، زمان و دماهای مختلف گرمخانه‌گذاری (۳۰، ۳۲، ۳۵، ۳۷ درجه سانتیگراد) با روش سطح پاسخ و با رسم منحنی های رشد جدایه‌ها و منحنی تولید باکتریوسین در طی ۵۸ ساعت انجام شد. این تغییرات منجر به افزایش کارایی تولید باکتریوسین به مقدار ۷۰٪ اولیه گردید. حداکثر مقدار تولید و فعالیت باکتریوسین در ۳۵ درجه سانتیگراد و مدت زمان ۴۸ ساعت در کشت حاوی پپتون، عصاره مخمر، توئین ۸۰ و گلوکز مشاهده شد. اثر مهاری باکتریوسین‌ها در هنگام تیمار با آنزیم‌های پروتئولیتیک به طور قابل توجهی کاهش یافت. باکتریوسین حاصل از جدایه ۱۰A با فعالیت ۶۴۰۰۰ Au/mL اثر مهاری بیشتری در مقایسه با نایسین از خود نشان داد، این مطالعه با موفقیت باکتریوسین ۱۰A را به عنوان یک نگهدارنده بیولوژیک برای کنترل رشد باکتری‌های مولد فساد و بیماری‌زای مواد غذایی توصیه می‌کند.

واژه‌های کلیدی: فعالیت ضد باکتریایی، نگهدارنده بیولوژیک، فراورده‌های تخمیری، *لاکتی پلانتی باسیلوس پلانتاروم*