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Application of Biotechnology for Production, Purification and Characterization of Peptide Antibiotic Produced by Probiotic *Lactobacillus plantarum*, NRRL B-227

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Abstract: This work was carried out in the course of a screening program for specification of the bioactive substances that demonstrated inhibitory affects against bacterial pathogens and application of oral administration of probiotic *Lactobacillus plantrum* NRRL B-227 *in vivo* using mices. Forty *lactobacillus* sp. selected from American agriculture culture collection. All these strains were screened for their antibacterial activity against pathogenic bacteria. Among the forty *Lactobacillus,* the broad spectrum strain *Lactobacillus* sp NRRL B-227 was active against bacterial pathogens, Gram positive bacteria viz *Staphylococcus aureus* ATCC 29213*, Micrococcus luteus* ATCC 9341, *Bacillus subtilis* NRRL B-543, *Bacillus subtilis* NCTC 10400, *Bacillus pumilus* NCTC 8214 and Gram negative bacteria viz *Escherichia coli* ATCC 25922*, Escherichia coli* NCTC 10416, *Salmonella typhi* NCIMB 9331, *Enterobacter cloacae* ATCC 13047, *Pseudomonas aeruginosa* ATCC 10145, *Klebsiella pneumonia* NCTC 9111, *Klebsiella pneumonia* ATCC 13883. From the taxonomic features, the *Lactobacillus* sp NRRL B-227 matches with *Lactobacillus plantarum* in the morphological, physiological and biochemical characters. Thus, it was given the suggested name *Lactobacillus plantarum* NRRL B-227. The nucleotide sequence of the 16S RNA gene of the most potent strain evidenced a 100% similarity with *Lactobacillus plantarum*. The active metabolite was precipitation using ammonium sulfate and its purification was performed using reverse-phase high performance liquid chromatography (RP-HPLC) on ODS Hypersil C18 column. The physico-chemical characteristics of the purified peptide antibiotic viz. heat & pH resistance and spectroscopic characteristics (UV, IR, Mass spectrum and amino acid analyzer) have been investigated. In conclusion, the collected data emphasized the fact that the purified peptide antibiotic may be belonging to bacteriocin antibiotic produced by *Lactobacillus plantarum* NRRL B-227. The application of oral administration of probiotic *Lactobacillus plantrum* NRRL B-227 *in vivo* using mice's had been investigated.

Key words: *Lactobacillus plantarum* NRRL B-227 · Antibacterial activity · Bacteriocin · 16S RNA · Fermentation · Precipitation and Purification · Probiotic *Lactobacillus plantarum in vivo*

INTRODUCTION

Lactic acid bacteria have been widely used in the food industry as starter culture for fermentation. *Lactobacillus* species play a crucial role in foodstuffs, because of their fermentative ability and their health and nutritional benefits [1]. Lactic acid bacteria can produce many compounds such as organic acids, hydrogen peroxide and bacteriocin during fermentation [2,3]. Bacteriocins are proteinaceous substances that display antimicrobial activity against species closely related to the producer strain and/or other bacteria [4,5]. A variety of antimicrobial agents that differ in their inhibitory spectra, mode of actions and biochemical characteristics is produced by *Lactobacillus* species. *L. plantarum* has been isolated from various habitats and several bacteriocins (antimicrobial peptides) have been described in strains from milk and cheese [6,7]. Bacteriocins are proteinaceous antibacterial compounds and exhibit bactericidal activity against species closely related to the producer *Lactobacillus* strains [8, 9]. Many bacteriocins are active against food-borne pathogens, especially against *Listeria monocytogenes* [10, 11]. Bacteriocins are heterogeneous and they are classified largely based on differences in their molecular weights [12]. Some bacteriocins are peptides consisting of only 13 to 37 amino acids. Some small bacteriocins contain unusual amino acids originating from modifications of conventional amino acids after translation [13]. Several types of bacteriocins from food-associated lactic acid

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bacteria have been identified and characterized, of which nisin, diplococcin, acidophilin, bulgarican, helveticins, lactacins, lactolin and plantaricins are the important bacteriocins [14,15]. Of these, bacteriocin and nisin produced by *Lactococcus lactis* sp. lactis, has been the most extensively characterized [16,17]. Nisin is the only bacteriocin commercially available [18]. It has been reported that nisin is more active against Gram-positive bacteria, particularly the spore-formers [19]. Other bacteriocins of *Lactobacilli* have been reported to be effective against closely related species of mesophilic *Lactobacillu*s and therefore considered as potential natural food preservatives [20, 21]. However, studies relating to the antibacterial properties of these organisms are limited and not fully exploited for use [22, 23]. Probiotic *lactobacilli* are known to confirm an array of health promoting activities on their host after either parenteral or oral administration [24]. Some of their beneficial effects include prevention of intestinal infection [25] anticarcinogenic activity [26] control of serum cholesterol and enhancement of immunity [27] growth enhancement of animals [28]. The mechanism by which these probiotics affects their host and bring about improvement in the gut barrier can be due to competition for adhesion site, production of inhibitory compounds and rebalancing of disturbed gastrointestinal microbial composition and metabolism [29].

The current work aimed to a screening program for specification of the bioactive substances produced by *Lactobacillus* sp. NRRL B-227 selected from American agriculture culture collection, which generates an antibacterial compound. The identification of this strain, based on the cultural, morphology, physiology and biochemical characteristics, as well as 16s rRNA methodology, is also reported. The bioactive substance was precipitation, purification and the physico-chemical characteristics have been investigated. Application of oral administration of probiotic *Lactobacillus plantrum* NRRL B-227 *in vivo* using mice was determined.

MATERIALS AND METHODS

Bacterial Culture and Media: Bacterial strain used in this study were selected from culture collection NRRL B- 227 grown in MRS broth (10 g Peptone, 8 g Meat extract, 4 g yeast extract, 20 g D(+) Glucose, 2 g Dipotassium hydrogen phosphate, 5 g Sodium acetate trihydrate, 2 g Triammonium citrate, 0.2 g Magnesium sulfate heptahydrate, 0.05 g Magnesium sulfate tetrahydrate, 1L Dist. water, Final pH 6.2) at 30°C for 48 h [30].

Indicator Bacterial Strains: Gram positive bacteria viz *Staphylococcus aureus* ATCC 29213*, Micrococcus luteus* ATCC 9341, *Bacillus pumilus* NCTC 8214, *Bacillus subtilis* NRRL B-543, *Bacillus subtilis* NCTC 10400 and Gram negative bacteria viz *Escherichia coli* ATCC 25922, *Escherichia coli* NCTC 10416, *Salmonella typhi* NCIMB 9331, *Enterobacter cloacae* ATCC 13047, *Pseudomonas aeruginosa* ATCC 10145, *Klebsiella pneumonia* NCTC 9111, *Klebsiella pneumonia* ATCC 13883.

Screening for Antibacterial Activity: The anti- microbial activity was determined by cup method assay according to [31].

Bacterial Identification: Selected strain was examined microscopically for cellular morphology and biochemical properties [32]. PCR was used to amplify the 16S ribosomal DNA gene of strain. The 16S ribosomal DNA sequence was determined by direct sequencing. Total DNA was isolated by using Wizard genomic DNA purification kit (Promega, Madison, USA). Primers used for PCR and DNA sequencing. The PCR amplification was performed with the primer pair SPO/SP6 targeted against regions of 16S ribosomal DNA StrepF 5-AAGAGTTTGATCCTGGCTCAG-3. and Strep R 5-CTACGGCTACCTTGTTACGA-3 [33]. Amplification of DNA was performed in a Mastercycler personal thermal cycler (Eppendorf). PCR conditions included a hot start at 96°C (5 min.), 35 cycles consisting of hybridation at 50°C (1 min), polymerisation at 72°C (2 min.), denaturation at 96°C (1 min) and a final extension at 72°C (2 min.). PCR products were resolved by electrophoresis in 1% (w/v) agarose gel and visualized by ethidium bromide (1 µl/10 ml) staining. 16S ribosomal DNA PCR applicants were purified following the microcon YM-100 kit (Bedford, MA, USA) and sequenced using the Big Dye Terminator V3.0 kit as specified by the supplier with primers while automated sequencing of both strands of the PCR products gene sequencer (ABI, Forster, USA). The sequences obtained (500-750 bp) were then assembled in silico (Vector NTI) using overlapping zones between the various sequences to form the contiguous sequence. Phylogenetic analysis was realized by an alignment of sequence consensus of the 16S ribosomal DNA genes collected in an international database (Gene bank). The results were then expressed in percentage of homology between the submitted sequence and the sequences resulting from the database [33].

Estimation of Total Protein: The protein content of the peptide antibiotic was determined according to [34].

Fermentation, Extraction, Precipitation and Purification

Fermentation: *Lactobacillus plantrum* NRRL B-227 was propagated when 1L MRS broth inoculated with 10 ml bacterial culture and incubation for 48 h in a static incubator at 30°C. MRS liquid medium composed of Peptone, 10.0; Meat extract, 8.0; yeast extract, 4.0; D(+) Glucose, 20.0 Dipotassium hydrogen phosphate, 2.0; Sodium acetate trihydrate, 5.0 Triammonium citrate, 2.0 Magnesium sulfate heptahydrate, 0.2; Magnesium sulfate tetrahydrate, 0.05 and distilled water up to 1000ml. The pH was adjusted at 6.2 before sterilization at 30°C for 48 h [35].

Extraction: A cell-free filtrate was obtained by centrifuging (10,000 rpm for 20 min. at 4°C, followed by filtration of the supernantant through a 0.2 um pore size cellulose acetate filter. Various organic solvents including iso–amylalcohol, chloroform, n-propanol, hexane, Diethyl ether, petroleum ether were added to purify antimicrobial substance in 1:1 ratio. The organic phase was concentrated to dryness under vacuum by using a rotary evaporator.

Precipitation: The precipitation process of the peptide antibiotic was carried out using ammonium sulfate. Culture supernatant was treated with solid ammonium sulphate to 0, 20, 40 and 60% saturation. The mixtures were stirred for 2 h at 4°C and later centrifuged at 9,000 rpm for 50 min at 4°C. The pellet was re-suspended in 25 ml of 0.05 M potassium phosphate buffer pH 7.0. Dialysis: was carried out against the same buffer for 18 h in spectrapor dialysis tubing. Assay of the antimicrobial activity was determined. This product was named CE1 [36].

Purification: The purification of the peptide antibiotic was carried out by using reverse-phase (RP) HPLC. This analytical technique has been shown to be extremely valuable for the analysis of this peptide antibiotic, since peptide antibiotic are generally resistant to different organic solvents used as mobile phases and the high pressures employed trough the chromatographic process. In the HPLC protocol, after the sample has been separated by cation exchange or SPE chromatography, the peptide antibiotic extract is loaded onto the HPLC column. In the purification protocols described by the authors listed in the references of this chapter, several types of RP columns bas been used, being the silica-based C4 to 18 ODS the most frequently employed. Although for peptide separation a 300 ? pore column is sometimes recommended, the frequent experimentation shows that 100 A pore columns are also effective for peptides purification. In the protocols used in our laboratory, purifications were performed by the frequent use of C18 column (hypersil ODS) a column. The inhibitory activity is found in fractions collected in between the retention time of the peptides (hatched area). The mobile phase consisted of (i) acetonitrile and (ii) HPLC-grade water containing 0.1% trifluoroacetic acid (TFA). The sample was loaded on the C18 column (hypersil ODS) and separated by a linear biphasic gradient of 20 to 80% acetonitrile over 30 min at a flow rate of 1.0 ml/min.

Physico-chemical Properties

Spectroscopic Analysis: The IR, UV and Mass spectrum were determined at the National Research Center Egypt and amino acid analyzer was determined at the Desert Research Center Egypt.

Separation of Antibacterial Agent by SDS-PAGE: The pure peptide antibacterial agent was checked by sodium dodecyl sulphate-polysaccharide gel electrophoresis (SDS-PAGE), with 10 % polyacrylamide gels as described by [37]. In the presence of 0.1 % SDS in pH 8.8 and 1 ml M-dithiothreitol, gels and gel buffer were prepared as described by [38, 39]. Gels were stained for protein using Coomassie Blue as described by [40].

Reaction of the Antibacterial Agent with Certain Chemical Test: For this purpose, the following reactions were carried out: molish's, fehling, sakaguchi, ninhydrin, ehrlish, nitroprusside, ferric chloride and mayer reactions [41].

Heat Resistance: purified peptide antibiotic (400 µl) was exposed to various heat treatments: 40, 60, 80, 100 and 120°C. Aliquot volumes of each fraction were then removed after 0, 30, 60 or 90 min [35].

pH Resistance: purified peptide antibiotic (400 µl) were adjusted to pH 2,4,6,8,10 and 12 with hydrochloric acid and sodium hydroxide, incubated for 4 h at room temperature [35, 42].

Characterization of the Antibacterial Agent: The peptide antibiotic produced by *Lactobacillus plantrum* NRRL B- 227 was identified according to the recommended international references [43, 45].

Application of Probiotic *Lactobacillus plantrum* NRRL B-227 *In Vivo* Using Mice

Animals: A total number of 18 male Egyptian mice weighing approximately 20 g were kept in separate cylindrical wire cages and were supplied with pellet food and drinking water.

Inoculation in Mice: The animals were divided into six groups, each group containing 3 mice. The first group of animals was kept without any treatment (the control group). Group of animals from B to D were orally challenged twice after disinfection of cages by ethyl alcohol 70 % with 5 µl per mouse of saline (NaCl 0.9 %) suspension mixture *(Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922 and *Salmonella typhi*), after 5 days stool samples were taken for analysis. The group F of animals was challenged twice orally with lyophilized culture of *Lactobacillus plantarum* with dose of 0.5 g per mice mouse once / day for 5 days.

Treatment Step: The group B of animals did not receive any antibiotic and lyophilize culture of *L. plantarum* NRRL B- 227. The C group of animals was treated with lyophilized culture of *L. plantarum* NRRL B- 227 only. respectively orally (Dose=0.5 g per mouse once/ day) for 5 days with disinfected cages by ethyl alcohol 70 % every morning. The group D of animals was orally treated with ximacef antibiotic (Dose 5.2 gm / kg b.wt per mice once day) for 5 days with disinfected cages by ethyl alcohol 70% every morning. The group E of animals was treated orally with lyophilized culture of *L. plantarum* NRRL B- 227 (Dose= 0.5 g per mouse once day) and Ximacef antibiotic (Dose= 5.2 g / kg b.wt per mice once day) for 5 days with disinfected cages by ethyl alcohol 70 % every morning.

Stool Culture and Body Weight: A stool sample was collected early from each mice cage in sterile container, used sterile inoculated lob for inoculation each stool sample on three selective plates media S.S. agar, MacConkey agar and Blood agar under aseptic condition, incubated at 37°C for 24 h then the plates were examined to determine the bacterial count. Sensitive balance was used for weighting mice ever day.

RESULTS

Screening for the Antibacterial Activities: The active metabolites produced by *lactobacillus* sp NRRL B-227 exhibited various degrees of activities against Gram positive and Gram-negative target strains, especially human intestinal bacterial pathogens (Table 1).

Bacterial Identification: The bacterial strain, were found to be Gram-positive bacilli and hydrolysis gelatine, whereas Catalase and nitrate reduction are negative. Their growth was weak pH of 4.4, while grew luxuriously at 37°C and pH of 9.0. The bacterial strain produced lactic acid and degradable of esculine. The bacterial strain fermented mannitol, melibiose, mannose, lactose, maltose, glucose, galactose, ribose, sucrose, rhamnose, raffinose and trehalose but they could not ferment xylose, arabinose and starch. On the basis of the previously collected data, it could be stated that bacterial strain, NRRL B-227 may be belonging to *Lactobacillus plantrum* NRRL B-227.

Molecular Phylogeny: The resulted sequence was aligned with available, almost compete sequence of type strains of family *lactobacilli* and then with corresponding sequences of representative *Lactobacillus* sepecies, in each case, the reference sequence was retrieved from the Gene Bank databases. The phylogenetic tree (diagram) revealed that the bacterial strain is closely related to *Lactobacillus plantrum* with similarity matrix of 100% (Fig. 1).

Fermentation of Antibacterial Agent: Lactobacillus plantrum NRRL B-227 was propagated when 1L MRS broth inoculated with 10 ml bacterial culture and incubation for 48 h in a static incubator at 30°C. A cell-free filtrate was obtained by centrifuging (10,000 rpm for 20 min. at 4°C, followed by filtration of the supernatant through a 0.2 um pore size cellulose acetate filter.

Extraction of Antibacterial Substance with Organic Solvents: Various organic solvents were tested for the extraction of peptide antibiotic produced by the *L. plantarum* NRRL B- 227. It was observed that extraction with polar solvents such as hexane, di-ethyl ether and petroleum ether did not result in the removal antibacterial substance produced at the aqueous phase to the organic phase, while chloroform extraction completely destroyed the antibacterial substance activity. However, when different alcohols such as n-propanol and Iso-amyl alcohol were used in the extraction procedure, antibacterial substance was removed from the aqueous phase and recovered from the organic phase (Table 2).

Table 1: Mean diameters of inhibition zones (mm) caused by 100µl of the antibacterial activities produced by *Lactobacillus* sp NRRL B-227 in the agar plate diffusion assay.

Test organism	Origin	*Inhibition zone (mm)
Staphylococcus aureus	ATCC 29213	21.0
Micrococcus luteus	ATCC 9341	22.0
Bacillus pumilus	NCTC 8214	21.0
Bacillus subtilis	NRRL B-543	21.0
Bacillus subtilis	NCTC 10400	20.0
Escherichia coli	ATCC 25922	22.0
Escherichia coli	NCTC 10416	22.0
Salmonella typhi	NCIMB 9331	20.0
Enterobacter cloacae	ATCC 13047	22.0
Pseudomonas aeruginosa	ATCC 10145	19.0
Klebsiella pneumonia	NCTC 9111	22.0
Klebsiella pneumonia	ATCC 13883	23.0

*The diameter of the used cup assay was 9 mm

Table 2: Extraction of antibacterial substance by organic solvents

	*Mean diameter of inhibition zones (mm)	
Organic solvent	Organic phase	Aqueous phase
Iso-amylalchohal	11.0	11.0
Chloroform	0.0	0.0
n- Propanol	10.0	0.0
Hexane	0.0	0.0
Di-ethylether	0.0	13.0
Petroleum ether	0.0	0.0

*The diameter of the used cup assay was 9 mm

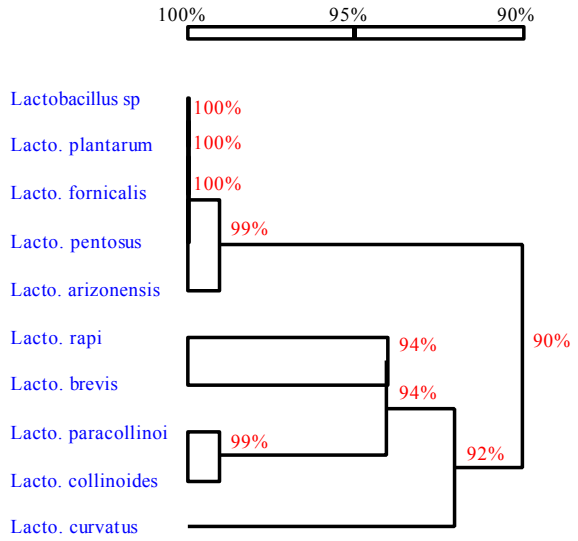

Fig. 1: The phylogenetic position of the *Lactobacillus* sp. strain among neighboring species. The phylogenetic tree was based on the pairwise comparisons of 16_S DNA sequences.

Precipitation of Peptide Antibiotic by Ammonium Sulfate: The peptide antibiotic produced by *L. plantarum* NRRL B- 227 was treated with ammonium sulphate 60% saturation. The mixtures were stirred for 2 h at 4°C and later centrifuged at 10,000 rpm for 50 min at 4°C. The pellet was resuspended in 25 ml of 0.05 M potassium phosphate buffer pH 7.0. Dialysis was carried out against the same buffer for 18 h in spectrapor dialysis tubing. Assay of the antibacterial activity was carried out increase of antibacterial activity comparing with culture supernatant. On the other hand the estimation of total protein by lowery method was carried out decrease of total protein content (Table 3).

Purification of Peptide Antibiotic by Reverse-phase (RP) HPLC: Partial purification steps of the peptide antibiotic are summarized in Table 4. During the purification procedure, each step resulted in a considerable loss of protein concentration while activities are increases. The fractions have been given from Reverse-phase (RP) HPLC are assayed to determined the antibacterial activity then taken the active fraction and estimated the total protein concentration. The active fraction was purified by a subsequent reverse phase chromatography and its amino acid sequence was determined (Fig. 2).

Amino Acid Composition of Peptide Antibiotic
The Amino Acid Content Was Carried out Using: Amino acid analyzer LC3000. The amino acid composition of the peptide antibiotic obtained after the final reverse-phase chromatography was determined. The calculation of the number of amino acid residues in the antibacterial substance revealed that it contained 13 amino acids (Table 4).

Separation Peptide Antibiotic by Polyacrylamide Gel Electrophoresis: The active proteins were homogenous in disc *polyacrylamide* gel electrophoresis and gave only one band of protein at molecular weight 3.8 KDa.

Spectroscopic Characteristics: The spectroscopic analysis of the purified of peptide antibiotic produced by *L. plantarum* NRRL B- 227, maximal IR spectra showed eight absorption peaks in the region of 1129.12; 199.51; 1400.7; 5.; 1650.77; 285.0; 2919.7and 3431.71 cm^{-1} (Fig. 3). The ultraviolet (UV) absorption spectrum are recorded a maximum absorption peak at 328 and 336 nm (Fig. 4). The Mass spectrum showed that the molecular weight at 381 (Fig.5).

Biochemical Reaction of the Peptide Antibiotic: The reactions revealed the detection of certain groups in the investigated molecule. The antibacterial peptide exhibited

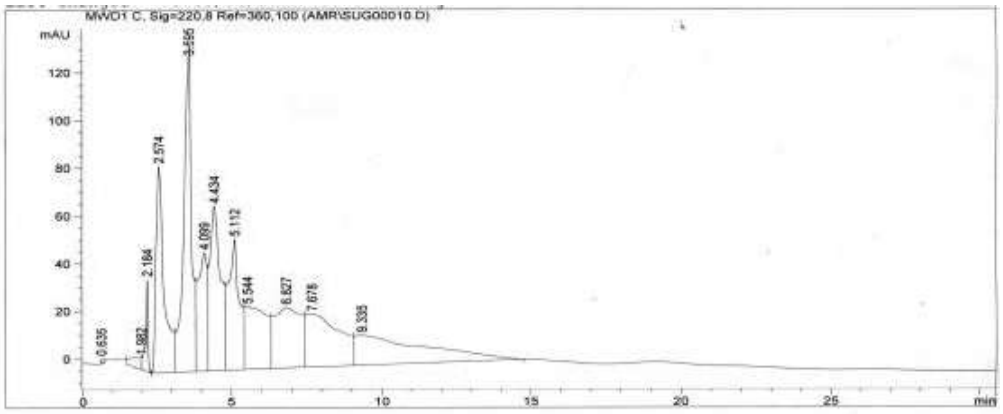

Fig. 2: Purification of peptide antibiotic produced by *Lactobacillus plantrum* NRRL B-227 using HPLC

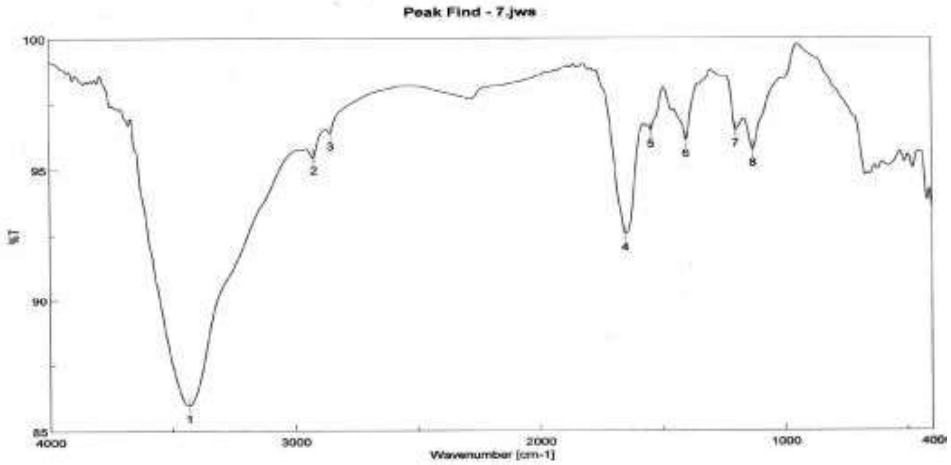

Fig. 3: I.R spectrum of peptide antibiotic produced by *Lactobacillus plantrum* NRRL B-227

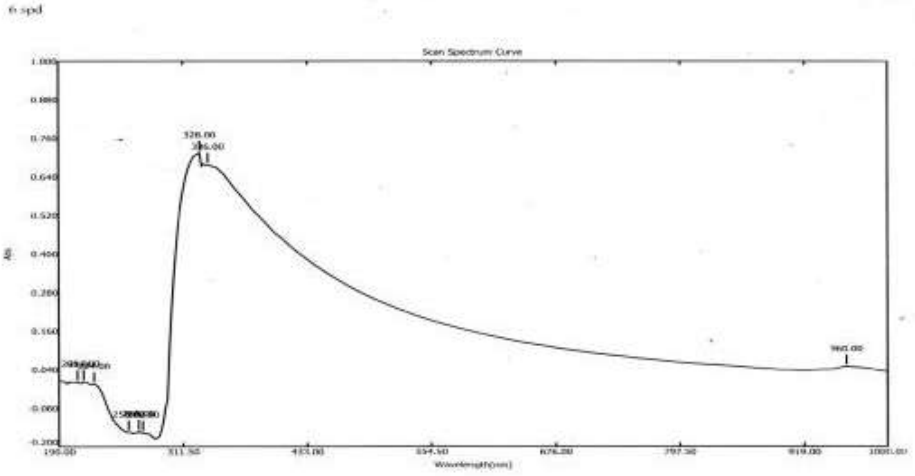

Fig. 4: Ultraviolet absorbance peptide antibiotic produced by *Lactobacillus plantrum* NRRL B-227

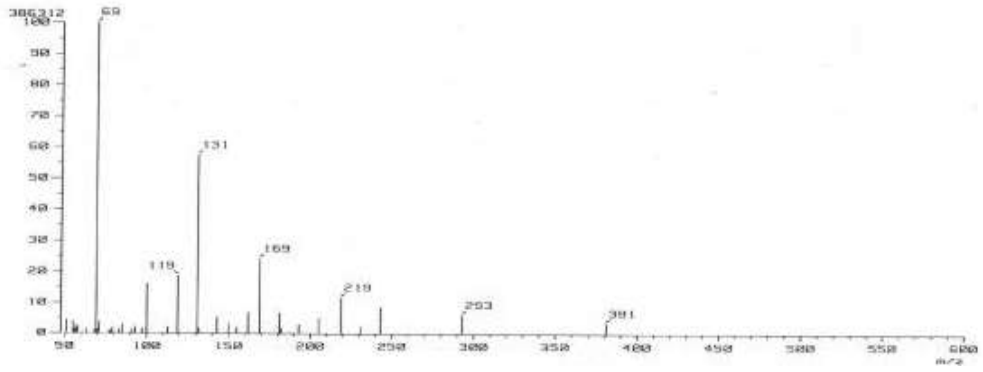

Fig. 5: Mass spectrum of peptide antibiotic produced by *Lactobacillus plantrum* NRRL B-227

Table 3: Precipitation and purification of peptide antibiotic produced by *Lactobacillus plantrum* NRRL B-227

Purification stages	Volume (ml)	IZ (mm)a	Protein (mg\ml)b	Recovery (%)c
Culture supernatant	1000	17.0	4.6	100
Amm.SO_4 ppt.(CE1)	20	35	2.5	55
HPLC	1.0	50	0.13	3.1

a. Inhibition zone including the diameter of the well 9 mm

b. Protein concentration was determined by the lowery method

c. Recovery percentage is the remaining protein concentration as a percentage of the initial protein concentration

Table 4: The amino acid composition of the peptide antibiotic produced by *L. plantarum* NRRL B- 227

No	R.T	Amino acids	%
1	11.57	Aspartic acid	0.399466
2	15.95	Therionine	0.162441
3	17.44	Serine	0.291149
4	19.59	Glutamic acid	1.790077
5	--	Proline	ND
6	24.70	Glycine	0.46701
7	25.77	Alanine	0.808896
8	--	Valine	ND
9	36.19	Methionine	7.65333
10	--	Isoleucine	ND
11	40.59	Leucine	1.318516
12	44.25	Tyrosine	2.822643
13	48.20	Phenylalanine	5.46419
14	56.23	Histidine	1.651119
15	--	Lysine	ND
16	65.77	Ammonia	76.87712
17	68.22	Argenine	0.294045

positive results with ninhydrin, ferric chloride and mayer reactions; whereas negative results with molish's, fehling, sakaguchi, ehrlish and nitroprusside reactions.

Heat Resistance: The effects of heat on peptide antibiotic were determined by using *Escherichia coli*, ATCC 25922 as indicator organism. Antibacterial agent produced by *Lactobacillus plantrum NRRL B-227* activity remained constant after heating at 121°C for 10 min.

pH Resistance: The effects of pH on peptide antibiotic were determined by using *Escherichia coli*, ATCC 25922 as indicator organism. Antibacterial agent produced by *Lactobacillus plantrum* NRRL B-227, it was found to be stable at pH 4 to 9.

Identification of the Peptide Antibiotic: On the basis of the recommended keys for the identification of antibiotics and in view of the comparative study of the recorded properties of the peptide antibiotic, it could be stated that the antibacterial agent is suggestive of being belonging Bacteriocin antibiotic.

Application of Probiotic *Lactobacillus plantrum* NRRL B-227 *in Vivo* Using Mice's: In the present studies, orally administered lactobacilli strains did flourish in the intestinal tract because they could overcome host defense mechanisms. The group C are infected with pathogenic bacteria could be recorded recovery healthy when treatment with probiotic *Lactobacillus plantarum* NRRL B-227 after 5 days and total mice's weight in group F that increase of body weight from 20 gm to 26 gm when consumed probiotic *Lactobacillus plantarum* NRRL

Table 5: The application of oral administration of probiotic *Lactobacillus plantrum* NRRL B-227 *in vivo* using mice's

	Infection with pathogenic bacteria			Treatment with *Lactobacillus plantrum* NRRL B-227 and/or ximacef antibiotic			
	E. coli growth Count	*S. typhi* growth Count	*S. aureus* growth Count	*E. coli* growth Count	*S. typhi* growth Count	*S. aureus* growth Count	
Groups	Report of stool culture after 5 days						Comment
A Control	No growth	No growth	No growth	No growth	No growth	No growth	Healthy control
Binfected without treatment	25000	25000	50000	Death	Death	Death	Death of all individual
C Infected and treatment with *L. plantrum*	25000	25000	100000	No growth	No growth	No growth	Recover healthy
D Infected and treatment with antibiotic	100000	25000	100000	Death	Death	Death	Death of all individual
E Infected and treatment with both *L. plantrum* and antibiotic	50000	30000	100000	Death	Death	Death	Death of all individual
F Treatment with *L. plantrum*	No growth	No growth	No growth	No growth	No growth	No growth	Healthy and Increase of body weight from 20gm to 26 gm

B-227 and significantly from the control, whereas the group B and D are death after 5 days for treatment when infected by bacterial pathogenic (Table 5).

DISCUSSION

Probiotics were reported as a food supplement and improvement for human health through production of useful compounds [46]. Forty *lactobacillus* sp were selected from American agriculture culture collection. All these strains were screened for their antibacterial activity against pathogenic bacteria, especially human intestinal bacterial pathogens. Among the forty *Lactobacillus* sp., the broad spectrum strain *Lactobacillus* sp NRRL B-227 was exhibited various degrees of activities against bacterial pathogens, Gram positive bacteria, *Staphylococcus aureus* ATCC 29213*, Micrococcus luteus* ATCC 9341, *Bacillus pumilus* NCTC 8214, *Bacillus subtilis* NRRL B-543, *Bacillus subtilis* NCTC 10400 and Gram negative bacteria, *Escherichia coli* ATCC 25922, *Escherichia coli* NCTC 10416, *Salmonella typhi* NCIMB 9331, *Enterobacter cloacae* ATCC 13047, *Pseudomonas aeruginosa* ATCC 10145, *Klebsiella pneumonia* NCTC 9111, *Klebsiella pneumonia* ATCC 13883 [47, 48]. The bacterial strains were found to be Gram-positive bacilli and hydrolysis gelatine, whereas catalase and nitrate reduction are negative. Their growth was weak at pH of 4.4, while, they luxuriously grew at 37°C and pH of 9.0. The strain produced lactic acid and degradable of esculine. The bacterial strain was fermented mannitol, melibiose, mannose, lactose, maltose, glucose, galactose, ribose, sucrose, rhamnose, raffinose and trehalose, but they could not ferment xylose, arabinose and starch [49, 50]. The resulted sequence was aligned with available, almost compete sequence of type strains of family *lactobacill*i and then with corresponding sequences of representative *Lactobacillus* species, in each case, the reference sequence was retrieved from the Gene Bank databases. The phylogenetic tree (diagram) revealed that the bacterial strain is closely related to *L. plantrum* with similarity matrix of 100%. The active metabolite of the peptide antibiotic was precipitated by ammonium sulphate (60% saturation), The mixtures were stirred for 2 h at 4°C and later centrifuged at 10,000 rpm for 50 min at 4°C. The pellet was resuspended in 25 ml of 0.05 M potassium phosphate buffer pH 7.0 [22, 23, 51]. The purification was performed using reverse-phase high performance liquid chromatography (RP-HPLC) on ODS Hypersil C18 column [52]. The fractions have been assayed to determined the antibacterial activities then taken the active fraction and estimated the total protein concentration [48]. The amino acid

composition of the peptide antibiotic revealed that it contained 13 amino acids [53]. A pure protein was subjected to polyacrylamide gel electrophoresis (10%) in the presence of sodium dodecyl sulphate (SDS). Proteins were localized by staining with coomassie blue. The active proteins were homogenous in disc gel electrophoresis and gave only one band of protein could be recorded at molecular weight 3.8 KDa. The spectroscopic analysis of the purified of antibacterial compound produced by *L. plantarum* NRRL B- 227, maximal IR spectra showed eight absorption peaks in the region of 1129.12; 199.51; 1400.7; 5.; 1650.77; 285.0; 2919.7 and 3431.71 cm^{-1}. The ultraviolet (UV) absorption spectrum are recorded a maximum absorption peak at 328 and 336 nm. The Mass spectrum showed that the molecular weight at 381[54]. The reactions revealed the detection of certain groups in the investigated molecule. The peptide antibiotic exhibited positive results with ninhydrin, ferric chloride and mayer reactions; whereas negative results with molish's, fehling, sakaguchi, ehrlish and nitroprusside reactions [41]. The effects of heat and pH resistances on antibacterial agent were determined by using *Escherichia coli*, ATCC 25922 as indicator organism was exhibited the activity remained constant after heating at 121°C for 10 min and its stable at pH 4 to 8 respectively. The identification of peptide antibiotic and in view of the comparative study of the recorded properties of the antibacterial peptide, it could be stated that the peptide antibiotic is suggestive of being belonging Bacteriocin antibiotic [15, 43, 44, 45]. Orally administered lactobacilli strains did flourish in the intestinal tract because they could overcome host defense mechanisms. The group C are infected with pathogenic bacteria could be recorded recovery healthy when treatment with probiotic *Lactobacillus plantarum* NRRL B-227 after 5 days and total mice's weight in group F that increase of body weight from 20 gm to 26 gm when consumed probiotic *Lactobacillus plantarum* NRRL B-227 and significantly from the control, whereas the group B and D are death after 5 days for treatment when infected by bacterial pathogenic [55].

It could be concluded that the bacteriocin antibiotic produced by probiotic *Lactobacillus plantarum* NRRL B-227 that demonstrated inhibitory affects against bacterial pathogenic. Probiotics had been used as growth promoters due to their ability to suppress the growth and activities of growth depressing microflora and their ability in enhancing absorption of nutrients through the production of digestive enzymes Chang.

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