New molecule of nonribosomal peptide synthesis mechanism from *Lactiplantibacillus plantarum* LMG100 probiotic bacteria

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Background

The huge increasing on gastrointestinal illness by spreading of resistance pathogens requires to develop alternative antimicrobial agents. Nonribosomal peptides are considered one of these alternatives which produced by wild spectrum of bacteria.

Objective

Detection of nonribosomal peptide synthesis from *Lactiplantibacillus plantarum* LMG100 probiotic strain isolated from traditional lactic fermenting foods as alternative antimicrobial agent is important to human health and immune system.

Materials and methods

The identification of isolated strains using 16S rDNA technique was performed and followed by bioinformatics analysis tools; AntiSmash, PKS-NRPS analysis website, LSI based A-domain function predictor, NRPS predictor2, clustering using PhyML 3.0 to detect adenylation domain substrate specificity of NRP synthetases genes cluster of *Lactiplantibacillus plantarum* LMG100. To prove the presence of the NRP synthetases genes cluster, degenerate primers protocol and three sets of primers covered the five gene cluster were designed based on the original reference strain *L. plantarum* WCFS1. Antibacterial activity of the isolated strain was detected against bacterial strains from coliform group of the enteric genera of *Escherichia*, *Salmonella* and *Shigella* which formed the ordinary gastrointestinal tract infection.

Results and conclusion

The isolated *L. plantarum* LMG100 strain showed 99.96% of identity to 16S rDNA partial gene sequence of *Lactiplantibacillus plantarum* WCFS1 strain and bioinformatics analysis tools revealed the presence of NRPS gene cluster of five genes; two biosynthetic genes *nps*A and *nps*B from the five genes encoded for polypeptide of six amino acids, but six different predictors programs couldn't assign the specificity of all adenylation domains except A4 serine and A5 glycine. The use of degenerate primers confirmed the presence of the NRPS in the isolated strain *L. plantarum* LMG100 compared to the standard strain WCFS1. Three sets of primers covering the five gene cluster were designed based on the original reference strain *L. plantarum* WCFS1 complete genome sequence confirmed the same organization of the putative gene cluster. In general, the approach of degenerated primers proved the presence of polypeptide NRPs gene presence in lactobacilli isolates. The new polypeptide NRP produced from the strain LMG100 showed maximum inhibition zones against G^{-ve} short-rods bacterial strains from the coliform group of the enteric genera of *Escherichia*, *Salmonella* and *Shigella* which formed the ordinary gastrointestinal tract infection, and the minimal inhibitory concentrations (MIC) for G^{-ve} bacterial strains was approximately of 125 mg.ml^{-1}. The obtained results revealed that the selected probiotic *Lactiplantibacillus* strain is suitable candidate for use as bio-preservative starter or probiotic for human consumption in food and pharmaceutical industries.

Keywords:
Lactiplantibacillus plantarum, nonribosomal peptide synthesis, probiotic bacteria

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Introduction

Lactic acid bacteria are considered play important roles in maintaining immune system and normal health in humans. Several members of Lactobacilli spp. are known to produce important metabolites from different synthetic mechanisms, one of the most important the nonribosomal peptide synthesis mechanism which produce molecules with antimicrobial activity against various pathogens live in the human female urogenital and intestinal tracts. These nonribosomal molecules act by interfering with

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adhesion to the epithelial cell's surfaces and biofilm formation [1].

Forty-six biosurfactant production from *Lactobacillus* spp. were reported, among them six were classified as cell free biosurfactant and the others fourteen species as cell associated biosurfactants and only 50% of those species' reports have described the structural composition which mainly are considered: proteinaceous, glycolipids, glycoproteins, or glyco-lipopeptides in nature [2]. *Lactobacillus* is a member of lactic acid bacteria which play a vital role in food preservation due to their unique features like nonpathogenic, production of lactic acid from carbohydrates fermentation, subsequently suppress pathogenic microorganisms and spoilage [3].

Analysis of genome-scale of lactic acid bacteria such as *Lactiplantibacillus* is an approach to study their physiological performance or predict of their adaptability to various environments as well as their putative probiotic potential [4]. Most of lactic acid bacteria that have antimicrobial properties and used as probiotics are of animal or human origin, but recently, lactic acid bacteria species with probiotic potential were obtained from unconventional sources like vegetables, fruits, grains, and other products [5]. Among *Lactobacilli* species, *Lactiplantibacillus plantarum* is one of the most potential candidates to use as a probiotic [6]. *Lactobacillus* biosurfactant are generally belonged to be surlactin type due to their proteinaceous nature with effective with strong action toward preventing the adherence of pathogens. Recently, Lactobacilli-derived biosurfactants are focused by researchers due to their antibiofilm properties. However, *Lactiplantibacillus plantarum* strains are well known with their probiotic application due to their functional activities such as, antioxidant, bio-protective, immune modulation, and antibacterial activity against both Gram-positive (G^{ve+}) and Gram-negative (G^{ve-}) and pathogenic bacteria, viz *Escherichia coli, Salmonella typhimurium, Staphylococcus aureus,* and *Listeria monocytogenes* [7].

Due to the increase of pathogens resistance to chemical antibiotics, it is of importance to develop alternative antimicrobial agents. Nonribosomal synthesized peptides are considered one of alternative antimicrobial compounds [8]. Nonribosomal peptides are microbial secondary metabolites with various bioactivities including antibiotics, surfactants, anticancer agents, anti-inflammatorily, toxins and siderophores [9,10]. Nonribosomal peptides are synthesis through nonribosomal peptide synthetases (NRPS) using multimodular enzymes and thiotemplate mechanism for assembling bioactive peptides [10]. NRPSs are produced by many microorganisms' lactic acid bacteria such as *Lactobacillus* species. *L. plantarum* WCFS1 was reported as the first NRPSs producer among lactic acid bacteria [9,11].

The advancement in computational tools and sequencing which led to abundance of the microbial genome data has helped to predict more novel natural products from different resources such as nonribosomal peptides (NRPs), polyketides (PKs), alkaloids and saccharides from unknown bacterial or plant sources [12]. On other hand, polypeptides are surface-active molecules with a double nature, both hydrophobic and hydrophilic, high biodegradability, humans' low toxicity and extreme conditions resistant, therefore it can be industrially used in various fields [13]. Nonribosomal peptides synthesized by *Lactobacillus* species principally consisted of polysaccharide, protein, and phosphate, which were characterized as glycolipids or glyco-lipoproteins. Nonribosomal peptides (NRPs)have antimicrobial activity against several pathogenic bacteria (*Escherichia coli, Staphylococcus saprophyticus, Klebsiella pneumoniae* and *Enterobacter aerogenes*) as well as antifungal effect such as *Candida albicans* [13,14]. However, various *Lactobacilli* species can live throughout the gastrointestinal tract, stomachs, duodena and colons of humans and animals, hence they have the ability to interact with host cells metabolism of gastrointestinal tract [9].

Lactobacilli plays a very vital role in shaping microbiota of gut, as well as intestinal pH reduction and other bacteria fitness modulation through production of propionic, lactic, and acetic acids. Furthermore, some species of *Lactobacillus* that have antimicrobial activities can reduce the number of bacteria in the digestive system [9,15]. Also, *Lactobacillus* sp. represents one of the most probiotic used to increase of farm animal weight [9]. *Lactobacillus plantarum* was registered as feed additives in European register of feed additives such as silage additive, preservative, and gut flora stabilizer [16].

Finally, this work aims to focus the significance of probiotic *Lactiplantibacillus* sp. derived nonribosomal peptides (NRPs) as suitable candidate for use as bio-preservative starter for human consumption in food and pharmaceutical industries.

Materials and methods

Bacterial strains and isolation condition

The *Lactiplantibacillus* sp. strain used in this work was isolated from traditional lactic fermenting foods according to the method described by [17]. One

loopful of the bacterial stock culture was injected into the culture broth of Man Rogosa Sharpe broth (MRS), and streaked onto agar plates containing the same media, then incubated for 48 h at 37°C. The growing colony was examined microscopically, and preserved in the same medium containing 15% glycerol at −20°C. The investigated foodborne bacterial strains were kindly obtained from the culture collection of National Research Center (NRC), Egypt. The pathogenic bacteria used in this study were selected as follows: two common strains including the G^{ve+}(*Bacillus cereus*) and G^{ve+} (*Escherichia coli* O157), two gram-negative bacterial strains of enteric poisoning pathogens involving *Shigella* sp., and *Salmonella typhi*, as well as two gram-positive bacterial strains of food poisoning pathogens (*Staphylococcus aureus* and *Streptococcus pyogenes*). The pre-cultures of all pathogenic bacteria were conducted by individual cultivation of each strain in Luria-Bertani [LB] medium at 37°C during 24 h of incubation. The method of disc-diffusion using Mueller-Hinton [MH] agar plates was followed as described according to the protocol of the clinical laboratory standards institute (CLSI) guidelines [18].

Identification of lactic acid probiotic bacteria

DNA isolation protocol was performed as described by Wizard Genomic DNA Purification Kit from Promega and 16S rDNA amplification was performed as described by [19], using the 16S rDNA bacterial universal primers 8 F; 5'AGAGTTTGATCCTGGCTCAG'3 and 1492 R 5'GGTTACCTTGTTACGACTT'3. PCR was lanced for 35 cycles in main steps of denaturation at 94°C for 1 min, hybridization at 50°C for 30 sec and elongation at 72°C for 1.5 min PCR products were separated on 1.2% agarose gel electrophoresis [20]. The Zymoclean^{TM} Gel DNA recovery kit (Epigenetics company) was used to purify16S rDNA fragments which were then ligated to pGEM-T Easy vector (Promega) and transformed into *E. coli* JM109 competent cells as described in pGEM- T Easy vector manual.LB media supplemented with Ampicillin/IPTG/X-Gal were used to culture the transformants colonies and incubated at 37°C for 24 hours till the appearance of white colonies. Plasmids were purified by Mini-Prep Plasmid Purification Kit (Promega) and then sent for sequencing. GenBank database (Blast) online software were used for sequence data alignment [20].

Fermentation protocol and purification process

Fermentation process was lanced in MRS medium, the 0.01 optical density at OD_{600} of fresh isolated *Lactiplantibacillus* sp. seed was injected in Erlenmeyer flasks with 70% filling volume, agitation rate 50 rpm and limited oxygenation incubated at 37°Cfor 30 h (stationary phase). After growth, planktonic bacterial cells were removed from culture growth media by centrifugation with 10.000 rpm for 15 min at 5°C. For NRPs peptides extraction, 0.5 ml supernatants sample was passing through prepared C_{18} cartridges (protocol of Alltech, Fr). The concentrations of NRPs peptide were determined by (HPLC) reverse phase spectrum and the peptide extracted after microbial fermentation in batch culture according to [21].

Detection of bacterial NRPs synthetases genes

The detection of NRPs synthetases genes were performed using AIb1-F 5' TYTYTGTTGGCA CTATTMTTCCA'3/TIb1-R 5' TGAYCYGSTA GCGACWTMCTT'3 as described by [22] depending on the conserved nucleic acids sequence by the alignment of thiolation and adenylation domains involved in biosynthesis of NRPs synthetases genes in *Lactobacillus*. PCR was lanced for 35 cycles of three main steps; denaturation at 95°C for 30 s; annealing for 30 sat 45°C, finally extension step of 45 s at 72°C.

In-Silico detection of putative bacterial (NRPs) gene cluster

NCBI Reference Sequence NC_004567.2 (GCA_000203855.3) for the complete genome sequence of *Lactobacillus plantarum* strain WCFS1 was used as model throughout this study. The NRPS gene clusters were firstly detected by using AntiSmash version 4.0.2 [23]. The catalytic domains involved in NRPSs biosynthesis genes were identified by NRPS/PKS analysis website [24]. The adenylation domain specificity prediction was lanced by using NRPS predictor 2 [25], LSI based A-domain function predictor websites [26] and PhyML 3.0 [27].

Genetic organization detection of putative NRPs synthetases gene cluster

Since genes responsible for biosynthesis of NRPS in *Lactobacillus plantarum* have not been reported, series of primers based on *Lactobacillus plantarum* strain WCFS1 gene cluster sequence were designed to detect the presence, the organization and arrangement of this gene cluster in the eight isolated *Lactobacillus plantarum* strains under study, these primers used are all illustrated in Table 1.

HPLC-Mass Spectrophotometry of synthetases NRPs molecule

The molecular composition, which frequently inserted in parent ion mass spectrometry, it was used to provide

Table 1 Primers used for the detection of NRPS synthetase gene cluster in in *L. plantarum*.

Primer	Sequence	Product size	Reference
*nps*A-*pan*DFwd	ATCACCAGTTGCGCACAAAA	1493 bp	This study
*nps*A-*pan*D Rev	AGGTAAGATTCACCGGGCAAC		
Phospho- *nps*B Fwd	TTCATCTTCCACCTCACATTT	2450 bp	This study
Phospho- *nps*B Rev	TCAAAACATATGGGGCTATCG		
*nps*B- *nps*C Fwd	AGCTAACACTGGTAAAAGCTTTATTTG	1650 bp	This study
*nps*B- *nps*C Rev	TGTATTACTTGAATAATCAGAGCATGT		

further information about composition structural. The isolated fractions were eluted from the high-performance liquid chromatography (HPLC) column and analyzed by mass spectrometry system coupled to ion Trap mass spectrometer (San Jose, CA, USA). The separation process was performed on 5 µm C_{18} column, 160×5.0 mm. The mobile phase included the polypeptide fraction were composite of acetonitrile (solvent A), formic acid1% (solvent B). After that, polypeptide fraction was eluted through the iso-citric gradient starting from: Solvent A (v/v) by (80:20), all positive experiments on positive ion modes were performed in the mass spectrometer [28].

Determination of antibacterial activity of purified NRPS molecule

The antimicrobial activity assay against pathogenic bacteria was conducted using Mueller-Hinton agar plates and disc-diffusion technique in accordance with the Baur and Kerby protocol as defined by (CLSI) guidelines. Briefly, the pathogenic bacteria were cultivated in MRS broth overnight and the density of cultures was modified to 0.5 McFarland turbidity standard units. The pathogenic bacterial strains were inoculated onto Mueller-Hinton agar plates by using sterile swab. While 0.6 mm sterile discs of Whatman filter Paper No. (1), were impregnated with each purified molecule (100 µl/disc), and placed on agar surface, discs impregnated with distilled water served as a negative control. All plates were overnight incubated for 18 h at 37°C, and the diameters of the inhibitions zones around discs (mm)were measured and considered as a measure of antibacterial activity. All experiments were performed in triplicate, and the results were expressed as means of inhibition zone diameter with their standard deviations. The complete inhibition of bacterial growth by the lowest concentrations ($mg\,ml^{-1}$) of the purified molecule was defined as the minimal inhibitory concentrations (MICs). Stock solutions of the fermentation culture were carried out by centrifugation at 1000 g for further final concentrations and then inoculated into the culture medium, the control consisted of clear media and growth culture (-/+ control) according to the standard (CLSI) guidelines protocol [18].

Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean values ± standard deviations. Data were subjected to statistical analysis using the SPSS (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp).

Results and discussion

Lactic acid probiotic bacteria isolation and identification

The dissimilar lactic acid probiotic standard colonies were isolated anaerobically on MRS agar specific medium from the traditional fermenting green olive samples. Colonies usually appeared on agar plates within two days. The bacterial colonies that exhibited positive result with Gram stain and belonged morphologically to non-spore forming motile long rode lactobacilli was selected [29]. The 16S rDNA amplified fragment of the selected LAB isolated strain was 600 bp in length, while Blast databases was used to compare and align these sequences which were found to belong to *Lactobacillus plantarum* which recently became *Lactiplantibacillus plantarum* according to the data listed in Table 2. The isolated *L. plantarum* strain16S rDNA partial gene sequence has shown identity of 99.67% with the *L. plantarum* WCFS1 (the reference strain). The presence of NRP synthetases genes involved in its genome was confirmed by bioinformatics tools and it was found that this is the only *Lactiplantibacillus* that harbors the NRPs genes, according to that this strain was used as a reference strain in this study.

The 16S rRNA gene sequence of the isolated bacteria with laboratory code LMG100 showed the same similarity percentage (99.21%) to the *Lactiplantibacillus plantarum* partial 16SrRNA genes of strains 3355, KCC48, G119, zrx03, LAH_19, NF3, AR514, XLP1733, TMPC10262, BSR19, HDC-06,

Table 2 Identity percentage of16SrRNA partial genes of isolated LAB using Blast nucleotide alignment

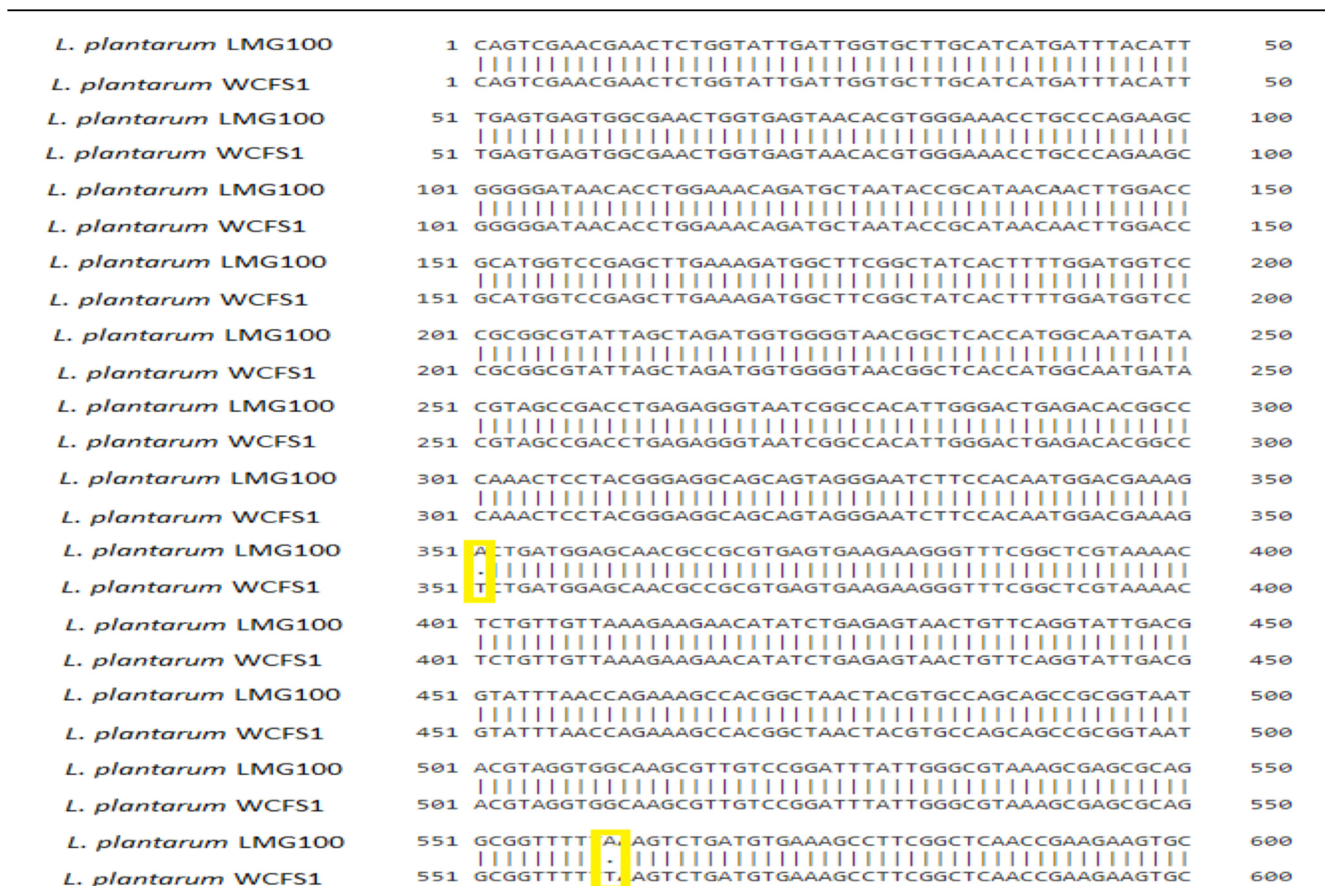
Description	Identity %	Accession
Lactiplantibacillus plantarum strain 3335 16S rRNA partial gene	99.21	MT613628.1
Lactiplantibacillus plantarum strain KCC48 16S rRNA partial gene	99.21	MT318652.1
Lactiplantibacillus plantarum subsp. *plantarum* strain GI19 16S rRNA partial gene	99.21	MT158596.1
Lactiplantibacillus plantarum strain zrx03 16S rRNA partial gene	99.21	MN784485.1
Lactiplantibacillus plantarum strain IAH_19 16S rRNA partial gene	99.21	MK990062.1
Lactiplantibacillus plantarum strain NF3 16S rRNA partial gene	99.21	MK418664.1
Lactiplantibacillus plantarum strain AR514 16S rRNA partial gene	99.21	MK311266.1
Lactiplantibacillus plantarum strain XLP1733 16S rRNA partial gene	99.21	MG983980.1
Lactiplantibacillus plantarum strain TMPC 10262 16S rRNA partial gene	99.21	OM267669.1
Lactiplantibacillus plantarum strain BSR19 16S rRNA partial gene	99.21	OK287090.1
Lactiplantibacillus plantarum strain HDC-06 16S rRNA partial gene	99.21	OK036472.1
Lactiplantibacillus plantarum strain F87 16S rRNA partial gene	99.21	MW217200.1
Lactiplantibacillus plantarum strain RHD13 16S rRNA partial gene	99.21	MW063509.1
Lactobacillus sp. strain LN18054 16S rRNA partial gene	99.13	MT754397.1

F87, and RHD13. While the 16S rRNA gene sequence of the isolated LMG100has similarity to other *Lactobacillus* sp. strain LN18054 (accession N°: MT754397.1) sequence by (99.13%). On other hand, the obtained data of similarity of the 16S rRNA gene refer to the high potentiated of belonging the isolated lactic acid bacteria to the species of *Lactiplantibacillus plantarum* according to the phylogenic tree Fig. 2.

Sequence analysis and adenylation domains specificity of NRPS encoding genes

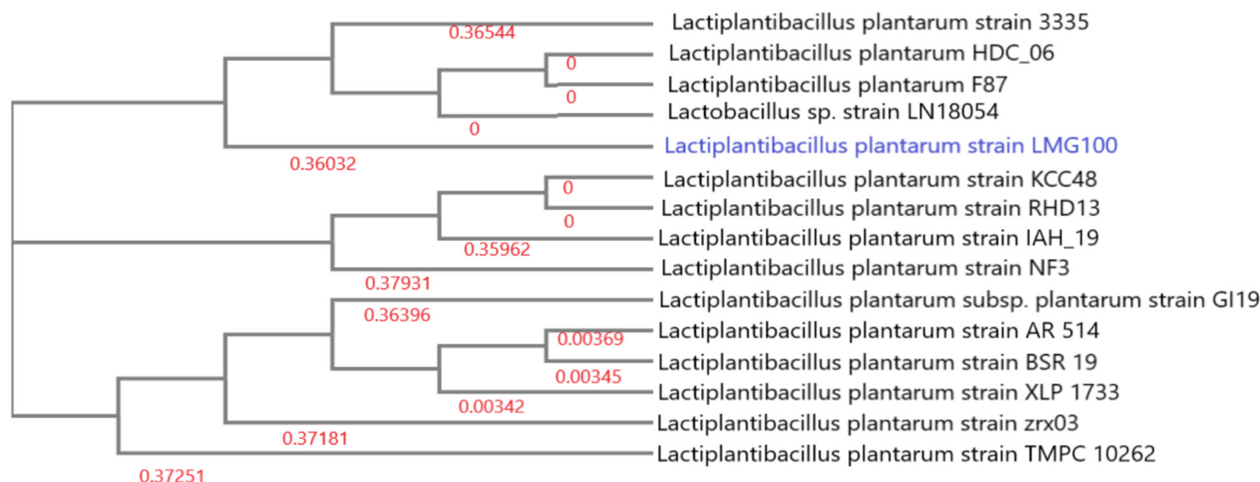
Since genes responsible for biosynthesis of NRPs in *Lactiplantibacillus plantarum* have not been reported except for *Lactiplantibacillus plantarum* WCFS1. Complete genome sequence was used for analysis after alignment of 16SrRNA partial gene of this reference strain and the isolated Lactiplantibacillus plantarum LMG100 as showed Fig 1. AntiSmash

Figure 1



EMBOSS-NEEDLE alignment of 16S rDNA partial gene sequence of the isolated Lactiplantibacillus plantarum LMG100 and the reference strain Lactiplantibacillus plantarum WCFS1 (99.67% identity).

Figure 2



Phylogenic tree based on aligned 16S rRNA partial gene sequences.

Table 3 Nonribosomal peptide synthetases genes (operon) detected in *Lactobacillus plantarum* WCFS1

Protein name	Accession no.	Protein product	Length (bp)	Length A.A
Nonribosomal peptide synthetase (npsA)	CCC78063.1	WP_011101060.1	15869	5289
MULTISPECIES: aspartate 1-decarboxylase (panD)	CCC78064.1	WP_011101061.1	392	130
bis(5\'-nucleosyl)-tetra phosphatase (symmetrical) YqeK (metal-dependent phosphohydrolase)	CCC78065.1	WP_011101062.1	92	330
AMP-binding protein (npsB)	CCC78066.1	WP_011101063.1	2417	805
MULTISPECIES: 4\'-phosphopantetheinyl transferase superfamily protein (npsC)	CCC78067.1	WP_011101064.1	644	214

version 4.0.2 was first used to detect the NRPs genes clusters in *Lactiplantibacillus plantarum* strain WCFS1 genome followed by the identification of their different domains by NRPS/PKS analysis website. These programs analysis detect the presence of five frequented genes: two NRPs genes *nps*A (nonribosomal peptide synthetase) and AMP-binding protein (*nps*B), aspartate-1-decarboxylase (*pan*D) or, metal-dependent phosphohydrolase and 4-phosphopantethenyl transferase (*nps*C) (Table 3).

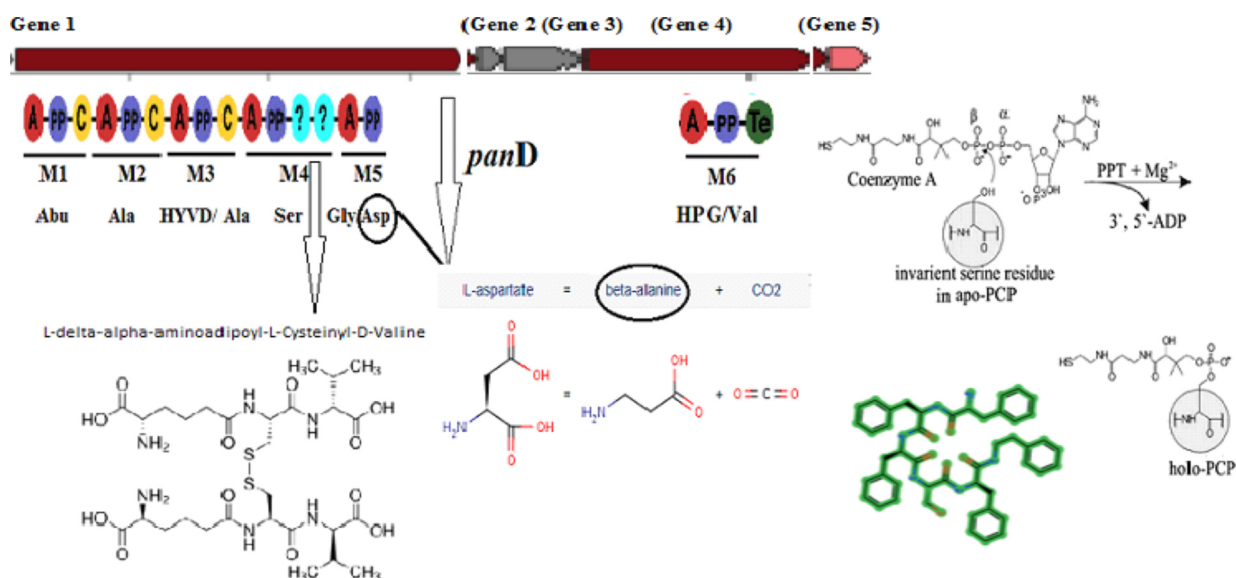
The first nonribosomal peptide synthetase gene *nps*A gene consists of five modules and 16 domains; 5 Adenylation domains (A), 5 peptidyl carrier protein or thiolation domains (T), 5 condensation domains (C) and two not identified domains. The second nonribosomal peptide synthetase gene *nps*B has one module with A, T and thioestrase TE domains and without C domain which carried on the previous gene *nps*A. This C domain most be carried by the *nps*A gene which is responsible of forming the peptide bond between the preceding module and its substrate. These kind of NRPS synthetases are rare and only found in thaxtomin [30] and in viomycin [31].To predict the peptide sequence of nonribosomal peptide synthetases genes from genome sequence, bioinformatics analysis of the specify of the A-domain of each module was predicted firstly by LSI based A-domain function predictor websites, these predictions are not confirmed because the similarity percentage is low which due to the highly difference in the sequence of the 8 amino acids residue binding pocket of these A domains which may refer to that we have discovered a new peptide with new amino acids structure. The 8 amino acids residue binding pocket of all A domains and their prediction listed in Table 4 and the putative peptide sequence Figs. 3 and 4.

Table 4 The 8 amino acids residue binding pocket of all adenylation domains detected by LSI based A-domain function predictor

NRPS	A-domain	Binding pocket	Prediction
*nps*A	A1	SVTQAAVA	ABU
*nps*A	A2	DSHGYGCV	ALA
*nps*A	A3	DVLIVAAV	HYVD/ALA
*nps*A	A4	DVWHFSLI	SER
*nps*A	A5	DITQMGIV	GLY/ASP
*nps*B	A6	VVYNTAV	HPG/VAL

Abbreviations for unusual hydroxyl and amino acid substrates: ABU: 2-amino-butyric acid. HYVD, D-2-hydroxyvaleric acid; HPG: Hydroxphenylglycine.

Figure 3



Modular organization of nonribosomal peptide biosynthetic cluster in *Lactobacillus plantarum* WCFS1; Two NRPS core genes (in rouge); nonribosomal peptide synthetase (*nps*A) and AMP-binding protein (*nps*B); one additional synthetase gene (in pink); 4'-phosphopantetheinyl transferase and two other genes; aspartate-1-decarboxylase (*pan*D) and metal-dependent phosphohydrolase.

Figure 4

A domain prediction tools						
PKS-NRPS Website	X	X	X	Ser	X	X
LSI based A-domain function predictor	Abu	Ala	HYVD/ Ala	Ser	Gly/ Asp	HPG/ Val
NRPS/PKS substrate predictor	Val	Val	Val	Ser	Val	Val
NRPS Predictor2	X	X	Gly/Ala	Ser	Gly	X
Clustering using PhyML 3.0	Ala	Leu	Ala/Pro	Ser	Gly	Ala
AntiSmash	X	X	X	D-Ser	Gly	X

All Adenylation domains substrate specificity of NRPS synthetases genes (*nps*A and *nps*B) from *L. plantarum* WCFS1 by specificity predictors; PKS-NRPS analysis website [25], LSI based A-domain function predictor [27], NRPS/PKS substrate predictor (NRPS predictor2 [26], clustering using PhyML 3.0 [28] and AntiSmash 4.0 [24].

Adenylation domains specificity of the *L. plantarum* NRPS were analyzed using six different prediction analysis programs (Fig. 5), PKS-NRPS analysis website, (X- X- X- Ser- X- X), LSI based A-domain function predictor (Val- Val- Val- Ser- Val –Val), NRPS predictor2 (X- X- Gly/Ala- Ser- Gly-X), clustering using PhyML 3.0 (Ala- Leu- Ala/Pro-Ser- Gly- Ala).

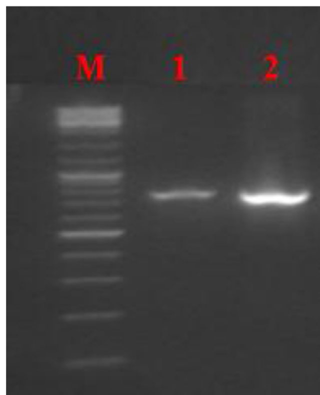
AntiSmash (X- X- X- Ser- D-Gly- X) only two amino acids serine (A4) and glycine (A5) of the *nps*A gene were confirmed by all except by PKS-NRPS analysis website and NRPS/PKS substrate predictor, while prediction of the chemical core scaffold structure of the putative nonribosomal peptide remains difficult with only four out six predicted A-domain specificities. Also, three predictors of them

AntiSmash 4.0 [23], PKS-NRPS analysis website [24] and NRPS predictor2 [25] could not assign any specificity to the majority of A domains, supporting the novelty of the NRPS system.

Detection of NRPs genes involved in L. plantarum LMG100 by degenerate primers

The primers designed before by [22] for NRPs genes detection in Lactobacillus strains were used in this study to detect the absence or presence of the NRPS genes in the isolated *L. plantarum* LMG100. [22] were reported before the efficiency of these primers AIb1-F/ TIb1-R in the detection of NRPS gene *nps*A in the reference strain *L. plantarum* WCSF1. We here detected a fragment of 750 bp of length in both the reference strain *L. plantarum* WCSF1 and in our isolated strain *L. plantarum* LMG100 which agree with [22] results concerning the reference strain, while he confirmed the absence of this NRPS gene in another four *Lactobacillus plantarum* tested strain in his study and which consider interesting results for our isolated strain (Fig. 5).

Figure 5



Amplicons of 750 bp generated by AIb1-F/ Tib1-R primers lane (1) *L. plantarum* WCSF1, Lane (2) *L. plantarum* LMG100, M=O'Gene Ruler standard.

The nonribosomal peptide synthetases genes presence in *Lactobacillus* was firstly detected in 2003 by the sequencing of the complete genome sequence of *L. plantarum* WCSF1 strain.The amplification of partial sequence of *nps*A gene by AIb1-F/ TIb1-R primers proved the nonibosomal peptide synthetases genes presence in this strain only as reported by [22,32] which confirmed the absence of nonribosomal peptide synthetases genes in all LAB except *L. plantarum* WCFS1. Also, [33] reported that strain *L .plantarum* JDM1 sequence nucleic similar 90%to *L. plantarum* WCFS1 and don't harbour any nonribosomal peptide synthetases genes.

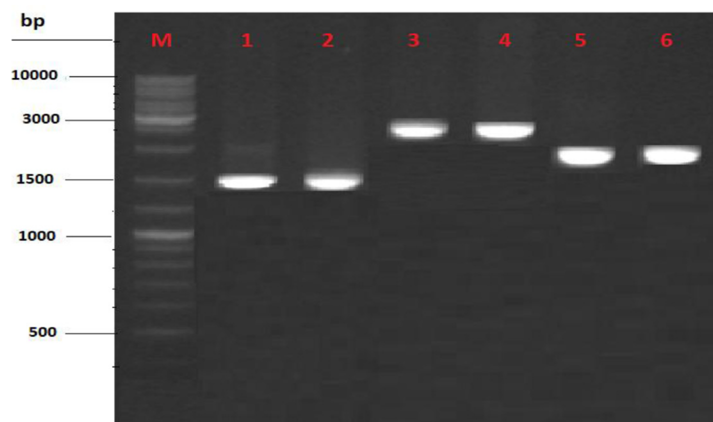
Detection of NRPs genes organization involved in *L. plantarum* LMG100

Different primers were designed to confirm the organization of the new NRPS operon in strain *L. plantarum* LMG100 compared to *Lactiplantibacillus plantarum* WSCF1. The three primer sets (*nps*A-*pan*D), (Phospho- *nps*B) and (*nps*B- *nps*C) amplified the expected fragments sizes of 1493, 2450 and 1650 bp, respectively in both reference and isolated strain which confirmed the presence of the same NRPS operon genes organization of the reference strain in the isolated strain *L. plantarum* LMG100 (Fig. 6).

The molecule production and HPLC-mass determination process

Microbial fermentation was lanced in Man Rogosa Sharpe broth (MRS)media for 48 hours (stationary phase), after the planktonic bacterial cells removed from growth media the new polypeptides supernatants samples were passing through clean C_{18}

Figure 6



Amplicons of *Lactiplantibacillus plantarum* WSCF1 and *Lactiplantibacillus plantarum* LMG100 generated by npsA-F/ panD-R primers lane (1,2); Phospho-*nps*B-F/ Phospho-*nps*B-R (lane 3,4); *nps*B-*nps*C-F/ *nps*B-*nps*C-R; M=O'Gene Ruler standard.

cartridges (protocol of Alltech, Fr). The concentration of detected polypeptide was calculated from the spectrum of (HPLC) reverse phase. The spectrum of second derivatives of UV-visible and the retention time of each peak was used to identify the new polypeptides molecules (Waters integrated PDA 996 diode array detector: Millenniums Software according to [21]. Firstly, the lactic acid bacteria (LAB) *Lactiplantibacillus plantarum* strain WSCF1 was recently used to produce one polypeptide with concentration of 109 mg.l^{-1}as illustrated before by [22,32]. This strain was given fragment size of 750 bp length for amplified nonribosomal polypeptides primers. The *Lactiplantibacillus plantarum* strain LMG100 was observed 203 mg.l^{-1} with the same fragment' size. Generally, the degenerated primers approach designed to achieve the peptides NRPs genes presence in lactobacilli isolates and this recently was confirmed by [34] who confirmed that the use of degenerated primers is certainly helpful in screening the NRPs in the *lactobacillus* strains harbor these genes. This trend is very supported to detect a new NRPs synthesis molecule and it facilitate the genetic potential knowledge of new polypeptides biosynthesis, these latest results proved that the *Lactiplantibacillus plantarum* strain LMG100 isolate have the ability to produce one type of polypeptide, it was also found that the isolate is more producing of the new polypeptide by the double production, it will make it known as good alternative of probiotic organism on animal and human inducers and perfect inducer for immunity system, however the primarily polypeptide fermentation experimentations under the study condition showed new type of polypeptides by the same *Lactiplantibacillus* strains under study [35].

The antibacterial activity of the new polypeptide NRPS molecule

The collected represented data in Table 5 referred to the confirmed inhibitory effects of extracted new polypeptide molecule verse the studied bacterial species groups with several formulas of polypeptide concentrations. From obtained data that the pathogenic bacterial inhibitions activities of the *Lactiplantibacillus plantarum* strain LMG100 formula which were more antibacterial efficiency by comparing with the extract formula from the strain WCSF1, the inhibition zones were dramatically increased by increasing the various formulas concentrations with observed the lowest levels of minimal inhibitory concentrations (MICs) and minimal inhibitory bacterial concentrations(MBCs) by 120 and 125 mg. ml^{-1}, respectively. The maximum inhibition zones ranged from 23.26 to 25.03 mm as maximum, these results are in accordance with those recorded by [36] that antibacterial activities are generally associated with microbial metabolites, mainly nonribosomal peptides. However, the new polypeptide especially those produced from the strain LMG100 showed maximum inhibition zones against the G^{-ve} short-rods bacterial strains from coliform group of the enteric genera of *Escherichia*, *Salmonella* and *Shigella* were (17.73, 23.26), (18.33, 24.87) and (19.83, 25.03) mm for WCSF1 and LMG100 extracts, respectively. However, G^{+ve} non-sporulated *Staphylococci* and *Streptococci* were (15.26, 20.09 and 15.83, 20.86) mm for WCSF1 and LMG100 extracts, respectively. While G^{+ve} bacterial strains from the genera of sporulated bacilli form by (14.46, 19.83) mm for WCSF1 and LMG100 extracts, respectively.

On other hand, the MIC ranged from 120 to 185 mg. ml^{-1} as maximum, and MBC ranged from 130 to 195 mg.ml^{-1} as maximum. In addition, the minimal inhibitory concentrations MIC for G^{-ve} bacterial strains from coliform group of the genus of *Salmonella*, *Shigella* and *Escherichia* were proximality of 125 mg.ml^{-1}, which formed the ordinary gastrointestinal tract infection, these results are also in accordance with [36]. Previous studies demonstrated

Table 5 The effect of *Lactiplantibacillus plantarum* WCSF1 and LMG100 extracts on the antibacterial activity

		Lactiplantibacillus plantarum WCSF1 Concentrations (mg.ml^{-1})				*Lactiplantibacillus plantarum* LMG100 Concentrations (mg.ml^{-1})			
				100	200			100	200
Group	Microbial strain	MICs	MBCs	Inhibition zone (mm±SD)		MICs	MBCs	Inhibition zone (mm ± SD)	
1	*E. coli*	160	175	$11.06±0.22_B$	$17.73±0.33_C$	125	145	$17.93±0.26_C$	$23.26±0.35_D$
	B. cereus	185	195	$8.76±0.34_A$	$14.46±0.35_B$	150	155	$14.89±0.32_B$	$19.83±0.28_C$
2	*S. typhi*	165	175	$11.56±0.41_B$	$18.33±0.28_C$	125	130	$19.06±0.41_C$	$24.87±0.26_D$
	Shigella sp.	155	160	$12.63±0.33_B$	$19.83±0.25_C$	120	125	$19.96±0.35_C$	$25.03±0.21_E$
3	*S. aureus*	170	190	$9.56±0.37_A$	$15.26±0.24_C$	140	155	$15.53±0.25_C$	$20.09±0.30_D$
	S. pyogenes	175	195	$9.77±0.29_A$	$15.83±0.31_C$	135	145	$16.02±0.31_C$	$20.86±0.26_D$

IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp. Values represent means±Standard division (SD) obtained from three treatments. The means in the same raw or column followed by different letters differ significantly, and when the means followed by the same letters do not differ significantly at ($P≥0.01$).

that, the complected fermentation process is characterized by various (LAB) development in the presence of other microorganisms, however in these conditions the (LAB) are subjected to high competition under high stress due to the hyper salts concentration allowed which makes these dominate strains should have potential biological properties to overcome these stress factors and consequently assure a successful lactic fermentation process of sour-treated products [37]. In the same inspect, a lot of studies have reported the pre-preservative properties of some strains of LAB isolated from fermenting products, mainly of the species *L. plantarum*, *L. pentosus* and *L. paraplantarum* [38].

Conclusion

Some of (LAB) group members are usually known to produce several antimicrobial substances. Nonribosomal peptides are considered one of amphipathic natural molecules that exhibit bactericidal and immunomodulatory properties. In our investigation, we aimed to focused on the antibacterial activities of a polypeptide produced by a LAB isolate strain LMG100 which classified as a member of the genus *Lactiplantibacillus plantarum*. Also, the recent approaches in detecting and predicting substrate specificity of adenylation domains involved in NRPS system considers of importance in facilitating and shorten the characterization of unknown NRPS. Predicting the structure of the secondary product remains complicated despite the detection of the substrate specificity of most of the A domains in any NRPS gene cluster. The major differences in predicting substrate specificity of adenylation domains involved in *L. plantarum* WCFS1 and LMG100, supporting the novelty of the NRPS systems in these strains. The recent polypeptide molecule that was purified using a combination of chromatographic filtration techniques and HPLC/MS analysis confirmed the presence of 6 amino acids composition assigned non-identified polypeptide class. However, the new polypeptide especially those produced from the strain *L. plantarum* LMG100 showed maximum inhibition zones against G^{-ve} short-rods bacterial strains from coliform group of the enteric genera of *Escherichia*, *Salmonella* and *Shigella* which formed the ordinary gastrointestinal tract infection, the obtained results revealed that the selected strain has potential application for pathogen intervention as probiotic in processing of food and pharmaceutical industries.

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Conflicts of interest

All authors declare that they have no conflict of interest.

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