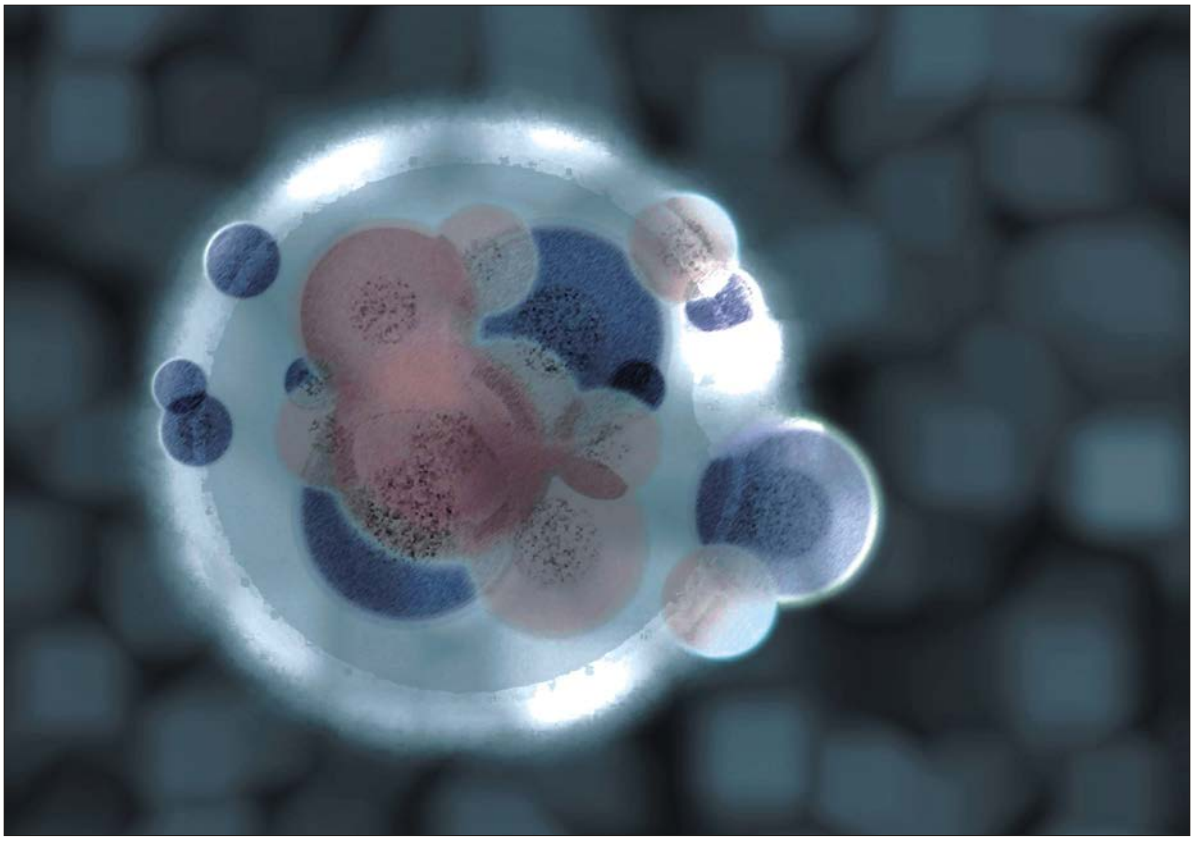

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Research Article
Isolation and Molecular Identification of Food Grade Lactic Acid Bacteria and Their Antifungal Activity

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Abstract

Background and Objective: The presence of pathogenic fungi in grains has been a global concern due to their economic effects. Recently, biological control methods have become an important strategy to control pathogenic fungi. Therefore, the aim of this study was to isolate and identify food grade lactic acid bacteria and screen their antifungal activity against pathogenic fungi isolated from peanuts. **Materials and Methods:** Lactic acid bacteria were isolated from dairy products. The lactic acid bacterial cell-free supernatants and bacteriocins were studied for the antifungal activity against fungal species isolated from peanut samples. The two lactic acid bacteria isolates and their bacteriocins that showed antifungal activity were identified using morphological, biochemical and molecular techniques. **Results:** A total of 20 lactic acid bacteria were isolated from traditional dairy products. Meanwhile, a total of 90 fungal isolates were isolated from peanut samples. *Aspergillus* (55%) was the dominant genus including five species namely, *A. parasiticus, A. flavus*, *A. ochraceus*, *A. terreus* and *A. niger*. *Fusarium*, *Penicillium* and *Trichoderma* genera were also isolated but in a lower frequency. Both lactic acid bacterial cell-free supernatants and their bacteriocins showed antifungal activity against fungal species isolated from peanut samples and were thus identified using the molecular 16S rDNA gene sequencing. Lactic acid bacteria isolates 11 and 12 showed 99% homology with *Enterococcus faecalis* and *Enterococcus faecium*, respectively. Their sequences were deposited in the GenBank databases under accession number MF000305.1 and MF000307.1. **Conclusion:** This study revealed the probability of using these *Enterococcus* isolates in food preservation. The partial or total sequencing of the 16S ribosomal DNA (rRNA) gene showed a fast technique for bacterial classification.

Key words: Peanuts, dairy product, fungi, bacteriocin, *Aspergillus*, antifungal activity

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Peanuts (*Arachis hypogaea* L., Family: *Fabaceae*) are a rich source of fat, proteins and vitamins and are grown on a great scale in tropical and subtropical countries. The environmental conditions such as high humidity, season drought stress and elevated soil temperature has led to fungal infection and aflatoxin contamination[1]. The infection of peanut occurs either during preharvest or postharvest and is considered a major problem that includes loss of germination, mustiness, moldy smell[2], that may lead to the decrease in yield and market value. The use of fungicides is a predictable method to prevent the fungal growth but it is associated with the hazardous impact on environmental contamination, public health, the high cost of agrochemicals and resistance progress among pathogens.

Biological control is another effective strategy for the control of fungal growth. The biological control of several lactic acid bacteria (LAB) against fungal growth was studied previously by Castellano *et al.*[3], to develop a suitable treatment to ensure food safety and consumer's health. Lactic acid bacteria have an important role in the food industry and benefit human and animal health. Moreover, their safety has been proved empirically and scientifically[4]. Previously, Hathout and Aly[5], reported that *Lactobacillus gasseri* followed by *Lactobacillus reuteri* were able to delay and/or decrease fungal contamination in Talbina (a cereal dairy product). *Enterococcus* genus is Generally Regarded As Safe (GRAS) lactic acid bacteria. Several investigators reported that *Enterococcus faecium* and *Enterococcus faecalis* have probiotic properties used to suppress carcinogenesis, reduce cholesterol level and prevent bacteria-associated diarrhea[6-8].

To correctly distinguish lactic acid bacteria, polymerase chain reaction (PCR)-based molecular methods have been used extensively in the past decades[9] and are considered a quick and delicate method for specific amplification of a definite segment of DNA. For the detection of bacteria, PCR techniques based on 16S rRNA genes have been used widely[10]. Vetrovsky *et al.*[11] reported that if two organisms present at a 16S rRNA gene and sequence identity are higher than 97%, then they can be considered closely related and thus belong to the same species. Identification based on the 16S rDNA sequence is of interest because ribosomal exists generally among bacteria and includes regions with species-specific predictability[12].

Fungi can cause serious phytopathological risks during pre-harvest and post-harvest stages, as well as in processed food products and can have a significant economic impact globally. Biological control using microbial antagonists has developed as a promising method for the control of fungi. Therefore, the aim of this study was to isolate and identify food grade lactic acid bacteria and screen their antifungal activity against pathogenic fungi isolated from peanuts.

MATERIALS AND METHODS

Chemicals: All chemicals were of analytical grade reagent and were used directly without further purification and purchased from Merck (NJ 07033, USA). Potato Dextrose Agar, M17 Agar and Kanamycin Aesculin Azide Agar were purchased from CONDA (Madrid, Spain). De Man Rogosa Sharpe Agar was obtained from Sigma-Aldrich, (St. Louis, MO 63103, USA).

Samples collection: Ten samples (250g) of unshelled peanuts and ten samples of traditional dairy products (cow milk, feta cheese and Ras cheese) were collected during April 2017, from different local markets in Cairo governorate.

Isolation of fungi: Fungi were isolated from peanut samples using the agar test methods according to Martins *et al.*[13]. The peanut samples were plated on Potato Dextrose Agar (PDA) and incubated for 7 days at 25±2°C. The individual isolates were transferred to new PDA plates in order to obtain pure cultures. All isolates were maintained on PDA and kept at 4°C for further analysis.

Identification of fungi: The isolated fungi were identified according to colony morphology and microscopic examination according to the keys of Klich[14], Nelson and Summerell[15], Barnett and Hunter[16] and Leslie and Summerell[17].

Isolation of lactic acid bacteria: The isolation of the LAB was done according to Lavanya *et al.*[18] as follows. Briefly, 1 mL (or 1 g) of the dairy products were homogenized with 9 mL of 0.85% w/v sterile sodium chloride. Then 0.1 mL sample of suitable dilution was plated into different types of media, i.e., De Man Rogosa Sharpe Agar (MRS), M17 Agar and Kanamycin Aesculin Azide Agar (KAA). The plates were incubated at 30°C for mesophilic LAB and at 40°C for the thermophilic LAB for 2-3 days. Colonies were selected according to their shape. The purity of isolates was checked by continuous streaking on the respective media. The isolates were purified and stored in a medium containing 10% skimmed milk and 20% glycerol at freezing temperature (-20°C).

Preparation of bacteriocins: The cultures were extracted from liquid cultures of LAB grown in MRS or M17 medium at the

optimum temperature for 24 h. The cells were removed by centrifugation at 4600 rpm for 15 min. The cell-free supernatant was adjusted to pH 7 and ammonium sulfate (concentration 60%) was added and stirred at 4°C for 18 h. Then, the mixture was centrifuged at 6400 rpm for 20 min and the bottom pellets were harvested and suspended in 10 mL sodium phosphate buffer (pH 6.5). One volume of the suspended product was added to 15 volumes of a methanol-chloroform mixture (1:2, v/v) and the mixture was stirred at 4°C for 1 h. The mixture was centrifuged at 4600 rpm for 20 min and the supernatant fraction was decanted and the pellets were air dried and suspended in 10 mL of distilled water and stored at -20°C[19].

Preparation of lactic acid bacterial cell-free supernatant: Lactic acid bacterial strains were stored and cultured twice in MRS broth before experimental use. They were cultured aerobically in MRS broth at 37°C for 18 h. Cells were removed by centrifugation at 10,000×g for 5 min and the cell-free supernatant was used.

Evaluation of the antifungal activity

Zone inhibition: Filter paper discs (5 mm diameter, Whatman No. 1) were saturated with 10 µL of each bacteriocin. The paper discs were placed on inoculated agar plates with the tested fungi and incubated at the appropriate temperature for five days. The diameter of the inhibition zones was measured, averaged and the mean values were recorded.

Mycelium dry weight: Fungal spore suspensions (10^6 spores/mL) were prepared in an aqueous solution of 0.1% Tween 80. About 1 mL of each of the bacterial cell-free supernatant for each of the strains was transferred into 250 mL conical flask containing 100 mL yeast extract (yeast extract 2%-Sucrose 20%) broth and inoculated with 1 mL fungal spore suspension. The cultures were incubated for 7 days at 28°C. The mycelium mats were collected by filtration through Whatman filter paper No. 4, washed twice with water and dried in an oven at 95°C until constant weight and weighed.

Morphological identification of LAB isolates: Identification of LAB isolates was performed by examination of cell morphology, gram staining, spore formation and motility. A Profile matching method based on Bergey's Manual of Systematic Bacteriology was used for characterization and identification of LAB isolates[20].

Physiological and biochemical identification of LAB isolates: Identification of LAB isolates were performed by examination for cell morphology, Gram staining, optimum pH, temperatures and salt tolerance[21]. Isolates were also characterized according to their biochemistry by the catalase and oxidase test[22]. Profile matching methods based on Bergey's manual of systematic bacteriology was used for characterization and identification of LAB isolates.

Molecular identification of LAB isolates

DNA isolation: The LAB isolates were cultivated on MRS agar plates at 37°C for 18 h.

Extraction of genomic DNA: Extraction of genomic DNA was carried out using Qiagen Kit catalog No. 51304 (Qiagen Sciences, Maryland, USA) according to the manufacturer's instruction manual.

Polymerase chain reaction amplification of 16S rDNA gene: The PCR reactions were done to amplify the 16S rDNA gene from LAB isolates. The 16S rDNA region was amplified by using the universal primer set; 8F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5-GGTTACCTTGTTACGACTT-3) according to Adimpong *et al.*[23]. PCR conditions were as follows: 5×GoTaq Flexi buffer, 10 µL, GoTaq Flexi DNA polymerase, 0.25 µL, $MgCl_2$, 25 mM, 2 µL, PCR nucleotide mix, 10 mM, 1 µL DNA, 1.5 µL double-distilled water mixed in a final volume of 50 µL. The program for PCR was as follows: 95°C for 5 min, 30 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 2 min and extension at 72°C for 7 min, +4°C%. The PCR amplification was performed using a PTC-100 thermocycler (MJ Research Inc., Watertown, Mass.).

Polymerase chain reaction fragment purification: The PCR products were eluted from agarose gels using Promega®'s Wizard® SV Gel and PCR Clean-Up System according to the manufacturer's instructions.

Data analysis: Ladder 1 Kb DNA Ladder (fermentas©) was used to identify the molecular weight of fragment. The dendrogram was designed using Unweighted Pair-Group Method with an Arithmetical average (UPGMA) with Cluster Method according to Faiza[24].

Electrophoresis: The PCR product was analyzed by electrophoresis in 1% agarose gel stained with ethidium bromide and photographed using gel documentation system (UV Trans-illuminator). The specific band was eluted from the gel Using Promega® PCR Purification Kit according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Isolation of fungi: Analysis of different species isolated from peanut samples for morphological and cultural characteristics showed that there was variation in the colony color, margins, texture and colony reverse colors. Ninety fungal isolates were isolated from peanut samples and they belonged to five genera. Results in Table 1 revealed the percentage of peanut contamination whereas *Aspergillus* species were the dominant genera and the percentage of infection reached 83.50% of the samples followed by *Fusarium* species (10.76%). Meanwhile, *Penicillium*, *Trichoderma* and *Gliocladium* species were detected in few samples with a percentage of infection recording 2.70, 1.89 and 1.50%, respectively. Among the *Aspergillus* species, the occurrence of *A. flavus* recorded 50% followed by *A. parasiticus* (16.00%), *A. niger* (16.00%), *A. ochraceus* (9.70%) and *A. terreus* (8.30%) in descending order (Table 2).

These results were confirmed by Magnoli *et al.*[25], who reported that *Aspergillus flavus* is generally the most destructive species in peanuts. These fungi as well as other fungal species increase the respiratory rate and accelerate grain deterioration[26]. Nakai *et al.*[27] and Fonseca[28] confirmed present study results and explained that peanuts are at a high risk of contamination with *A. flavus* and *A. parasiticus*, due to harvest during rainy periods and long drying periods and consequently aflatoxin production. Moreover, Sahab *et al.*[29] isolated *Aspergillus* species from Egyptian corn and recorded *A. flavus* and *A. parasiticus* as the dominant fungi. Current results were in good harmony with those reported by Marcos-Filho[30] who found that the incidence of the storage fungi is higher in peanut, whereas the most common fungi were *Penicillium* and *Aspergillus* genera. These differences are due to the variation of weather conditions, especially temperature and water humidity. The higher occurrence *A. flavus* and *A. parasiticus* in existing results are considered vital because these species are known to produce different types of aflatoxins especially aflatoxin B_1, which is the most potent carcinogen and causes mycotoxicosis to human and animals[31].

Isolation of lactic acid bacteria: Twenty local lactic acid bacteria (LAB) were isolated from milk and traditional dairy product samples. Two isolates showed antifungal activity and they were named as isolate (11) and isolate (12). The colony of the isolates was tested at different growth media. MRS was used for the isolation of *Lactobacillus* species[32], KAA for Streptococci (Enterococci)[33] and M17 for Streptococci[34]. These results were in agreement with Anacarsoi *et al.*[35], who also used these media to isolate 22 LAB belonging to *Lactococcus*, *Lactobacillus* and *Enterococcus* from plants, flowers and vegetable matrices. Similar observations were reported by Abo-Sereih *et al.*[12], who used these media to isolate ten LAB belonging to *Enterococcus* and *Pediococcus* from milk and traditional dairy products.

Evaluation of the antifungal activity: The bacteriocins produced by the lactic acid bacteria isolates were tested for their ability to inhibit fungal pathogens namely *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus ochraceus*, *Aspergillus niger*, *Fusarium* sp. and *Penicillium* sp. The bacteriocins of the two isolates showed significant antifungal activity in variable degrees. The mean values of zone inhibition diameter of *A. niger* recorded 22.3 mm by bacteriocin produced by isolates 11 (Table 3), whereas

Table 1: Percentage of infection and frequency occurrence (%) of fungi associated with peanut samples

Fungal genera	Number of isolates*	Infection (%)	Frequency occurrence (%)
Aspergillus	50	83.50	55.55
Fusarium	20	10.76	22.22
Penicillium	12	2.70	13.33
Trichoderma	5	1.89	5.55
Gliocladium	3	1.50	3.33

*Total number of isolates = 90

Table 2: Frequency occurrence (%) of *Aspergillus* species associated with peanut samples

Aspergillus species	Number of isolates*	Frequency occurrence (%)
A. flavus	25	50.00
A. parasiticus	8	16.00
A. niger	8	16.00
A. ochraceus	5	9.70
A. terreus	4	8.30

*Total Number of *Aspergillus* isolates = 50

Table 3: Antifungal activity of bacteriocin against various fungal isolates

Bacteriocin*	Zone inhibition (mm)					
	Aspergillus flavus	*Aspergillus parasiticus*	*Aspergillus ochraceus*	*Aspergillus niger*	*Fusarium* sp.	*Penicillium* sp.
11	15.6±1.20	16.2±1.54	10.6±1.56	22.3±1.44	11.5±1.40	11.7±1.50
12	17.4±1.87	19.5±1.19	21.2±1.60	18.9±1.37	14.7±1.80	15.6±1.30

Results are Mean±SD (n = 2). Bacteriocins were produced from isolates 11 and 12

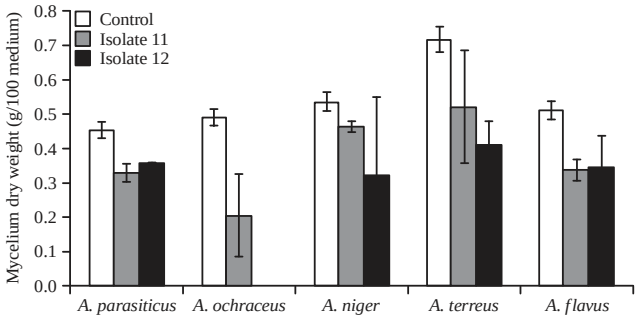

Fig. 1: Effect of lactic acid bacteria on mycelium dry weight. Results are Mean±SD (n = 2)

Table 4: Percentage of inhibition (%) of different *Aspergillus* species affected by the two lactic acid bacteria cell-free supernatant

Aspergillus species	Isolate 11	Isolate 12
A. parasiticus	27.42	20.92
A. ochraceus	57.95	100.00
A. niger	13.24	39.73
A. terreus	27.36	42.68
A. flavus	34.14	32.88

Table 5: Characterization of the two bacterial isolates

Test	Isolate 11	Isolate I2
Morphological		
Gram staining	+	+
Spore formation	-	-
Motility	-	-
Physiological		
Optimum (°C)	30-45	30-40
Optimum pH	6-9	6-9
Growth at NaCl (% w/v)		
0.0	+	+
1.0	+	+
2.0	+	+
4.0	+	+
6.0	+	+
8.0	+	-
10.0	+	-
Biochemical		
Oxidase	-	-
Catalase	-	-

A. ochraceus was highly inhibited by bacteriocin produced by isolate 12 and zone inhibition diameter recorded 21.2 mm. Bacteriocins produced by isolates 11 and 12 showed lower antifungal activities against *Fusarium* sp. and *Penicillium* sp.

Results in Fig. 1 revealed that both isolates extracts no. 11 and 12 greatly decreased mycelium dry weight, whereas extracts of isolate 12 reduced *A. niger*, *A. terreus* and completely inhibited *A. ochraceus* (100%). The percentage of inhibition ranged from 13.24-57.95% when using isolate 11 extracts (Table 4). On the other hand, the percentage of inhibition of isolate 12 extracts ranged from 20.92-100%. These results are in good harmony with those reported by Kang *et al.*[36], who reported that *E. faecalis* were found to have antimicrobial activity against some other pathogens. Meanwhile, Zheng *et al.*[37] reported that *Enterococcus faecium* was not active against *Aspergillus niger*. These variations in the results are due to the molecular and biochemical characterization of each isolate. Our results are in good harmony with those reported by Hadj-Sfaxi *et al.*[38], who stated that the antifungal activity of the two species may be due to the production of bacteriocins. Also, Svetoch *et al.*[39] recorded different bacteriocins as antifungal agents. Zheng *et al.*[37] added that *Enterococcus* have a bactericidal and bacteriostatic effect, which, lead to the delaying and/or disappearance of fungal growth. In a similar study by Kivanc *et al.*[40], the authors reported that the antifungal activity of *Enterococcus durans* could be due to the production of proteinaceous substance and organic acids, as they are known to produce the bacteriocin duracin and have antifungal activity. However, recently, the concerns on the safety of the genus in food and feed have been raised.

Morphological, physiological and biochemical characterization of lactic acid bacteria: The data in Table 5 summarized the morphological, physiological and biochemical characterization of the two LABs isolates that showed antifungal activity. The bacterial isolates were gram-positive, have the ability to grow in optimum temperature (30-40°C) and pH (6-9). The two isolates had the ability to grow at NaCl (0, 1, 2, 4 and 6% w/v). Both bacteria were oxidase and catalase negative and cocci shaped. Similar observations were reported by Holzapfel *et al.*[41] and Khalid[42] who found that LAB was Gram-positive, with negative catalase, microaerophilic, resistant to acid and could be fermented. These LABs are involved in the acidification of food and feed products. Similar observations were reported by Syah *et al.*[43], who identified 30 bacterial isolates as a LAB with Gram-positive, catalase-negative and rod-shaped characteristics. Lechardeur *et al.*[44] reported that LAB has a somewhat "simple" metabolism resulting in one or few fermentation end products. Accordingly, it was clear that LAB have a very diverse metabolic capacity, which enables them to adapt to a variety of conditions.

Molecular characterization of lactic acid bacteria: The fragment of the 16S rRNA gene was amplified using universal primers 8F and 1492R to amplify ~1.5 kb size fragment in all of the selected isolates 11 and 12. The amplified fragment (~1.5 kb) was purified and sequenced and compared with that of NCBI databases using BLAST

ACTGGCGGCGTGCTATACATGCAAGTCGTACGCTTCTTTTTCCACCGGAGCTTGCTCCACCGGAAAAAGAAGAGTGGCGAACGGGTGAGTAACACGTGGGTAAC
CTGCCCATCAGAAGGGGATAACACTTGGAAAGAGGTGCTAATACCGTATAACAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTGTCGCTGATG
GATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACA
CGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAA
ACTCTGTTGTTAGAGAAGAACAAGGATGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATAC
GTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGG
AAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTG
GTCTGTAACTGACGCTGAGGCTCGAAAGCTTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGTTAAGTGTTGGAGGGTTTCC
GCCCTTCAGTGCTGCAGCTAACGCATTAACCATTCCGCCTGGGGAGTACGACCGCAAGGTTGAAATTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAG
CATGTGGTTTAATTGGAAGCAACGGGAAGACCCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTCCCCTTCGGGGGCAAAGTGCCAGGTGGT
GCATGGTGGTCGTCAGTTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCCATCATTCAGTTGGGCATTTTAGCAAGACT
GCCGGTGCCAAACCGGAGGAAGGTGGGGAGGACGTCAAATCATCATGCCCCTTATGCCCTGGGTTACACACGTGTTCCAATGGGAAGTACAACGAGTTGGGAA
GTCGGGAGGTTAAGTTAATTTCTTAAAGTTTTTTTCAGTTCGGATTCCAGGCTGCAATTCGCCTGCAGGAAGCCGGAATCGTTAGTAATCGCGGATCACCACGCC
GCGGGGAATACGTTCCCGGCCCTTGTCCACACCGCCCGTCACACCACGAGAGTTTGTACCACCGGAAGTCGGTGAGGTAACCTTTTGGAGCCAGCCGCCTAAGG

Fig. 2: Nucleotide sequence of 16S r RNA gene of *Enterococcus faecalis* strain EFN1, partial sequence

CCCGAGTGCTTGCACTCAATTGAAAGAGGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTACCCATCAGAGGGGGATAACACTTGGAAACAGGTGCTAAT
ACCGCATAACAGTTTATGCCGCATGGCATAAGAGTGAAAGGCGCTTACGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCT
CACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTCGGGAGGCAGCAGTAGGGAATCTTCGG
CAATGGACGAAAGTCTGACCGAGCAACGCCGCGTAGTGAAGAAGGTTTTCGGATCGTAAAACGCTGTTGTTAGAGAAGAACAAGGACGTTAGTAACTGAACGT
GCCCTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCCTTGTCCGGATTTATTGGGCGTAAAGCGA
GCGCAGGCGGTTTCTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCAT
GTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGTGGCTCGAAAGCGGGGGAGCAAACAG
GATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGGG

Fig. 3: Nucleotide sequence of 16s r RNA gene of *Enterococcus faecium* strain EfN2, partial sequence

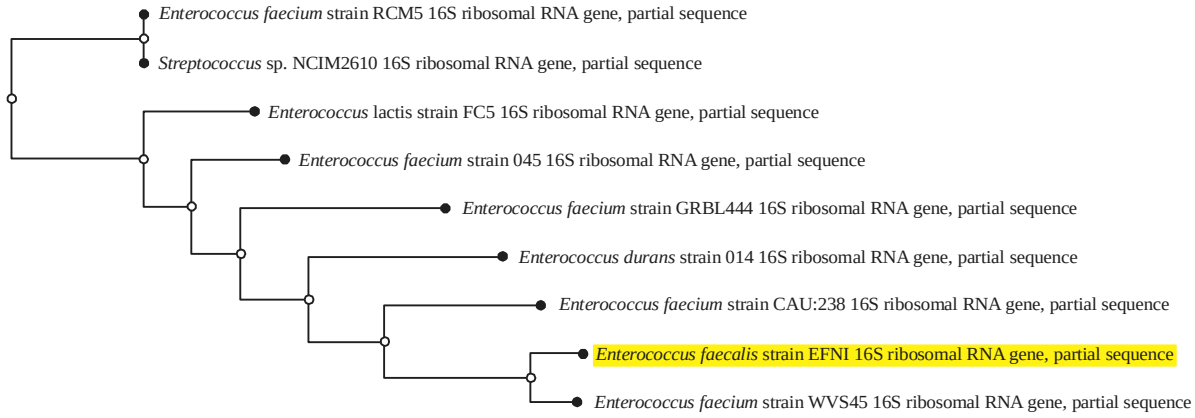

Fig. 4: Phylogenetic tree showing relationship of closely related species constructed using the neighbor-joining method and based on 16S rRNA gene sequences. Isolate 11 is closely related to *Enterococcus faecalis* strain EFN1 (highlighted)

network and the partial sequences of 16S rRNA and aligned with the available 16S rRNA sequences (Fig. 2 and 3). The phylogenetic tree was also constructed using the neighbor-joining (N-J) method based on the 16S rRNA sequences. The phylogenetic tree based on partial 16S rRNA was used for differentiation of *Enterococcus* species[45]. The 16S rRNA gene sequence analyses showed that isolate 11 was most closely affiliated with members of the genus *Enterococcus* and *Streptococcus* (Fig. 4). In the phylogenetic tree based on the neighbor-joining algorithm, isolate 12 was related to *Enterococcus faecium* strain EfN2 (Fig. 5). Molecular sizes of the DNA of bacterial species were estimated by the fluorescence intensity and comparison of the distance traveled with that of the molecular weight of marker standard as measured using gel electrophoresis (Fig. 6). A banding pattern which matched 8F and 1492R primers has been amplified with an approximate size of 1500 bp.

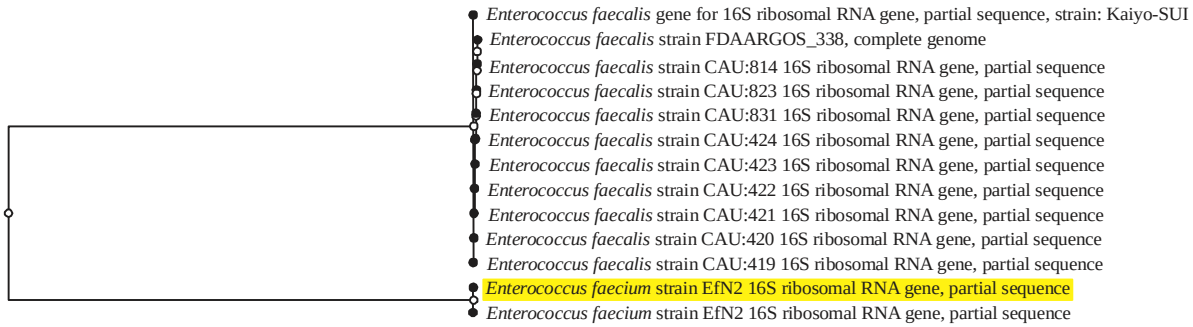

Fig. 5: Phylogenetic tree showing relationship of closely related species constructed using the neighbor-joining method and based on 16S rRNA gene sequences. Isolate 12 is closely related to *Enterococcus faecium* strain EfN2 (highlighted)

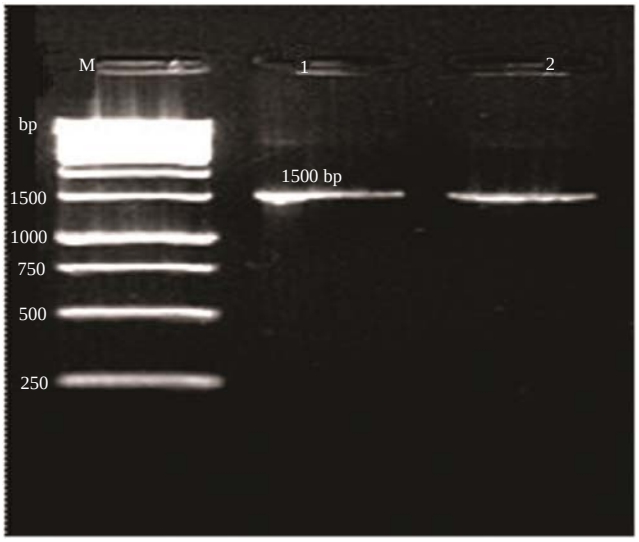

Fig. 6: Polymerase chain reaction-amplification of 16S rRNA gene using 8F and 1492 R primers; Lane M: Gene Ruler DNA Ladder 1 kb, Lane 1: 16S rRNA gene fragment of isolate 11, Lane 2: 16S rRNA gene fragment of isolate 12. DNA molecular weight marker Band sizes: 250, 500, 750, 1000 and 1500 bp

The 16S rRNA gene is currently the only widely used taxonomic marker that proved to be sufficiently informative and has been compiled in comprehensive and quality-controlled databases along with reliable taxonomic information[46,47]. The comparative analysis of 16S rDNA gene sequences enables the establishment of taxonomic thresholds that are useful not only for the classification of cultured microorganisms but also for the classification of the many environmental sequences[48]. Cibik *et al.*[49] showed that 16S rDNA gene sequencing is a powerful tool that has been used to trace phylogenetic relationships between bacteria and to identify bacteria from various sources, such as environmental and fermentation specimens. This technology is used today as a routine identification method, especially for slow-growing, unusual or fastidious bacteria and bacteria that are poorly differentiated by conventional methods. The phenotypic and genotypic characterizations of isolates were used as the first step in the selection and identification of potential bacteria[50]. It was also reported that genotype-based techniques e.g. 16S rRNA gene are more accurate, robust to identify bacteria at the species level as a complement or alternative to phenotypic methods[12].

Recently, new molecular tools have been applied for the routine identification of microbes and have led to an increase in the number of identified bacteria[51-53]. Most of the advance molecular methods are based on 16S ribosomal DNA sequences, complete or partial genomes[54].

For determining phylogenetic positions of species and genera, ribosomal RNA (rDNA) is more suitable, because the sequence contains both properly-conserved and less-conserved regions. It's far now possible to determine the sequence of a long stretch of rRNA (~1500 bases of 16S rDNA) from bacteria[55,56]. Comparisons of those sequences are currently the maximum powerful and correct technique for determining phylogenetic relationships of microorganisms[57]. With this technique, a clearer photo of the phylogeny of lactic acid bacteria is emerging.

The ability of LAB isolates to inhibit fungal growth, including both *A. flavus* and *A. parasiticus* which are known to produce different types of aflatoxins especially aflatoxin B_1, which is the most potent carcinogen is considered important. LAB also offers an excellent source for active metabolites. Future investigations could include the use of LAB isolates in food preservation.

CONCLUSION

In this study, LAB isolated from dairy products was identified and the gene sequence was deposited in GenBank

database. The LAB isolates and their bacteriocins showed a high rate of antifungal activity, which could be useful for the biocontrol of food. Further investigations to purify and identify extracted bacteriocins should be considered.

SIGNIFICANCE STATEMENT

The study revealed that the lactic acid bacteria isolated from dairy products showed antifungal activities against the fungal species isolated from peanut. The results confirmed the antifungal properties of the lactic acid bacterial isolates and the extracted bacteriocins. These lactic acid bacteria could be used as food preservative as they are Generally Regarded As Safe (GRAS). This study would help the researchers in determining the mechanism of the antifungal activities of lactic acid bacterial strains. Thus the best theory on the antifungal activity could be due to the production of proteinaceous substance and organic acids, as well as the production of bacteriocin.

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