Efficacy of Some Probiotics Bacteria Against Staphylococcus aureus food borne pathogen

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Abstract

The food industry has devoted intense research efforts to counteract the emergence of outbreaks caused by foodborne pathogenic bacteria. The aim of this study was to isolate, identify and evaluate the in vitro antagonistic activities of lactic acid bacteria isolated (probiotic) from the Raw milk and Yoghurt Samples in Egypt on foodborne pathogenic microorganism. (Staphylococcus aureus). Different biochemical tests were performed to identify isolated bacteria. Then, cell-free supernatant (CFS) was prepared from MRS culture and used in an antimicrobial assay that was performed by agar diffusion method. The effects of pH, and temperature, on antimicrobial activity were evaluated in the same test.. Simultaneously, the effects of lactic acid bacteria on antimicrobial materials production were also evaluated from Raw milk and Yoghurt. A total of 5 lactic acid bacteria were isolated; among them, one lactic acid bacteria(L5), belonging to the Lactobacillus plantarum. The cell-free supernatant of those five isolates exhibited the highest antibacterial activity, with the inhibition zone was 22 mm diameter l effect against tested foodborne pathogenic bacteria . Both these cultures were subjected to Biochemical and molecular identification using 16S rRNA gene sequencing highlighted the presence of species: Lactiplantibacillus plantarum, isolate (Size 1200pb acc. no. LC727688) showed a similarity > 98% to L. plantarum (acc.no.MT152629.1) and the food borne pathogenic S.aureus(Size 1200pb acc. no. LC727687) showed a similarity > 99% to S. aureus(acc. no. LC606406.1) . It maintained/retained antimicrobial activity in a wide range of pH 3.0–11, and temperature degree from 15-45°C on MRS medium . CFS exhibited antibacterial activity against S. aureus, as confirmed by the increase in absorbance value between 0.312 - 0.821 and 0.422-0.821 OD600-nm respectively .The highest activity were obtained at pH6 and temperature degree 30°C. Also, concentrations of bile salts and Sodium chloride. (gm /100ml) 1 and 0.6 respectively. Identification of the antimicrobial substances assay produced from L. plantarum and the SDS–PAGE analysis were able to produce bacteriocin with molecular weight 3.73 KDa . Evaluation the effect of Potasium sorbate used as food preservative, individually and incombination with the anitimicrobial compound produced from L.plantarum under the optimized conditions showed significant differences in most of food processing industries inhibiting growth of pathogenic bacteria in foods and enhance extension of shelf life of food products.

Keywords: *Antibacterial activity, Probiotic, Bipreservative, food industry..*

INTRODUCTION

Food is defined as any substance that is edible and portable too, originating either from plant or animal source. Food is ingested by each and every living being on the planet Earth and that is why it poses a great threat for disease transmission. Food sustains life and helps in providing energy, growth and maintenance of health of body (David et al., 2012). The Centers for Disease Control (CDC 2021) and Prevention reported that S. aureus is the most common foodborne pathogen (Deweymattia et al. 2018). Raw cow milk is consumed in different regions of the world and has been subjected to various studies (De Almeida et al. 2015 and Mulaw, et al. 2019). The primary food sources of LAB probiotics are raw milk and artisanal dairy products(Terzi et al. 2020). Some lactic acid bacteria are confirmed to be probiotics, while others are likely to be potential probiotics . Some of these include Lactobacillus acidophilus, L. rhamnosus, L. reuteri , L. Pontis, L. plantarum and L. pentosus (Oranusi et al.,2014). Many strains of LAB isolated from yoghurt, which are involved in the fermentation of milk, also provide many other antimicrobial compounds such as hydrogen peroxide, diacetyl, fatty acids, reuterin ethanol, and bacteriocins (Yazdi et al., 2017).Lactic acid bacteria (LAB) classified as “generally recognized as safe” (GRAS) microorganisms are one of the most important groups of bacteria in the food industry (Shehata et al. 2016).They can prevent the development of pathogenic bacteria (Ljungh et al. 2006 Boular et al. 2012) and are able to produce antimicrobial compounds such as lactic acid, acetic acid, diacetyl, acetoin, hydrogen peroxide, and bacteriocins (Aymerich et al 2000,Topisirovic, et al 2006 Prabhurajeshwar and Chandrakanth 2017). Acid production is thought to be the most important mechanism by which LAB inhibit pathogens; bacteria are gradually inactivated as the pH gets lower (Guo et al 2017). LAB can use various mechanisms to overcome the damage in response to stress by acid, including maintaining intracellular pH and cell membrane functionality and inducing stress-response proteins (Wu et al., 2014). Those mechanisms vary by species and exogenous factors, including the growth media and incubation conditions (Madureira et al., 2011 and .Wemmenhove et al 2017). The pH variation did not appear to have a significant effect on growth rate. The low temperature decreased the growth of all the LAB tested, confirming that low temperature is a limiting factor (Ammor & Mayo, 2007).The conditions applied (temperature, pH and salt variations, concentration of nitrite, nitrate and FOS) were limiting for L. casei strains growth (Bis-Souza et al 2020). Lactic acid bacteria play an important role in the preservation of food products by impeding the growth of spoilage/pathogenic microbes and also enhance the organoleptic attributes of foods (Hasan et al., 2014). Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria that inhibit the growth of similar or closely related bacterial strains (Nishie et al., 2012). Bacteriocins production depends on several environmental factors, such as temperature and pH, cell biomass, and the initial pH of culture media significantly influences growth (Yang et al., 2018).Bacteriocins are generally defined as ribosomally synthesized peptides produced by bacteria that have bacteriostatic or bactericidal activity against other related and unrelated microorganisms (Balciunas et al., 2013). Bacteriocins are a heterogeneous group and are usually classified into peptides that undergo significant post-translational modifications (class I) and unmodified peptides (class II) (Cotter et al.,

2013).Bacteriocins from lactic acid bacteria are considered safe additives, useful to control the frequent development of pathogens (S.aureus, E.coli, Salmonella …etc as broad range of antibacterial activity (Parada et al., 2007 and Pei Gee et al ., 2022). In order to satisfy the consumers' demands and restore their confidence in the safety of food products, the food industry was motivated to look for natural alternatives that exhibit strong antimicrobial and/or antioxidant properties (Ahmad et al., 2019). Some natural antioxidants/antimicrobials are able to extend the shelf life of food products. (Salleh et al., 2021).Therefore, the aim of this study was to screen ,characterize morphological ,biochemical and molecular identification of isolated strains. Evaluate the in vitro antagonistic activities of lactic acid bacteria isolated from, Raw milk and Yoghurt samples against pathogenic bacteria isolated from different foods (Toffees, Chocolate, Cake and Biscuits),then determine the effects of pH and temperatures on the antibacterial activity of the bacteriocin produced and using the selected strains in the safety of food products and shelf life of food.

MATERIAL AND METHODS

Food borne pathogenic bacterium used for experimental study.
from different product (Toffees, Chocolate, Cake and Biscuits).

Identification of S. aureus

The first step in the identification of suspected colonies is the Gram-staining, microscopic examination of the morphology,From among biochemical tests catalase test and also β-haemolysis surrounding colonies on the sheep-blood agar [Kérouanton et al. (2007), Normanno et al. (2007), Akineden et al. (2008) ,Rall et al. (2008) and Thaker et al. (2013)..However, the most reliable way to identify a suspicious colony as S. aureus is to investigate the presence of highly specific genes by the use of PCR technology .So from among the most employed genes, there is the possibility to detect the presence of 16S rRNA sequence(Tamura et al., 2007).

Probiotic sample Collection

Raw milk and Yoghurt samples used for the study was purchased from local different super markets in El obour city.

Isolation and maintenance of pure cultures

Ten grams of each sample was homogenized with 90 ml of 0.85% (w/v) saline and serially diluted. One hundred microliters of the desired sample suspension was spread on MRS agar media(0.1% glucose, and 50 µg/ml of cycloheximide).After plating the samples, the plates were incubated both aerobically and anaerobically at 37°C for 24 hrs. Colonies were selected randomly and purified by re streaking. Purified strains were stored as stock at − 4°C till further use [Harley, Prescott 2002and Sharma2009].

Purification of the antimicrobial compound from probiotic bacterial isolates

The cell-free culture supernatant for each probiotic isolate was saturated with 70% ammonium sulfate (Carl Roth, Germany) and kept at 4℃ for 4-5 hr to precipitate out the proteins. After precipitation, the pellet was controlled by centrifugation at 10,000 rpm for 30 min at 4℃. The pellet was re-suspended in25 ml of 0.05 M potassium phosphate buffer (PH 7.0) Further, it was applied on diethyl amino ethyl-cellulose column (1.5×40.0 cm) equilibrated with 0.1mol/L Tris-HCl buffer(pH 9) and eluted with linear salt gradient of Na Cl (0-1 mol/L). The active fractions were pooled together, concentrated

by ammonium sulphate, loaded on Sephadex G-75 column (1.594 0 cm) equilibrated with 0.1 mol /L Tris- HCl buffer (pH 9) and eluted with same buffer at a flow rate of 0.5 mL/min and then eluted fractions were assayed for antimicrobial activity against the pathogenic bacteria (S.aureus) (Nieto Lozano et al.,1992).

Preparation of Cell-Free Supernatants.

Strains to be tested for antimicrobial activity were incubated in MRS broth for 48 h at 37°C. Bacterial cells were removed by centrifugation of the culture at 5000 × g for 20 min at 4°C.The pH values of supernatants were adjusted to pH 6.5–7.0 by the addition of 1N NaOH. (e supernatants were membrane filtered (Millipore, 0.22 μm) and stored at 4°Cuntil use (Arena et al 2016).

Screening for antibacterial material from probiotic bacterial isolates using agar well diffusion assay

The antagonistic activity of isolated strains was determined by the agar well diffusion assay as described by Ivanova et al.,(2000)and Ogunbanwo et al., (2003). Twenty milliliters of nutrient agar was inoculated with 100 μL of each suspension bacteria and poured into a Petri dish. Then, wells of 5mm in diameter were perforated , and 50μL of an overnight culture of the LAB strain was loaded into the wells. Subsequently, each antagonistic activity was related to the area (square millimeter) of the clear zone observed surrounding wells after incubation at 37°C for 24 hr. Strains showing the highest antibacterial activity were then tested .

Identification and Biochemical Characterization of LAB.

According to Kumar Colony morphology, cell morphology, arrangement, and Gram staining were done for phenotypic characterization.Then, finally, the biochemical test was used to identify the isolated LAB species (Kumar. and Kumar,.2015).

Effect of different pH values and temperatures on the activity of antibacterial compound produced from L. 5 isolate

Selected probiotic bacterium (L. 5) were assayed against S.aureus by well diffusion method at different pH values (3, 4, 5, 6, 7, 8,9,10 and 11) and temperature degrees (5, 15, 25, 30, 37, 45 and 65°C) for 24-48 hr. Growth was measured using spectrophotometer as optical density at 600 nm as the method of (Ivanova et al., 2000 , Erdorul and Erblr 2006, and Hoque et al ., 2010.).

Effect of the different performed media on the activity of antibacterial compound produced from probiotic L5 isolate

0.5 ml of purified antimicrobial compound was inoculated into 4.5 ml of foure different performed media MRS, Nutrient Broth, Luria Broth, Trypticase Soy Broth) then incubated at 37°C for 30 min. The activity were assayed against S.aureus by well diffusion as the method of (Subhas et al., (2019).

Tolerance of the probiotic L5 isolate to different concentrations of bile salts and Sodium chloride

The Effect of different conc of bile salts (0.5, 1, 1.5, and 2 gm/100ml) and different conc of Sodium chloride (1, 2, 3,4,5,6 and 7gm/100ml) on probiotic bacterium L. 5 . Bacterial growth measuring absorbance at 600 nm as the method of Lundeen and Savage)1990(.and Hoque et al .,(2010).

Identification using 16srRNA sequence for the probiotic bacterium L.5 and S.aureus

DNA extraction and PCR amplification

The 16S r RNA genes of the two studied bacterial isolates (S. aureus and probiotic bacterium L. 5) were amplified using Bact 27F (5'-AGAGTTTGATCACTGGCTCAG-3') and Bact1492R (5'-TACGGCTTACCTTGTTACGACTT-3'). The amplifications were performed on ledom A 200 gradient thermocycler (Long Gene) in a final volume of 50 µl reaction mixture containing 0.5µM of each primer, 10mM of an equimolar dNTPs mix, 10X PCR buffer supplemented with 20mM MgCl2, 1.25 U of Taq DNA polymerase and, template 1µl of genomic DNA with a concentration between 0.1 to 10 ng. The Taq polymerase, dNTPs and PCR buffer were purchased from Thermo Scintific. The PCR steps was carried out as primary denaturation for 5min at 94°C; 30cycles of denaturation at 94°C for 30s; annealing at 58°C for 30 s, and extension at 72°C for 60s; and an additional reaction for 5min at 72°C. The PCR products were detected on 0.8% agarose gel with ethidium promide stain to confirm its purity, quantity, and size. The DNA sequencing reaction of PCR amplification was carried out using an ABI Big Dye Terminator V3.1 cycle sequencing kit and 3500 Genetic Analyzer Applied Biosystem , USA (Tamura et al., 2007). The resulted DNA amplicons ,almost 1200 pb, were eluted from agarose gel and purified using Q1Aquick Gel Extraction Kit (Cat.3:28704).The purified PCR fragment were ligated into pGEMR-T according to its manufacture. From LB/Amp/X gal plates, white colonies were selected and inoculated overnight at 33°C with shaking for stabilizing the plasmid inside the transformed cells. The alkaline method of Birnboim and doly (Birnboim and doly,1979) was used to isolate the plasmid .To confirm the recombinant plasmids the purified plasmids were examined by electrophoresis on 1.2% agarose gel using GeneRulerTM 1kb DNA ladder (Cat.3:SM0313). The obtained sequence for L5 AND T18 were examined for vector contamination using the VecScreen tool (http://www.ncbi,nlm.nih.gov/tools /vecscreen). While jalview software (http://www.jalview,org) was used to show single nucleotide polymorphism (SNPs) and consensus resulted from the alignment of our obtained sequences and the nearest strain in NCBI database(Waterhouse,2009). Construction of the phylogenetic tree was done using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and MEGA7 software (Kumar, et al., 2016). Determination of phylogenic relationships was analyzed by the program Phylogenic Analysis megAlign of DNAstar version 7.

Evaluation of potassium sorbate individually and In combination with antimicrobial material on the growth of S.aureus. under the optimized conditions.

1ml of Potasium sorbate at different concentrations w/v (0.1, o.2, 0.3, 0.4, 0.5, and 0.6) was added to 9 ml nutrient broth medium, then 1 ml aliquots of the selected bacterial culture was inoculated in broth media supplement with different potasium sorbate concentrations and then incubated at 37°C for 24 hr. Turbidity of growing bacterial culture was determined at 600 nm(Scott, 2011). Then 1ml of the tested extracted antimicrobial material was added to the previous mixture, then turbidity of growing bacterial cultures were determined at 600 nm(Rania., 2015).

Identification of the antimicrobial substances produced from L plantarum (L 5)

Under the optimized conditions the well-selected probiotic isolate was examined for the production of antimicrobial substances like, bacteriocins, organic acids and hydrogen

peroxide using agar well-diffusion technique with slight modification of Toure et al., (2003). The 25-ml grown culture on MRS broth was divided into equal fractions for different assays. For Bacteriocin assay, 5 ml of supernatant was treated with mg/ml pronase or 1 mg/ml trypsin. For organic acids assay, 5 ml of supernatant was adjusted to pH 6.5 ± 0.1 using 1 N NaOH, for hydrogen peroxide assay, 5 ml of supernatant was treated with 0.5 mg/ml of catalase (Hi-Media Pvt Ltd) and treated supernatants were filtered with 0.22 µm pore-size filters (Axiva Pvt Ltd) for bacteriocin assay. A volume of 50–100 µl of each supernatant was placed in 7-mm diameter wells and the plates were swabbed with 1% (v/v) overnight culture of tested pathogen S.aureus . Inhibitory characteristics were observed and the zone of inhibition was noted after 24 h of incubation at 37 °C.

Molecular Weight Determination of Protein by SDS PAGE

SDS PAGE separation of probiotic bacterial protein 10 mg of the precipitation pellets were dispersed in 1ml SDS 10% with 100-µl β-mercaptoethanol for 15 min with vortexing every 5 min. The extract was centrifuged at 10,000 rpm for 10 min. A mixture of 20 µl-extracted protein with 20µl of SDS-loading sample buffer (SDS 4%, β-mercaptoethanol 3%, glycerol 20%, Tris HCl 50 mM pH 6.8 and bromophenol blue traces) was heated at 96°C for 3 min. 10µl of the mixture was electrophoresed by SDS-PAGE according to Laemmli (1970).

Statistical Analysis

All experiments were carried out in triplicate. Statistical analysis was performed using SPSS V.20. For quantitative data, the mean and standard deviation (SD) were calculated. For qualitative data, number and percentages were calculated. Comparison of qualitative data between two groups was performed using one way analysis of variance (ANOVA) test was used. Differences were reported at a significance level of $p \leq 0.05$.

Results

Morphological and biochemical characters of the selected indicator S.aureus isolate

Data in Table1 showed that the isolate (S.aureus) Gram positive reaction, appear circular ,entire shape of colony with smooth texture under light microscope. Also positive for catalase, coagulase, urease, nitrate reduction and carbohydrate fermentation tests with β-hemolysis blood agar. While was negative for motility, oxidase and H2S production tests.

Table 1 Morphological and biochemical characters of the selected indicator S.aureus isolate

Test	Result
Gram reaction	+
Shape of colony	Circular and entire
Motality	–
Catalase	+
Coagulase	+
Oxidase	–
Urease	+
H_2S production	–
Nitrate reduction	+
Carbohydrate fermentation	+
Hemolysis blood agar	*β-hemolysis*

-Negative result +Possitive result

Antibacterial potential of labcfs on pathogenic organism.

Table 2 clearly indicated that Five isolates which grew on MRS agar isolated from different dairy products (Raw milk and yoghurt) collected from different super

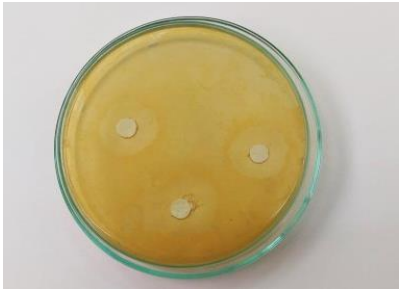
markets in El Obour city , Egypt. Then Supernatants were tested against S. aureus using the agar well-diffusion method The isolate L5 exhibited the superior antibacterial activity with 22 mm diameter inhibition zones, followed by the isolates L1 and L2 (12,13 mm respectively). The least activity was recorded for the isolates L3 and L4 (11 mm). against S. aureus.

Table 2: Probiotic isolates sources, their codes and antibacterial activity against pathogenic bacteria (S.aureus).

Probiotic isolate source	Isolate code	Inhibition zone (mm)
Raw milk	L_1	12± .11547
Raw milk	L_2	13± .20817
Raw milk	L_3	11± .20000
Yoghurt	L_4	11± .15275
Yoghurt	L_5	22± .11547

Results are mean of three determination (n=3), significantly different (P<0.05).

Figure 1: Antibacterial activity of probiotic bacterium L5 against pathogenic bacteria S.aureus



Effect of different pH values and temperatures on the activity of antibacterial compound produced from Probiotic bacterium (L5).

Table3: showed that the tested antibacterial compound produced was active in wide range of pH, but the maximum activity was observed at pH 6 and7 with inhibition zone (24.7 and 24.1 mm) respectively .While less activation occurred at pH 3 and 10 with inhibition zone(13.7 and 12.7 mm)respectively. Antibacterial compound could retain its antimicrobial activity partially when there was a shift to acidic or basic range. Data also ,showed that Antibacterial compound was active in wide range of temperature till 100 °C, but the maximum activity was observed at 30-40°C with inhibition zone(24.7 and 24.2mm) respectively. While less activation occurred at temperature 90 and 100°C with inhibition zone (14.2 and 11.7mm) respectively .Antibacterial compound loss its antimicrobial activity partially with increasing in temperature degree.

Table 3: Effect of different pH values and temperatures on the activity of antibacterial compound produced from Probiotic isolate(L5).

pH values	Inhibition zone (mm)	Temperatures	Inhibition zone (mm)
3	13.7± .05774	30	24.7± .05774
4	14.4± .11547	40	24.2± .05774
5	19.4± .05774	50	20.7± .05774
6	24.7± .05774	60	19.2± .05774
7	24.1± .05774	70	17.2± .05774
8	16.2± .05774	80	15.4± .05774
9	14.7± .05774	90	14.2± .05774
10	12.7± .05774	100	11.7± .05774

Results are mean of three determination (n=3),significantly different (P<0.05).

Effect of the different performed media on the activity of antibacterial compound produced from Probiotic bacterium (L5).

Data shown in Table 4 revealed that the antibacterial compound of L. plantarum when grown on the most widely used selective broth medium (MRS),showed maximum antibacterial activity against S. aureus with the diameter of inhibition zone was 15mm, followed by Nutrient broth medium with inhibition zone was lesser (11mm). One interesting finding was observed in Luria Broth which was also known as Lysogeny broth that antibacterial production was very less(6mm). In trypticase soy broth, no antibacterial production.

Table 4: Effect of the different performed media on the activity of antibacterial compound produced from Probiotic isolate(L5).

MEDIA	INHIBITION ZONE (MM)
MRS(CONTROL)	15± .05774
NUTRIENT BROTH	11± .05774
LURIA BROTH	6± .05774
TRYPTICASE SOY BROTH	-

(-) No inhibition zone

Results are mean of three determination (n=3),significantly different (P<0.05).

Effect of different pH values and incubation temperatures on the growth of the. Probiotic bacterium (L5).

The obtained results in Table 5 showed that the tested isolate L5 able to grow at pH ranging from 3 to 11 , but the best growth showed at pH 5-7 while the lowest growth showed at PH (3,4,10 and 11).Also it showed moderate growth at pH 8-9.Temperatures ranging from 15°C to 45°C and the best growth showed at 37˚C while the lowest growth showed at 45 ˚C . Also it showed moderate growth at 25 ˚C. On the other hand the tested strain L5 showed no growth at temperature degree (5, and 65 ˚ C).

Table 5: Effect of different pH values and incubation temperatures on the growth of Probiotic isolate(L5).

pH values	O.D	Temperature °C	O.D
3	0.312	5	--
4	0.369	15	0.422
5	0.622	25	0.631
6	0.711	30	0.711
7	0.821	37	0.821
8	0.562	45	0.332
9	0.421	65	--
10	0.411		
11	0.366		

O.D Optical density -- No growth

Tolerance of the Probiotic bacterium (L5) to different concentrations of bile salts and Sodium chloride

Table 5 showed that the tested Probiotic isolate(L5) was capable of growing and surviving till (1gm/100ml) bile salt concentration. The highest growth was observed at 0.2 % bile salt concentration with O.D >0.7. While the lowest growth was observed at 1% bile salt concentration with O.D >0.3. Also, tested isolate L5 was capable of growing and surviving at 1-6% Sodium chloride concentration. The highest growth was observed at 1 % Sodium chloride concentration with O.D >0.6. While the lowest growth was observed at 6% Sodium chloride concentration with O.D >0.3. However, isolated bacteria showed no significant growth against 7% NaCl concentration after 24 hours period.

Table 6: Tolerance of the Probiotic isolate (L5) to different concentrations of bile salts and Sodium chloride

Bile salt Conc. (gm/100ml)	O.D	Sodium chloride Conc. (gm/100ml)	O.D
0.2	0.733	1	0.678
0.4	0.611	2	0.623
0.6	0.521	3	0.514
0.8	0.421	4	0.412
1	0.366	5	0.355
		6	0.321
		7	0.00

O.D: Optical density at 600 nm.

Identification using 16srRNA sequence for the probiotic bacterium L.5 and food borne pathogenic bacteria S.aureus

DNA extraction and PCR amplification

Biochemical and molecular identification using 16S rRNA gene sequencing highlighted the presence of species: lactobacillus plantarum, isolate (Size 1200pb acc. no. LC727688) showed a similarity > 98% to l. plantarum (acc.no. MT152629.1) and the food borne pathogenic S.aureus(Size 1200pb acc. no. LC 727687) showed a similarity > 99% to S. aureus (acc. no. LC606406.1) as shown in Figures 2,3&4. Determination of phylogenic relationships was analyzed by the program Phylogenic Analysis megAlign of DNAstar version 7.

Figure 2: Agarose gel electrophoresis of the amplified PCR products of 16SrRNA gene of two strains (Staphylococcus aureus and lactobacillus plantarum).

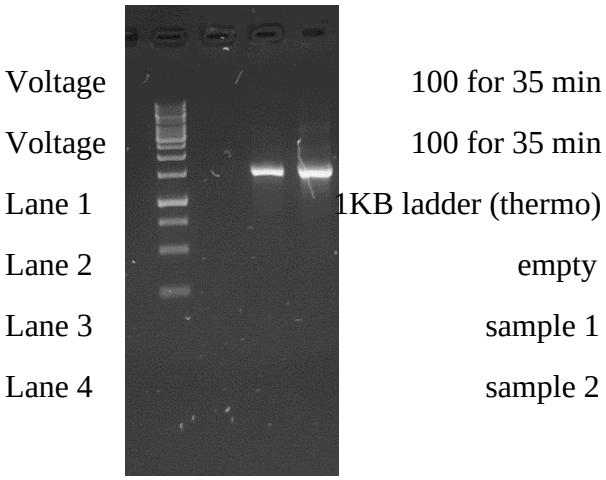


Figure 3: Phylogenetic tree based on neighbour-joining method of 16S rRNA gene sequencing of lactobacillus plantarum.

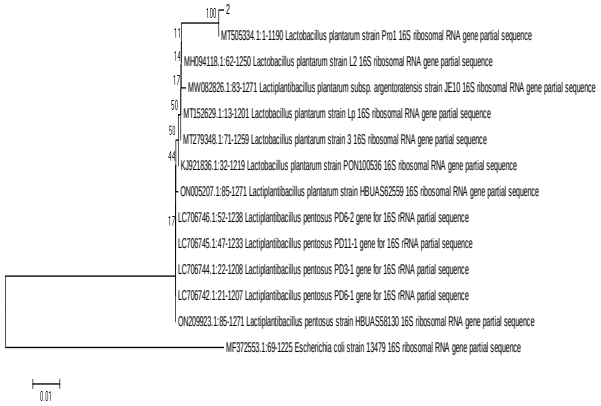
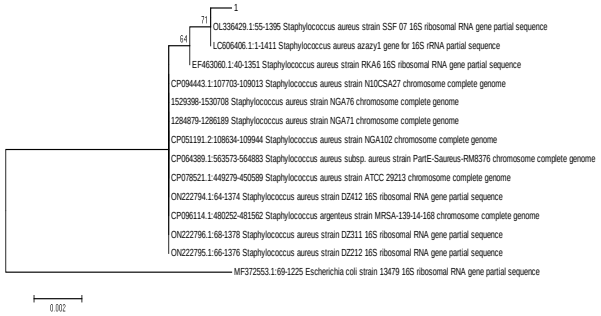


Figure 4: Phylogenetic tree based on neighbour-joining method of 16S rRNA gene sequencing of S.aureus



Evaluation of potassium sorbate individually and In combination with antimicrobial substance on the growth of S.aureus. under optimized conditions.

The results in Table 7 showed that the increase of potassium sorbate concentrations caused a recognizable decrease in the population of the tested strain (S. aureus) till 0.4 conc., where there is no growth of bacteria at potassium sorbate concentrations 0.5. On the other hand the results also showed that S. aureus populations were reduced when mixed potassium sorbate with antimicrobial substance of L. plantarum L5 till 0.2 conc., where there is no growth of bacteria at potassium sorbate concentrations 0.3 unlike when added potassium sorbate individually where bacteria continue to grow till 0.4 conc.

Table 7: Effect of potassium sorbate individually and synergistically with antimicrobial substance produced from L. plantarum (L5) on the growth of S.aureus.

Conc.(gm/100ml)	O.D.	O.D.
	Individually	Incombination(1ml antimicrobial substance)
Control	0.821	0.821
0.1	0.633	0.023
0.2	0.512	0.011
0.3	0.036	-
0.4	0.026	-
0.5	-	-
0.6	-	-

O.D: Optical density at 600 nm. Individually: preservative only

Incombination: preservative with antimicrobial substance

(-) No Growth

Identification of the antimicrobial substances produced from L. plantarum (L5).

Effective Lactobacillus plantarum isolate was considered for the characterization of inhibitory substances like Bacteriocin, organic acid and hydrogen peroxide. The antimicrobial substance produced by the selected L. plantarum L5 isolate was characterized by agar well-diffusion assay against selected indicator strain S.aureus. Culture supernatant treated with pronase (1 mg/ml) or trypsin (1 mg/ml) not gave inhibitory activity against the indicator strain S.aureus. This indicates that the inhibitory effect of Lactobacillus isolate was due to bacteriocin production. Culture supernatant treated with catalase did not affect the inhibitory activity of the Lactobacillus isolate against indicator S.aureus . This showed that inhibition by the Lactobacillus isolate was not due to hydrogen peroxide production. Also, neutralized supernatant (pH 6.5) of the selected Lactobacillus isolate did not affect the inhibitory activity of the Lactobacillus isolate against indicator strain isolate S.aureus.it showed that inhibition by theLactobacillus isolate was not due to organic acid production .Hence, this study concludes that inhibitory substance of the selected Lactobacillus isolate(L5) was bacteriocin.

Table 8: Identification of the antimicrobial substances produced from L. plantarum

Antimicrobical assay	Substances	Inhibition zone (mm)
Antimicrobial substance without any treatment (control)		22
Bacteriocin assay(treated with enzymes)	\	-
Organic acid assay(adjust pH to 6.5)		22
Hydrogen peroxide assay(treated with catalase)		22

(-) No inhibition zone

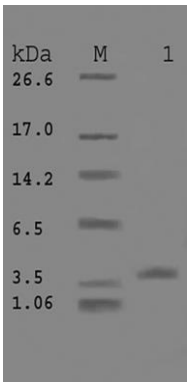
Results are mean of three determination (n=3), Non significantly different (0.871).

Molecular Weight analysis of purified bacteriocin produced from L.plantarum .

The SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) of bacteriocin produced from L. plantarum are shown in Figure5.The molecular weight was investigated showed a single band with an estimated molecular weight of 3.73 KDa in comparison with protein marker.

Figure 5: Tricine SDS–PAGE analysis of purified bacteriocin produced from L.plantarum.

Lane 1: bacteriocin sample .



DISCUSSION

Foodborne pathogens are serious challenges to food safety and public health worldwide. Many studies have reported that lactic acid bacteria (LAB) can have significant antimicrobial effects. Five lactic acid bacteria isolated from Raw milk and Yoghurt Samples in Egypt were identified based on its micromorphological, biochemical, and fermentation abilities test result. Biochemical and molecular identification using 16S rRNA gene sequencing highlighted the presence of species: Lactiplantibacillus plantarum, isolate (Size 1200pb acc. no. LC727688) showed a similarity > 98% to l. plantarum (acc.no. MT152629.1) and the food borne pathogenic S.aureus(Size 1200pb acc. no. LC 727687) showed a similarity > 99% to S. aureus (acc. no. LC606406.1) .This is similar to Alzahrani, (2021) who found by molecular identification for her tested probiotic isolate that it belong to lactobacillus plantarum with similarity percentage 99%. .Then screened for its antibacterial activities against the pathogenic and foodborne pathogenic organisms. The isolation of the respective Lactobacilli especially L. plantarum) from Raw milk and samples, was not surprising, as the presence of the Lactobacilli has been reported in Raw milk and Yoghurt fermentation. Amongst the several Lactobacillus spp. isolated from Raw milk and Yoghurt, L. plantarum exhibited the highest antimicrobial activity (22mm) against S. aureus during the screening test . This observation is similar to those reported by Ali et al.,(2012). Bukola and Onilude (2008) who indicated that 64.29% of L. plantarum isolates were active producers of extracellular antimicrobial polysaccharides As confirmed by inhibitory zones in the agar well-diffusion assay Pediococcus,Enterococcus, Leuconostoc, and Weissella were used to investigate the antagonistic activity. As

reported previously, several LAB have shown potential antibacterial effects against a number of pathogenic and foodborne pathogenic organisms (Ogunbanwo,et al 2003 and Darsanaki,et al 2012).The antibacterial activity of the four LAB strains confined from fermented beverage (Borde) and finfish has beenexamined primarily in vitro, and studies have especially centered on the inhibitory activity against the growth of Gram-negative and Gram-positive pathogens. An antibacterial substance of CFS produced by Enterococcus, Pediococcus, Leuconostoc, and Weissella strains exhibited inhibitory activity against all four bacterial strains S. aureus, E. coli, K. pneumonia, and P. aeruginosa. A recent review has similarly demonstrated that isolated species of Lactobacillus exert antagonistic impact in vitro against pathogenic and foodborne pathogenic organisms by creating antibacterial metabolites, most of which remain to be recognized (Ryan,et al 2011 & Sanlibaba and uçer 2015). there is expanding prove that the antibacterial action of LAB includes various mechanisms of action, including the generation of lactic acid and antimicrobial substances like bacteriocins and non bacteriocin molecules (Stoyanova et al 2012 and Ren, et al 2018). The antagonistic activity of CFS illustrated by the four isolates was too pH-dependent. (e foremost antagonistic activity was displayed in the acidic pH range of 2 to 5,whereas loss of antagonistic was observed in alkaline pH condition (pH > 10). (e same result was reported by Pehrson et al., (2015). in which lactic acid bacteria, specifically L. acidophilus ATCC 4356 obtained from the research center, appeared to have the most elevated antagonistic activity and stability at pH 2 and 4 (Pehrson, et al.,(2015). The antimicrobial material of L.plantarum when grown on MRS media which showed maximum antimicrobial activity against S.aureus . Nutrient broth, also observed antimicrobial activity against S.aureus but less than that in MRS broth. Low antimicrobial activity against S.aureus was observed in Luria Broth which is also known as Lysogeny broth. In trypticase soy broth, no antimicrobial production. Trypticase soy broth is known to support the growth of aerobic bacteria only and it supports LAB growth only when the media is supplemented with surfactants. These results with agreement of Subhas et al.,(2019) who found that maximum bacteriocin activity noted on MRS media then on nutrient broth but less than on MRS media while no bacteriocin activity showed on trypticase soy broth. Optimization of cultures conditions that affected on the growth of L. plantarum L5 such as incubation temperature , pH , Sodium chloride and bile salt tolerance were studied. Results showed that the tested isolate L. plantarum L5 able to grow at temperature degree ranging from 15°C to 45°C and the best growth was showed at 37˚C while the lowest growth showed at 45 ˚C . Also it showed moderate growth at 25 ˚C. On the other hand the tested strain L. plantarum L5 showed no growth at temperature degree (5 and 65˚C). This agrees with The study conducted by Rawal et al. (2013) who reported that the optimal temperature for growth and bacteriocin production was 37°C. Similarly, Karthikeyan and Santosh (2009) reported that the maximum activity of L. plantarum bacteriocin was 12,800 AU/ml at 37°C with best growth of L. plantarum .Tested isolate L. plantarum (L5) was exposed to different pH values (3, 4, 5, 6, 7, 8,9,10 and 11). Then the growth was measured by optical density at 600 nm. Results obtained showed that the tested isolate L. plantarum L5 able to grow at pH ranging from 3 to 11 , but the best growth showed at pH 5-7 while the lowest growth showed at pH (3,4,10 and 11) . Also it

showed moderate growth at pH 8-9. This results are in a good agreement with those obtained by Sankar et al. (2012), who found that L. plantarum was found to be active at a wide range of pH from 5-8 with higher inhibition zones of produced bacteriocin

Bacteriocins are ribo- somally assembled peptides that show antimicrobial properties to closely or distantly related bacteria (Ng et al 2020). They are either bacteriocidal or bacteriostatic to obliterate or repress the development of different micro-organisms as a way of competition and survival in the microbial community (Dabour et al 2009). The diluted crude bacteriocin extract recovered from this isolate displayed a very high antimicrobial activity at pH 2 compared to the antimicrobial activity displayed at pH 4, 6 and 8. No antimicrobial activity was displayed by the serially diluted extract at pH 10. This observation could be due to the fact that the producer organism (L. plantarum) has a high tolerance to low pH(Daeschel and Nes 1995).This observation is similar to that reported by [Ogunbanwo et al.2003] which indicated that purified bacteriocin extract recovered from L. plantarum was more active at pH 6 and 7, than at pH 10. The increased sensitivity of S. aureus to the pH amended crude bacteriocin extract could be as a result of the cell wall and membrane physiology of the bacterium.This observation is in agreement with a report by Malini et al., 2012 Ohenhen et al., (2015) who observed that the antibacterial activity of purified bacteriocin extracted from L. paracasei subsp. tolerans was more active against S. aureus. The diluted crude bacteriocin extract at pH 10 exhibited no antimicrobial activity against the indicator organisms. This could indicate that high pH value had a negative effect on the antibacterial activity of the extract. The L. plantarum isolate was resistance to different concentrations of both Sodium chloride and bile salt, hence are capable of being probiotic.

Also, Ogunbanwo et al.2003 recorded similar results for purified bacteriocins recovered from L. plantarum which retained their stability when stored for 60 days at -20ºC. the antimicrobial compound of L. plantarum was viable till temperature degree 100˚C with inhibition zone 11.7mm, which shows that the antimicrobial compound is thermostable in nature as it can withstand high temperature. Thermos ability of antimicrobial compound at high temperature makes it feasible to used in foods .This result is similar to Ravi et al., (2012) who found that antibacterial compound (bacteriocin)was more active at pH 6 and stand temperature over 100˚C.. In the present study, potassium sorbate was added as preservative material at different concentration (0.1 ,0.2, 0.3, 0.4 ,0.5 ,0.6) that decrease the growth of bacteria with increasing in concentration of added preservative material till no growth of bacteria occur at concentration of 0.5. While by combination of bacteriocin and preservative material decreasing the growth of bacteria till no growth occur at concentration of 0.3 .This is similar to Rania Mohamed., 2015) who found that combination of any preservative material with bacteriocin decreasing the growth of bacteria more than using preservative material individually .Culture supernatant treated with pronase (1 mg/ml) or trypsin (1 mg/ml) not gave inhibitory activity against the indicator strain (S.aureus). This indicates that the inhibitory effect of L. plantarum was due to bacteriocin production. Culture supernatant treated with catalase did not affect the inhibitory activity of the L. plantarum against indicator strain (S.aureus). This showed that inhibition by the L. plantarum was not due to hydrogen peroxide production. Also, neutralized supernatant (pH 6.5) of the

selected L. plantarum gave inhibitory effect on the indicator strain (S.aureus). This showed that inhibition by the L. plantarum was not due to organic acid production. Hence, this study concludes that inhibitory substance of the selected L. plantarum isolate was bacteriocin this is similar to (Prabhurajeshwar, and Chandrakanth ., 2018) where they found that culture supernatants of the isolated Lactobacillus isolates (Y10 and Y13) and the reference strain treated with pronase (1mg/ml) ortrypsin (1 mg/ml) affect their inhibitory activities against the indicator strains. This indicates that the inhibitory effect of Lactobacillus isolates were due to bacteriocin production. The molecular weight of bacteriocin produced from L. plantarum was investigated by SDS-PAGE which showed a single band with an estimated molecular weight of 3.73KDa in comparison with protein marker. Similar molecular weight has been reported by Todorov and Dicks.,(2010) for bacteriocin ST414BZ (3.7 kDa) from L. plantarum ST414BZ which is supported by the present findings. recommended to food processing industries to enhance extension of shelf life of food products, and inhibiting growth of pathogenic bacteria in foods. According to Scopes (1993), the salt is relatively inexpensive and more readily available compared to PEG hence it is capable of large-scale commercial applications in protein purifications. Besides, ammonium sulphate is very soluble in water and the protein precipitate formed is stable against proteolysis and bacterial degradation.

CONCLUSION

Raw milk and Yoghurt are a viable source of several Lactobacilli which are capable of producing several antimicrobial compound such as bacteriocin. Itis recommended that the crude bacteriocin extract recovered from L. plantarum should be further purified and its potential antimicrobial activities against a variety of bacterial isolates and spoilage microorganisms investigated. This could improve the hygiene and safety of the food products so produced. Also, recommended to food processing industries to enhance extension of shelf life of food products

Reference

Ahmad, F.T., Lani, M.N., Nazari, S.A., Hajar, N.H.M., Hasan, K.N.A.M., Razak, S.B.A. and Hassan, Z. (2019). Antioxidant and antimicrobial properties of honey propolis and bee bread of stingless bee (Geniotrigona thoracica). Asian Journal of Agriculture and Biology, 7(Special Issue), 76-85.

Akineden O, Hassan A, Scheider E, Usleber E (2008) Enterotoxigenic properties of Staphylococcus aureus isolated goats' milk cheese. Int. j. food microbiol. 124: 211- 216.

Ali,F. S., Saad O. A. and Salwa A. H. (2012). Production of probiotic yoghurt. Minia International Conference for Agriculture and Irrigation in the Nile Basin Countries, El-Minia, Egypt, 26th to 29th March

Alzahrani N.h,.Antibacterial Activity of Bacteriocin Produced by Lactobacillus plantarum Isolated from Horse Milk.Int j pharm phytopharm res. 2021,11(1):34-40.

Ammor, M. S., & Mayo, B. (2007). Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: An update. Meat Science, 76,138–146.

Arena, M. P. A. Silvain, G. Normanno et al., “Use of Lactobacillus plantarum strains as a bio-control strategy against food-borne pathogenic microorganisms,” Frontiers in Microbiology, vol. 7, p. 464, 2016.

Aymerich M. T., Garriga M., Monfort J. M., Nes I., and Hugas,M. "Bacteriocin-producing lactobacilli in Spanishstyle fermented sausages: characterization of bacteriocins," Food Microbiology, 2000, 17, 33-45

Balciunas EM, Martinez FAC, Todorov SD, de Melo Franco BDG, Converti A, de Souza Oliveira RP. Novel biotechnological applications of bacteriocins: A review. Food Control. 2013;32:134-142.

Birnboim, H.C. and Doly, J. (1979) A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA. Nucleic Acids Research, 7(6), 1513-1523.

Bis-Souza C. V., Penna A. L. B., Silva Barretto A. C(2020). Applicability of potentially probiotic Lactobacillus casei in low-fat Italian type salami with added fructooligosaccharides: in vitro screening and technological evaluation Meat Science168: 108-186.

Boular´es M., Aouadhi C., Mankai M., Ben Moussa O., Essid, I. and Mnasser,H. "Characterisation, identification and technological properties of psychotrophic lactic acid bacteria originating from Tunisian fresh fish," Journal of Food Safety, vol. 32, pp. 333–344, 2012.

Bukola CA, Onilude AA. Screening of lactic acid bacteria strains isolated from some Nigerian fermented foods for EPS production. World Appl. Sci. J. 2008;4(5): 741-747.

CDC, Foodborne Germs and Illnesses, Center for Disease Control and Prevention, Atlanta, GA, USA, 2021, https:// www.cdc.gov/foodsafety/foodborne-germs.html.

Cotter PD, Ross RP, Hill C (2013). Bacteriocins-aviable alternative to antibiotics? Nat Rev Microbiol 11: 95-105.

Dabour N, Zihler A, Kheadr E, Lacroix C, Fliss I (2009) In vivo study on the effectiveness of pediocin PA-1 and Pediococcus acidilactici UL5 at inhibiting Listeria monocytogenes. Int J Food Microbiol 133(3):225–233

Daeschel MA, Nes IF. Lactobacillus plantarum: Physiology, genetics and applications in foods. In: Hui, Y.H. and Khachatourians, G.G. (Eds) Food Biotechnology Microorganisms. VCH Publishers, Inc. New York; 1995.

Darsanaki,R. M. Rokhi, Morteza, A. Aliabadi, and K. Issazadeh, "Antimicrobial activities of lactobacillus strains isolated from fresh vegetables," Middle-East Journal of Scientific Research, vol. 11, pp. 1216–1219, 2012.

David RJ, Jaako M and Katie AM.(2012): The importance of food. Arch Pediatr. Adolesc Med. 2012;166(2):187–188.

Deweymattia, D., K. Manikonda, A. J. Hall, M. E. Wise, and S. J.Crowe. 2018. Surveillance for foodborne disease outbreaks—United States, 2009–2015. Morb. Mortal. Wkly. Rep. Surveill. Summ. 67:1–11.

Erdorul Ozlemand Erblr Feryal (2006) . Isolation and characterization of lactobacillus bulgaricus and lactobacillus casei from various foods. Turk J Biol;30:39e44.

Guo, L., Y. M. He, M. Li, D. W. Chen, X. Chen, Y. J. Huang, and R. X. Gu. 2017. Inhibitory effects of single and mixed lactic acid bacteria on intestinal pathogens. Food Sci. Technol. 42:26–30

Harley JP, Prescott LM. Laboratory Exercises in Microbiology. 5th Edn. Mc Graw Hill, New York; 2002.

Hasan MN, Sultan MZ, Mar-E-Um M. Significance of fermented food in nutrition and food science. J Sci Res. 2014; 6:373-386.

Hoque M, Akter F, Hossain K, Rahman M, Billah M, Islam K (2010). Isolation, identification and analysis of probiotic properties of Lactobacillus spp. from selective regional yoghurts. World J Dairy Food Sci 5:39–46.

Ivanova I, Kabadjova P, Pantev A, Danova S, Dousset X (2000). Detection, purification and partial characterization of a novel bacteriocin substance produced by Lactococcus lactis subsp. lactis b14 isolated from Boza- Bulgarian traditional cereal beverage. Biocatalysis 41: 47-53.

Karthikeyan, V., and Santosh, S. W. (2009). Isolation and partial characterization of bacteriocin produced from Lactobacillus plantarum. Afr. J. Microbiol. Res. 3, 233–239.

Kérouanton A, Hennekinne J, Letertre C, et al. (2007) Characterization of Staphylococcus aureus strains associated with food poisoning outbreaks in France. Int. j. food microbiol.115: 369-375.

Kumar A. and Kumar, D. "Characterization of Lactobacillus isolated from dairy samples for probiotic properties," Anaerobe,vol. 33, pp. 117–123, 2015.

Laemmli, U.K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage. Nat., 227 : 680-685.

Ljungh A. and Wadstr¨om, T. "Lactic acid bacteria as probiotics,"Current Issues in Intestinal Microbiology, vol. 7, pp. 73–89, 2006.

Lundeen SG, Savage DCJJob1990. Characterization and purification of bile salt hydrolase from Lactobacillus sp. strain 100–100. J Bacteriol 172:4171–4177.

Madureira, A. R., Amorim, M., Gomes, A. M., Pintado, M. E., & Malcata, F. X. (2011).Protective effect of whey cheese matrix on probiotic strains exposed to simulated gastrointestinal conditions. Food Research International, 44, 465–470.

Malini M, Savitha J. Heat stable bacteriocin from Lactobacillus paracasei subsp.tolerans isolated from locally available cheese: An in vitro study. E3 J. Biotech.Pharma. Res. 2012;3(2):28-41.

Mulaw G. Mulaw, T. Sisay, D. Muleta, and A. Tesfaye, "In vitro evaluation of probiotic properties of lactic acid bacteria isolated from some traditionally fermented Ethiopian food products," International Journal of Microbiology, vol. 2019 Article ID 7179514, 11 pages, 2019

Nieto Lozano JC, Meyer JN, Sletten K, Peláz C, Nes IF. Purification and amino acid sequence of a bacteriocin produced by Pediococcus acidilactici. J Gen Microbiol. 1992;138:1985–1990

Nishie M., Nagao J.and Sonomoto K. "Antibacterial peptides "bacteriocins": an overview of their diverse characteristics and applications". Biocontrol Science17.1 (2012): 1-16.

Ng ZJ, Zarin MA, Lee CK, Tan JS (2020) Application of bacteriocins in food preservation and infectious disease treatment for humans andlivestock: a review. RSC Adv 10(64):38937–3896

Normanno G, LaSalandra G, Dambrosio A, et al. (2007) Occurence, characterization and antimicrobial resistance of enterotoxigenic Staphylococcus aureus isolated from meat and dairy products. Int. j. food microbiol. 115: 290-296.

Ogunbanwo ST, Sanni AI, Onilude AA.Characterization of bacteriocin produced by Lactobacillus plantarum F1 and Lactobacillus brevis OG1. Afr. J.Biotechnol. 2003;2(8):219-227.

Ohenhen R. E. , Isibor J. O. , Emonfonmwan G. and Enabulele S. A.(2015) Antibacterial Activity of Bacteriocin Produced by Lactic Acid Bacteria Isolated from OGI British Microbiology Research Journal 9(3): 1-9,

Oranusi SU, Atolagbe OM, Olopade BK. Probiotics in the management of diseases: a review. Int J Curr Res. 2014; 2(8):138-158.

Parada JL, Caron CR, Medeiros ABP, Soccol CR (2007). Bacteriocins from lactic acid bacteria: purification, properties and use as biopreservatives. Braz arch biol technol 50: 512-542.

Pei Gee Yap, Zee Wei Lai and Joo Shun Tan2022 Bacteriocins from lactic acid bacteria: purification strategies and applications in food and medical industries: a review Beni-Suef Univ J Basic Appl Sci 11:51(2-18).

Pehrson,M. I. Mancilha, and C. Pereira, "Antimicrobial activity of probiotic Lactobacillus strains towards gramnegative enteropathogens," European International Journal of Science and Technology, vol. 4, pp. 136–149, 2015.

Prabhurajeshwar,C.and Chandrakanth,K(2018). Evaluation of antimicrobial properties and their substance against pathogenic bacteria in-vitro by probiotic Lactobacilli strains isolated from commercial yoghurt Clinical Nutrition Experimental xxx (2018) 1-19.

Prabhurajeshwar C. and Chandrakanth,R. K.(2017 "Probiotic potential of lactobacilli with antagonistic activity against pathogenic strains: an in vitro validation for the production of inhibitory substances," Biomedical Journal, vol. 40, no. 5,pp. 270–283,.

Rall V, Vierira F, Rall R, et al. (2008) PCR detection of staphylococcal enterotoxin genesin Staphylococcus aureus strains isolated from raw and pasteurized milk. Vet. microbiol.132: 408-413.

Rania Mohamed Ali ., 2015: Microbiological study on the effect of certain chemical and physical agents on some food borne pathogenic isolates , doctor of philosophy in microbiology, Faculty of science , zagazig university.

Ravi Sankar. N, Deepthi Priyanka.V, Srinivas Reddy.P, Rajanikanth.P, Kiran Kumar.V and Indira.M (2012). Detection, Purification and Characterization of Bacteriocin Produced. by Lactobacillus plantarum Isolated from Cow Milk, International Journal of Microbiological Research 3 (2): 133-137, 2012 ISSN 2079-2093

Rawal, K., Bhavsar, N., Raol, G., Raol, B. V., and Patel, J. D. (2013). Bacteriocin: production and optimization by Lactobacillus species. J. Microbiol. Biotechnol. Res. 3, 64–76.

Ren,D. J. Zhu, S. Gong, H. Liu, and H. Yu, "Antimicrobial characteristics of lactic acid bacteria isolated from homemade fermented foods," BioMed Research International, vol. 2018 Article ID 5416725, 9 pages, 2018.

Ryan L. A. M., E. Zannini, F. Dal Bello, A. Pawlowska,P. Koehler, and E. K. Arendt, "Lactobacillus amylovorus DSM 19280 as a novel food-grade antifungal agent for bakery products," International Journal of Food Microbiology,vol. 146, no. 3, pp. 276–283, 2011.

Salleh, F., Lani, M.N., Chilek, T.Z.T., Kamaruding, N.A. and Ismail, N. (2021). Lactic acid bacteria producing sorbic acid and benzoic acid compounds from fermented durian flesh (Tempoyak) and their antibacterial activities against food borne pathogenic bacteria. Applied Food Biotechnology, 8(2), 121-132.

Sankar, N.R., V.D. Priyanka, P.S. Reddy, P.Rajanikanth, V.K. Kumar and M. Indira(2012). Purification and characterization of bacteriocin produced by Lactobacillus plantarum isolated from cow milk. Int. J.Microbiol. Res., 3 (2): 133-137. DOI: 10.5829/idosi.ijmr.2012.3.2.62182.

Sanlibaba P. and Y. G¨uçer, "Antimicrobial activity of lactic acid bacteria," Journal of International Scientific Publications, vol. 3, pp. 451–457, 2015.

Scopes, R. K. (1993). Protein purification: principles and practice: Springer-Verlag.

Scott., 2011. Measurement of Microbial Cells by Optical Density. Journal of Validation Technology 17.1 (2011): 46-48.

Sharma K. Manual of Microbiology. Ane Books. Pvt. Ltd. New Delhi; 2009.

Shehata,M. G. , El Sohaimy S. A., El-Sahn, M. A. and Youssef, M. M. "Screening of isolated potential probiotic lactic acid bacteria for cholesterol lowering property and bile salt hydrolase activity," Annals of gricultural Science, vol. 61,no. 1, pp. 65–75, 2016.

Stoyanova L., E. Ustiugova, and A. Netrusov, "Antibacterial metabolites of lactic acid bacteria: their diversity and properties," Prikladnaia biokhimiia i mikrobiologiia, vol. 48,pp. 259–275, 2012.

Subhas Chandra Jana, Madhusree Chatterje, and Utpal Raychaudhuri (2019). "Optimization of Media and Culture Conditions for Improved Production of Bacteriocin by Using Conventional One-Factor-At-A-Time (OFAT) Method". EC Microbiology 15.4 (2019): 251-258.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Molecular Biology and Evolution, 24, 1596-1599.

Terzi ,A. ´c-Vidojevi´c, K. Veljovi´c, M. Tolinaˇcki et al., "Diversity of non-starter lactic acid bacteria in autochthonous dairy products from Western Balkan Countries-technological and probiotic properties," Food Research International, vol. 136,Article ID 109494, 2020.

Thaker HC., MN Brahmhatt, and JB Nayak (2013). Isolation and identification of Staphylococcus aureus from milk and milk products and their drug resistance patterns in Anand, Gujarat. Veterinary World, 6(1): 10 – 13.

Todorov, S.D. and Dicks, L.M.T. (2010). Characterization of Bacteriocins Produced by Two Strains of Lactobacillus plantarum Isolated from Beloura and Chouriço, Traditional Pork Products from Portugal. Meat Science, 84, 334-343.

Topisirovic, L., Kojic,M. , Fira,D,. Golic N., Strahinic, I. and Lozo, J. "Potential of lactic acid bacteria isolated from specific natural niches in food production and preservation," International Journal of Food Microbiology, vol. 112, no. 3, pp. 230–235, 2006..

Toure R, Kheadr E, Lacroix C, Moroni O, Fliss I.(2003). Production of antibacterial substances by bifidobacterial isolates from infant stool active against Listeria monocytogenes. J Appl Microbiol 2003;95(5):1058e69.

Waterhouse, A.M., Procter, J.B., Martin, D.M.A., Clamp, M and Barton, G.J. (2009) Jalview version 2 – A multiple sequence alignment editor and analysis workbench. Bioinformatics 25.(9) 1189-1191.

Wemmenhove E., van Valenberg H.J.F, van Hooijdonk A.C.M, Wells-Bennik M.H.J and Zwietering M.H. (2017).Factors that inhibit growth of Listeria monocytogenes in nature-ripened Gouda cheese: A major role for undissociated lactic acid. Food control (84) 413-418.

Wu, C., He, G., & Zhang, J. (2014). Physiological and proteomic analysis of Lactobacillus casei in response to acid adaptation. Journal of Industrial Microbiology & Biotechnology,41, 1533–1540.

Yang.E , Fan. L , Yan. J, Jiang. Y, Doucette.C, Fillmore.S and Walker B. , Influence of culture media, ph, and temperature on growth and bacteriocin production of bacteriocinogenic Latic Acid Bacteria, AMB Express, vol. 8, 2018, pp. 01-14.

Yazdi M. K. S. , Davoodabadi A , Zarin H. R. K. , Ebrahimi M. T. , and Dallal, M. M. S. “Characterization and probiotic potential of lactic acid bacteria isolated from Iranian traditional yoghurt,” Italian Journal of Animal Science, vol. 16,no. 2, pp. 185–188, 2017.