

Selection and Characterization of Two Probiotic Lactic Acid Bacteria Strains to be used as Starter and Protective Cultures for Food Fermentations

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This study focused on selection and characterization of lactic acid bacteria (LAB) strains to be used as starter and protective culture for food fermentations. Thirty LAB isolates were isolated from different fermented foods onto MRS agar medium. Two LAB isolates only CK21 and PP17 showed the highest medium acidification values pH 3.2 and 3.6 respectively and inhibited many Gram positive and Gram negative pathogenic bacteria. CK21 and PP17 isolates were selected due to their desired properties to be used as starter cultures. Based on biochemical properties as well as sequences of 16S rRNA genes, CK21 and PP17 isolates were identified as belonging to *Enterococcus faecium* CK21 (*E.faecium*) and *Lactobacillus fermentum* PP17 (*L.fermentum*). Both strains tolerated NaCl up to 10%, bile salt up to 3% and pancreatin up to 2%. Also, these strains have the ability to grow at wide pH range (2-9) and temperature range (20-50°C). These strains inhibited many pathogenic bacteria such as *Bacillus cereus* (*B.cereus*), *Streptococcus pyogenes* (*S.pyogenes*), *Staphylococcus aureus* (*S.aureus*), *Escherichia coli* (*E.coli*), *Listeria monocytogens* (*L.monocytogens*), *Acinetobacter baumannii* (*A.baumannii*) and *Salmonella typhimurium* (*S.typh*). The antibacterial compound from culture supernatants of both strains was found to be proteinacious in nature, heat stable at 100°C for 30min, and active over a wide pH ranges (2-8). All this characters coupled with the definitions of bacteriocins, so the antibacterial compound could be classified as bacteriocin. Both cell free supernatants (CFS) and neutralized CFS (NCFS) of lactic acid bacterial strains CK21 and PP17 inhibited *E.coli* and *S.pyogenes* distinctively *in vitro*.

Keywords: Lactic acid bacteria (LAB), Starter cultures, Probiotics, Food, Bacterial pathogens.

Lactic acid bacteria (LAB) include a wide group of Gram-positive fermenting bacteria which are generally non- sporulating and non-motile. They are widespread in nature, in soil, vegetables, meat, milk and the human body¹. Bio preservation refers to extending shelf life and enhanced safety of foods using microorganisms and/or their metabolites². LAB are mainly used in this purpose as they play important role in enhancing shelf life and microbial safety, improve texture, flavor and contribute to the pleasant sensory profile of the end product³.

Starter cultures are microbial preparations of large numbers of cells of at least one or more microorganisms to be added to a raw material to produce a fermented food by accelerating and steering its fermentation process³. LAB are used as natural or selected starters in food fermentations and apply the antimicrobial effect due to different metabolic processes (lactose metabolism, proteolytic enzymes, citrate uptake, bacteriophage resistance, bacteriocin production, polysaccharide biosynthesis, metal-ion resistance and antibiotic resistance)⁴.

Recently, new starter cultures of LAB bacteria with an industrially important functions are being developed and provide microbial safety and nutritional, or health advantages. Examples

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are LAB bacteria that produce antimicrobial substances, sugar polymers, sweeteners, aromatic compounds, vitamins, or useful enzymes, or that have probiotic properties. Nowadays the food products are considered healthy due to the characters of specific strains of LAB they contained⁵. There are characters of bacterial strains to be used as starter and probiotic cultures. For performing the probiotic functions, LAB have to face the low pH environment of the gastrointestinal tract. Therefore, acid resistance of LAB is important not only for their own growth, but also for fermentation and preparation of probiotic products⁶. Also the LAB strains must have the ability to produce antimicrobial substances such as organic acids (mainly lactic and acetic acid), hydrogen peroxide and also other compounds, such as bacteriocins and antifungal peptides⁷. Starter cultures consisting of LAB produce bacteriocins and other antimicrobial substances aid in extending the shelf life of food, modifying food sensory attributes and improving texture⁸. Also, LAB strains that can be used as probiotic and contribute in food industry as starter culture must tolerate bile salt as it is one of the most effective stress factors and it is very undesirable for survival of the probiotic strains during gastrointestinal tract⁹. The main aim of this study was to select LAB strains to be used as starter and protective cultures with probiotic characters in order to improve the quality of food fermentations

MATERIAL AND METHODS

Collection of food samples and isolation of LAB

Thirty samples were collected from different food types such as pickles (olive, pepper, lemon, carrot and mixed samples), fermented dairy products as Kareesh cheese, white cheese and mesh, fermented fish, grape fruit and tomato salsa. All the samples are collected from different markets at Sharkia Governorate 100Km north Cairo, Egypt in the period from 1/5/2016 till 1/11/2016. Isolation of LAB was carried out using the microbial pour plate method. Different dilutions of the samples prepared in saline (0.85% NaCl) and pour plated onto MRS agar medium¹⁰ and incubated at 37°C, for 24h. Colony forming units (CFU/mL) of LAB were determined. The presumptive LAB species were purified by re-streaking on MRS

agar plates and incubated to obtain single pure colony. After incubation the colonies were stored in refrigerator on MRS agar slants/stabs throughout the experiments.

Growth of LAB isolates at wide pH values and medium acidification

Growth of bacterial isolates at different pH values and their ability to acidify medium were tested. MRS broth was prepared and adjusted with either 0.1M HCL or NaOH to different pH values (3, 5, 7, and 9). The tubes were inoculated by 1% V/V fresh overnight LAB culture and incubated at 37°C for 24h. The growth was measured by reading optical density at 600nm using spectrophotometer (New Brunswark Scientific CO.) and also final pH was detected by using pH meter^{11,12}.

Antagonistic effect of LAB isolates

The antimicrobial activity of the isolated LAB against pathogenic bacteria (*E. coli*, *S. aureus*, *L. monocytogenes*, *B.cereus*, *A.baumannii*, *Salmonella typhi* and *S.pyogens*), was performed by the well diffusion assay. The pathogenic bacteria were provided from laboratory of Applied Bacteriology, Faculty of science, Zagazig University, Egypt. The pathogenic bacteria were propagated in nutrient broth at 37°C for 24 h and streaked on to BHI (oxoid) agar plates. The plates were left for solidification then stored for 2h in a refrigerator. After that wells were made by using sterile cork porer and filled with cell-free supernatants (CFS) of LAB isolates that were prepared by centrifugation of LAB cultures at 10,000 rpm for 20 min. CFS was adjusted at pH 7 to avoid inhibition due to acidity and sterilized by filtration (0.45Mm, Milipore, Amican). After incubation at 37°C for 24h the antimicrobial activity was determined by measuring the diameter of the inhibition zone around each well (mm)¹³.

Identification of bacteria

Both LAB isolates CK21 and PP17 which showed the highest medium acidification and inhibited most of pathogenic bacteria were identified according to standard clinical laboratory methods recommended by Bergey's Manual of Systematic Bacteriology^{14,15}.

Also the identification of both CK21 and PP17 isolates was confirmed by the fingerprinting of 16S rRNA gene(s). Total DNA was extracted from both CK21 and PP17 according to Sambrook and Russel (2001). The gene

coding for 16S rRNA was amplified from each tested isolate by PCR using the forward primer 5'AGAGTTGATCMTGGCTCAG3' and reverse primer 5' TACGGYTACCTTGTTACGACTT 3'¹⁶. Molecular size of amplified fragments should be approximately 1485 bp. The amplification conditions were as follows: 94°C for 10 min and 35 cycles of denaturation at 95°C for 30s, annealing extension at 56°C for 1 min, 72°C for 1 min and an extension at 72 °C for 10 min. the PCR products were subjected to agarose gel electrophoresis and the bands of amplified 16S rRNA were cleaned up using gene purification Kit (Centrisep). The amplified DNA fragments were partially sequenced at GATC Biotech (Metabion Germany) using ABI 3130, USA DNA sequencer. Sequence analysis and their comparison to deposited data in gene bank was made using the Basic Local Alignment Search Tool (BLAST) Program at <http://ncbi.nlm.nih.gov/blast>¹⁷.

Sodium chloride tolerance by selected LAB bacterial strains

Ability of both CK21 and PP17 strains to tolerate sodium chloride was tested. Number of test tubes contained MRS broth were adjusted to different NaCl concentrations (2, 4, 6, 7.5, 10, 12.5 and 15 %). After sterilization, each tube was inoculated by 1% V/V fresh overnight LAB bacterial strains and incubated for 24h at 37°C. After incubation, the growth of bacteria was measured by spectrophotometer at 600 nm¹².

Bile salt and pancreatin tolerance by LAB strains

Sterile MRS broths were supplemented with bile (oxgal) at concentrations of (0.1, 0.5, 0.7, 1, 1.5, 2, and 2.5%) and pancreatin at concentrations of (0.5, 0.7, 1, 1.5, and 2 %) separately. After sterilization, 1% V/V of overnight culture of both CK21 and PP17 strains were inoculated into the MRS broth and incubated at 37°C for 24h. The survival rates of LAB isolates were measured by taking absorbance at 600nm using the spectrophotometer (New Brunswick Scientific CO.)^{18,19}.

Antibiotic susceptibility test

The susceptibility of antibiotic discs was done by disc diffusion method as described by Bauer et al. (1966). Selected LAB strains CK21 and PP17 were inoculated into MRS broth and incubated at 37 °C for 24h. Then, LAB

bacterial suspension was spread onto the surface of the MRS agar plate. The antibiotic discs of fourteen antibiotics listed in Table 4 were placed onto the surface of MRS agar plates and incubated at 37°C for 24h. After incubation, inhibition zone diameters around the disc were measured using millimeter ruler²⁰. The sensitivity, resistance and intermediacy of both LAB strains were calculated according to CLSI (2008) and NCCLS (2009).

Growth of CK21 and PP17 at wide temperature range

MRS broths were prepared and inoculated by 1%V/V of both LAB strains. The inoculated media were incubated at different temperatures (25, 30, 35, 40, 45, 50°C) for 48h. After incubation, the growth was detected by measuring the absorbance at 600 nm using spectrophotometer (New Brunswick Scientific CO.).

Characterization of inhibitory substances produced by both LAB strains (CK21 & PP17) Preparation of cell free supernatant

Cell-free supernatants (CFSs) were prepared by cultivating LAB strains in MRS broth for 16h at 37°C and centrifuged for 10 min at 4°C. The CFSs were used to inhibit indicator bacteria used. In another experiments, this CFS was neutralized by both 0.1N HCl and 0.1N NaOH at pH 7.0 and filter sterilized (0.45 milipore, Amican) and designated NCFS²¹.

Factors affecting inhibitory substances

The proteinaceous nature of the inhibitory compounds in NCFS was confirmed by testing their sensitivity to proteolytic enzymes. Aliquots of NCFS was treated by 1mg/mL from proteinase K, chymotrypsin, trypsin, pepsin, α -amylase and catalase (Sigma, USA). Antimicrobial activity was determined by using the well diffusion method as described above in Materials and Methods^[22].

The pH on stability of inhibitory substance in NCFS was tested by adjusting CFS of both strain at different pH values 2,4,6,8 by using 1N HCl and 1N NaOH and incubated overnight at room temperature, pH was adjusted at pH 7 for all test and then antimicrobial activity was examined by using well diffusion method²³

To test the thermal stability of inhibitory substance, aliquots of sterilized NCFS of each strain was exposed to different temperature values viz. 70, 80, 90°C, for 5 min, 100°C for 5, 10, 20, 30 min and 121°C for 15 min. Activity of inhibitory

substances was detected by using well diffusion method²³.

To study the effect of organic solvents on activity of the inhibitory substance, 1ml from sterilized NCFS of each LAB strain was mixed with 10% (v/v) from different organic solvents as chloroform, benzene, ethyl ether, and acetone and diethyl ether. Controls were NCFS without adding organic solvents. To allow solvents to evaporate each of samples and controls were incubated overnight at 60 °C. Then, antimicrobial activity was determined by using well diffusion method²⁴.

Inhibition of some pathogenic bacteria by CFS and NCFS from *L. fermentum* PP17 and *E. faecium* CK21

The effect of CFS and NCFS of CK21 and PP17 on the selected pathogenic bacteria: *S.pyogens* and *E.coli* were investigated, *in vitro*. BHI broths (Oxoid) were cultivated by either *E.coli* or *S.pyogens* bacteria as they were the most sensitive organisms at concentrations of about 2×10^5 CFU/mL. One mL from filter-sterilized CFS and NCFS of *L.fermentum* was added separately to 9ml from growing cells of *S.pyogens* and also 1ml from CFS and NCFS of *E.faecium* was added to *E.coli*. Control cells contained indicator organism without either CFS or NCFS of both LAB strains tested. After incubation at 37°C, samples were taken at time intervals; 0, 6, 12, 24, 48, 72, 96h and CFU/mL were determined at each time. The percentage of cell viability was determined in relation to control²⁵.

RESULTS AND DISCUSSION

The recent studies focused on selection of LAB isolates to be used as starter and protective culture in food fermentations or with probiotic capability^{26, 27}. LAB also used in bio preservation process in order to extended shelf life and enhanced safety of foods². In this present study, Thirty LAB isolates were isolated from different fermented food types such as pickles and fruits onto MRS agar medium. These LAB cultures were assayed for medium acidification, as production of acids is a desired property for probiotic capability and used of strain as starter cultures as most of food-borne pathogens cannot grow at acidic conditions²⁶. Fourteen LAB isolates out of 30 tested decreased pH values to acidic levels. LAB isolates CK21,

and PP17 were the most powerful in acidification of medium, they decreased pH value to almost 3.5 within 24h. This is a promising result in using of these two isolates as starter cultures for food fermentations because they could consume sugars from food matrix converting them to acid via fast fermentation process. Such produced acids are also attractive criteria for both strains to be protective cultures where pathogenic bacteria cannot grow. By consumption of food fermented by, these LAB can balance pathogenic bacteria in small intestine and improve intestinal ecosystem by their produced acids. So both CK21 and PP17 may be used as probiotics²⁸. This is in confirm with Banwo et al.²⁹ who reported ability of *E. faecium* to make shift in pH until 3.85 after 48-h incubation. The acidic medium formed due to production of lactic acid is not suitable for survival of spoilage bacteria that may found in fermenting substrate³⁰

The fourteen LAB isolates were tested for growth at different pH values (3, 5, 7, and 9). All of them grew well at various pH levels with higher growth value in pH range (6-7) also they were able to tolerate and grow at acidic pH values (3, 5) as shown in Table (1). It is of interest to recall herein that both CK21 and PP17 LAB isolates showed fast growth at the pH values tested. Such results enable both of these isolates to possess broader applications in food fermentations at different pH values. Similarly, Kumari et al.³¹ found that different LAB isolates were tolerant acidic conditions until pH 3. Also this coupled with the work of Enan et al.^{32, 33}

To investigate the probiotic capabilities of the 14 LAB tested, their antimicrobial activities against many Gram positive and Gram negative food –borne pathogen were studied. Results are given in Table (2) *S.aureus* and *S.pyogens* were inhibited by all LAB tested, but *S.typhimnum* was not inhibited by any of them. Most of tested LAB isolates inhibited *L.monocytogens*, *B.cereus* and Gram negative strains: *E.coli* and *A.baumannii*. This inhibition of this sensitive bacteria could be due to the inhibitory metabolites produced by LAB isolates such as lactic acid and other organic acid, hydrogen peroxide, diacetyl, carbondioxide, ethanol and bacteriocin³⁴. This greatly show the necessity of LAB in food fermentations as the antimicrobial compounds produced by these bacteria play a promising role in ensuring the safety and extending

the shelf life of produced fermented food ³² .

The LAB isolates; CK21 and PP17 showed the highly medium acidification, grew at wide pH range (3-9) and inhibited all the indicator bacterial pathogens tested expect *S. typhimurum*, they were subjected to identification using the criteria of biochemical identification that given in Bergey's Manual of Systematic Bacteriology ^{[14],[15]}.

The isolates CK21 and PP17 could be identified as belonging to *E.faecium* and *L.fermentum* respectively. Because biochemical identification showed sometimes speculative results the two isolates were subjected to molecular identification using 16S rRNA cataloging analysis²⁶. The 16S rRNA genes of CK21 and PP17 isolates were polymerized using PCR technique, electrophoresed

Table 1. Growth of LAB isolates at different initial pH values and their final pH values

LAB isolates No. & Code	pH 9		pH 7		pH 5		pH 3	
	Final pH	OD600						
GF7	8	1.391	5.1	1.656	5	0.415	3	0.294
PL8	7.4	0.605	5	1.33	4.8	0.822	3	0.528
PO10	6.9	0.574	5	0.7	5.4	0.477	3	0.344
PO11	6.4	1.092	4.5	1.717	4.8	0.8	3	0.399
PO12	6.2	0.872	5.1	1.23	5	0.952	3	0.275
PM14	6	1.11	5.3	1.787	4.7	0.652	3	0.362
PC15	6.2	0.917	5.4	1.717	5	1.775	3.1	0.354
PP17	6	0.752	3.4	1.23	4	0.952	2.3	0.433
CK18	7.3	0.682	5	1.181	5.1	0.53	2.8	0.381
CK19	7.2	0.41	4.2	1.398	4.2	0.897	3	0.273
CK21	5.8	0.524	3.5	0.966	4	0.708	2.5	0.393
CM25	7	0.76	5.2	0.943	5.4	0.42	2.9	0.276
CV28	6	0.31	5.1	0.86	4.2	0.523	3	0.3
CV30	5.3	0.672	5	1.272	4.8	0.73	3.1	0.37

GF: Grape fruit, PL: Pickles (lemon), PO: Pickles (olive), Pickles (mixed), PC: Pickles (carrot), PP: Pickles (pepper), CK: Cheese (Karessh) CM: Cheese (mesh), CV: Cheese (veta)

Table 2. Antimicrobial activity of LAB isolates against some pathogenic bacteria

LAB isolates	Diameter of inhibition zone (mm)						
	Gram Postive				Gram negative		
	<i>B. cereus</i>	<i>S. pyogens</i>	<i>L.monocytogens</i>	<i>S.aureus</i>	<i>E.coli</i>	<i>A.baumannii</i>	<i>S.typhi</i>
GF7	18	10	20	12	0	17	0
PL8	10	17	15	0	0	18	0
PO10	20	13	10	10	0	3	0
PO11	18	12	17	15	20	15	0
PO12	0	9	10	10	10	0	0
PM14	10	10	8	8	10	9	0
PC15	10	0	0	12	13	0	0
CK18	0	0	10	10	15	0	0
PP17	20	25	22	18	20	25	0
CK19	0	0	0	12	12	0	0
CK21	18	20	19	15	25	27	0
CM25	10	0	0	12	0	10	0
CV28	12	0	10	17	0	0	0
CV30	0	12	0	11	5	9	0

Table 3. Characteristics needed for LAB strains to be used as starter and protective culture

Parameters	Growth of bacterial strain (O.D)	
	<i>L. fermentum</i>	<i>E. faecium</i>
Sodium chloride Conc. %		
2	0.957	0.875
4	0.791	0.686
6	0.353	0.567
7.5	0.296	0.332
10	0.043	0.150
12.5	0	0.055
Bile salt Conc. %		
0.1	0.935	1.06
0.5	0.580	0.981
0.7	0.520	0.761
1	0.362	0.729
1.5	0.206	0.435
2	0.240	0.350
2.5	0.206	0.123
3	0.133	0.055
Pancreatin Conc. %		
0.5	1.130	1.014
0.7	0.947	0.947
1	0.755	0.654
1.5	0.350	0.223
2	0.124	0.089

via agarose gel and showed molecular mass of about 1485bp Fig.(1). They were sequenced and both sequences of two strains CK21 and PP17 were submitted to GenBank (<http://www.ncbi.nlm.nih.gov/GenBank/update.htm>) under accession numbers MH802621 and MH807546 respectively Fig.(2) By using Basic Local Alignment Search Tool (BLAST) they showed similarities e⁹⁹ 98 % to *E.faecium* (CK21) and *L.fermentum* (PP17) ¹⁷ . The phylogenetic tree was displayed using treeview program Fig. (3a, b)

NaCl is an inhibitory substance that prevent the growth of certain types of bacteria. Also salt is the most widely preservative factor used in many food industry. Therefore, it was essential to test the NaCl tolerance of lactic acid bacteria ^[35]. In this present study *L.fermentum* was able to grow at MRS broth supplemented with 7% NaCl concentrations while the growth was decreased at 10% NaCl concentration or no growth detected Table (3). Meanwhile, *E.faecium* strain had the ability to grow in MRS broth supplemented with 12% NaCl concentrations. These results were similar to that obtained by Forhad et al.³⁶ who reported that *L.fermentum* grew well until 7% NaCl concentrations but failed to grow at 10% NaCl concentration.

Table 4. Antibiotic sensitivity of LAB strains *E. faecium* CK21 and *L. fermentum* PP17

Antibiotic	Symbol	Coc.mg /disc	Sensitivity and diameter of inhibition (mm)	
			CK21	PP17
Amikacin	AK	30	R (0)	R (0)
Amoxicillin/Clavulanic acid	AMC	20	R (0)	S (25)
Ampicillin/sulbactam	SAM	15	R (0)	R (0)
Ampiclox	AX	15	S (20)	S (38)
Azithromycin	AZM	15	M (14)	S (19)
Ciprofloxacin	CIP	15	R (12)	M (17)
Ceftriaxone	CRO	30	R (0)	R (8)
Cephadrine	CEP	15	R (12)	S (20)
Doxycycline	DO	30	S (35)	S (21)
Erythromycin	E	15	R (0)	M (15)
Imipenem Nitrofurantoin	IPMF	1030	S (30)	S (40)
Sulphamethoxazol/trimethoprim	SXT	25	S (20)	S (30)
Vancomycin	VA	30	R (0)	R (0)

Ranges of zone of inhibition diameters exhibited by bacteria considered sensitive (5S), moderate (M), or resistant (5R) to each antibiotic

Furthermore, CK21 and PP17 strains were tested for their ability to tolerate bile salt and pancreatin. Both strains showed high tolerance to bile salt up to 3% and pancreatin at 0.5-2% Table (3). Also this previous work³⁷ reported that *L.fermentum* showed good tolerance to bile until 2.5% bile concentration. Also, Banwo et al.²⁹ found that *E.faecium* tolerate bile salt up to 1% bile concentration. Differences in bile salt tolerance among LAB strains are related to their hydrolase activity. Tolerance to bile salt is one of the criteria used to select probiotic that would be able to act effectively in human gastrointestinal tract, and colonize small intestine and produce their metabolic activity³⁷. In this study both LAB strains tolerate pancreatin up to 1.5%. Such criteria is necessary to viability of any bacterial strain to be probiotic.

Table 5. Characterization of the inhibitory substance produced by LAB strains CK21 and PP17

Sodium chloride	Diameter of inhibition zones (mm)	
Enzymes	PP17	CK21
Pepsin	0	0
-chemotrypsin	0	0
Trypsin	0	0
Catalase	20	22
Thermostability		
70 °C For 30 min	28	25
80 °C 30 min	28	25
90 °C 30 min	28	25
For 5 min	28	25
10 min	28	25
100 °C 20 min	25	23
30 min	25	20
120 °C 15 min	0	0
Organic Solvent		
Acetone	28	28
Chloroform	28	28
Ethyl lchol	28	25
Diethyl ether	25	28
Butanol	28	25
pH		
2	13	15
4	19	25
6	25	28
8	15	11
9	0	5

In the current study, both strains were able to grow at wide temperature growth ranged from 25°C to 50°C Fig. (4). The maximum growth of *L.fermentum* and *E.faecium* was achieved at 40°C and decreased by increasing temperature up to 50°C. Van den Berghe et al.³⁸ reported that the optimum temperature for *E.faecium* was 40°C and growth observed until 50°C. The bacterial ability to grow at high temperature is a good characteristic, as it could be interpreted as showing an increased rate of growth and lactic acid production. Growth of LAB strains at wide temperature range enable the organisms to be used in food fermentations at wide temperature growth range. Moreover, the fermentation that require high temperature decreases the opportunities of contamination by other microorganisms³⁹.

Moreover, the sensitivity of CK21 and PP17 strains to antibiotics was tested. *L.fermentum* (PP17) showed sensitivity and moderate sensitivity to most of antibiotic tested while it was resistant to Amikacin, Ampicillin/ sulbactam, Ceftriaxone, Sulphamethoxazol/ trimethoprim and Vanomycin Table (4). While, for *E.faecium* (CK21) the rate of antibiotic resistance is higher than that in the PP17 strain. CK21 strain was sensitive to Vanomycin, Nitrofurantoin, Imipenem, Doxycycline and Ampiclox. Our results similar with this previous work⁴⁰ which

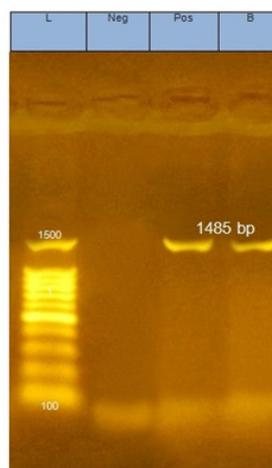


Fig. 1. Agarose gel electrophoresis of PCR products including the amplified 16S rRNA gene of two LAB isolates. Lane 1, DNA marker; Lane 2, negative control; Lane 3, *E.faecium* CK21; Lane 4 *L.fermentum* PP17

> *Enterococcus faecium* strain CK21, 16S ribosomal RNA gene, partial sequence
 TCAGGGCGGGTCTATACATGCAAGTCGAACGCGTTGGCCCAATTGATTGATGGTGTGCTTGCACCTGATTGATTTG
 GTCGCCAACGAGTGGCGGACGGGTGAGTAACACGTAGGTAACCTGCCAGAAGCGGGGGACAACATTTGGAAC
 AGATGCTAATACCGCATAACAACGTTGTTTCGCATGAACAACGCTTAAAGATGGCTTCTCGCTATCACTTCTGGAT
 GGACCTGCGGTGATTAGCTTGTGGTGGGGTAATGGCCTACCAAGGCGATGATGCATAGCCGAGTTGAGAGACT
 GATCGGCCACAATGGGACTGAGACACGGCCATACTCTACGGGAGGAGCAGTAGGGAATCTCCACAATGGG
 CGCAAGCCTGATGGAGCAACCGCGTGTGAGTAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAA
 CACGTATGAGAGTAACTGTTACATCGTTGACGGTATTTAACAGAAAGTACGGCTAACTACGTGCCAGCAGCCGC
 GGTAAACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGAGAGTGCAGGCGGTTTTCTAAGTCTGAT
 GTGAAAGCCTTCGGCTTAAACCGGAGAAGTGCATCGGAACTGGATAACTTGAGTGCAGAAGAGGGTGTGGAAC
 TCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACAGTGGCGAAGGCGGCTACCTGGTCTGCAACT
 GACGCTGAGACTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAACGATGAGTG
 CTAGGTGTTGGAGGGTTTTCCGCCCTTCAAGTCCGCGAGCTAACGCATTAAAGCACTCCGCTGGGGAGTACGACCGC
 AAGGTTGAACTCAAAGGAATTGACGGGGGCCCGCACAAAGCGGGGGAGCATGTGGGTTAATTCCAAAGCTA
 CGCGAAAGAACCTTACCAGGGTCTTGACATTCTTGCCCCAACCCCTAAAGAATAGGGCGGTTTTCTTCCGGGA
 AACGCCAATGGACAGGGTGGGGGCATGG

(a): CK21

Lactobacillus fermentum strain PP17, 16S ribosomal RNA gene,
 partial sequence

GGGGGGGGGGCTATACATGCAGTCGAACGCGTTGGCCCAATTGATTGATGGTGTGCTTGCACCTGATAG
 ATTTTGTGCGCCAACGAGTGGCGGACGGGTGAGTAACACGTAGGTAACCTGCCAGAAGCGGGGGACA
 ACATTTGGAAACAGATGCTAATACCGCATAACAACGTTGTTTCGCATGAACAACGCTTAAAGATGGCTTC
 TCGCTATCACTTCTGGATGGACCTGCGGTGCATTAGCTTGTGGTGGGGTAATGGCCTACCAAGGCGAT
 GATGCATAGCCGAGTTGAGAGACTGATCGGCCACAATGGGACTGAGACACGGCCATACTCTACGGG
 AGGCAGCAGTAGGGAATCTCCACAATGGGCGCAAGCCTGATGGAGCAACACCGCGTGTGTAAGAA
 GGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACACGTATGAGAGTAACTGTTACATCGTTGACG
 GTATTTAACAGAAAGTACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTA
 TCCGGATTTATTGGGCGTAAAGAGAGTGCAGGCGGTTTTCTAAGTCTGATGTGAAAGCCTTCGGCTTAA
 CCGGAGAAGTGCATCGGAACTGGATAACTTGAGTGCAGAAGAGGGTGTGGAACCCATGTGTAGCG
 GTGGAATGCGTAGATATATGGAAGAACACAGTGGCGAAGGCGGCTACCTGGTCTGCAACTGACGCTG
 AGACTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAACGATGAGTG
 CTAAGGTGTTGGCGGGTTTTCCGCCCTTCAAGTCCGCGGAGCTAACGCATTAAAGCACTCCGCCCTGG
 GGGAGTAACGACCCGAAGGGTTTGAACCTCAAAGGAAATTGAACGGGGGGCCCGCAGCAAGCC
 GGAGGTAGCCATGTGGCTTTAATTTCAAAGCCTACCGCGGAAGAAACCTTACCAGGGTCTTTGA
 ACATTTGGGCGGCCAACCCCTAAAAAATAAGGGGCGTTTTCCCTTCCGGGAACCCCAAATTGGA
 CCAGGGTGGCTGGCCATGGGGTTCGTTCAAGCCTCCGGGTTCAATGGAACAATGGTTAGGGGA
 TAAAGGATCCCGGCTAACTGAATCCGCCA

(b): PP17

Fig. 2. Nucleotide sequences of 16SrRNA genes of the purified PCR products of LAB isolates (A); CK21 and (B); PP17

found that *E. faecium* was sensitive to Vanomycin, Nitrofurantoin and resistant to Erythromycin and ciprofloxacin similar with our results. Melo et al.⁴¹ reported that *L. fermentum TCUESC01* was resistant to Amikacin, Sulphamethoxazol/trimethoprim and Vanomycin and sensitive to most of antibiotic that PP17 strain sensitive to it. Knowledge of antibiotic sensitivity is very

important by considering three important factors: the rare possibility of infection by Lactobacillus, the risk of horizontal transfer of resistance genes to other microbes, and the link between probiotic bacteria and antibiotic treatment⁴². The resistance profiles of our strains has the advantage that these strains can be introduced in the human diet in the same time of antibiotic treatment. Also,

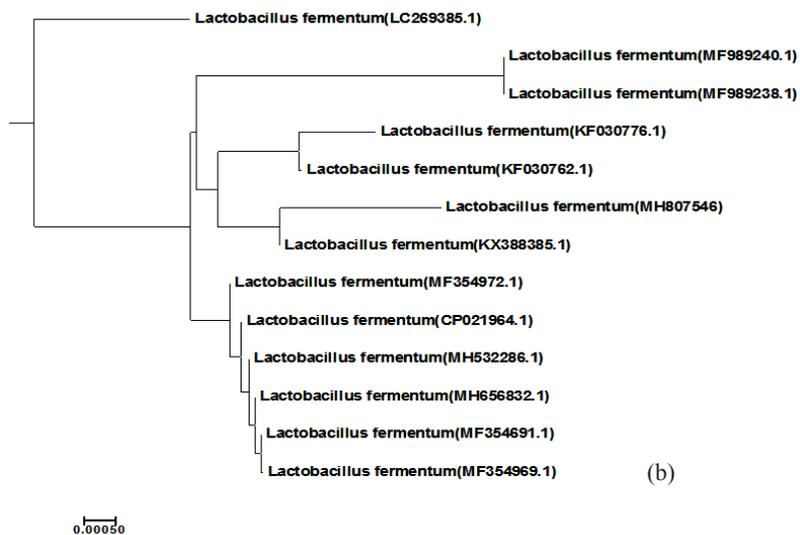
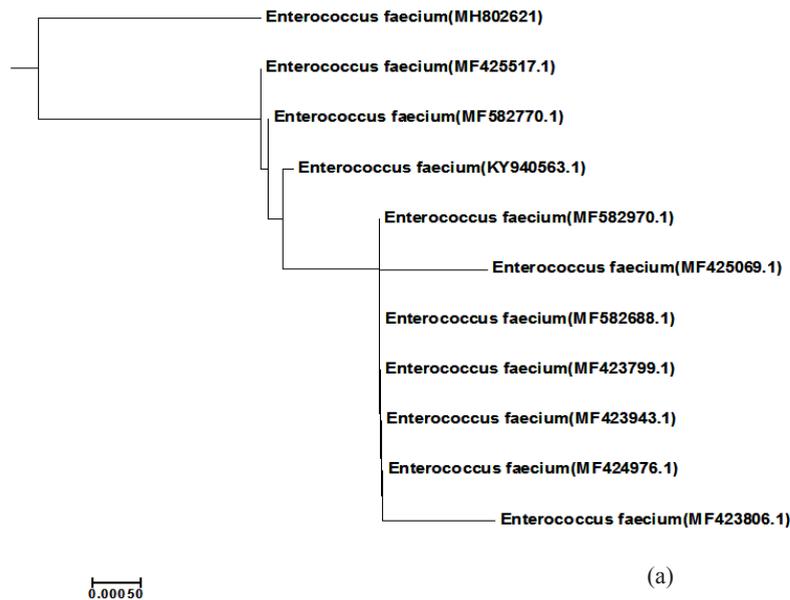


Fig. 3. Phylogenetic tree of both LAB strains CK21(a) and PP17(b)

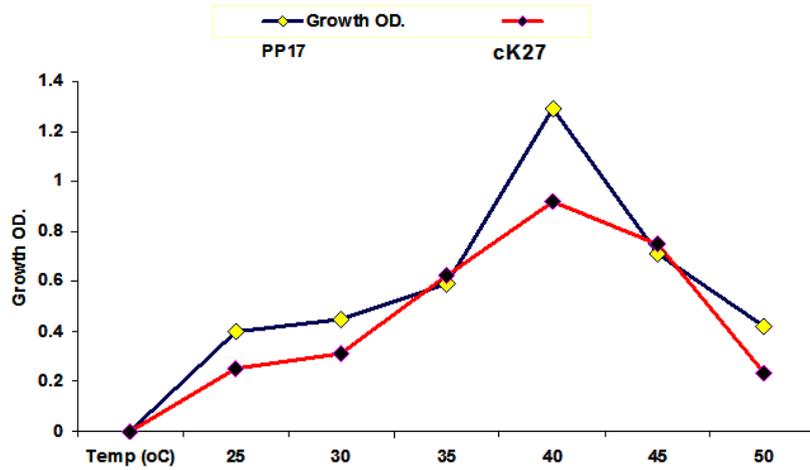


Fig. 4. Growth of LAB strains CK21 and PP17 at wide temperature range

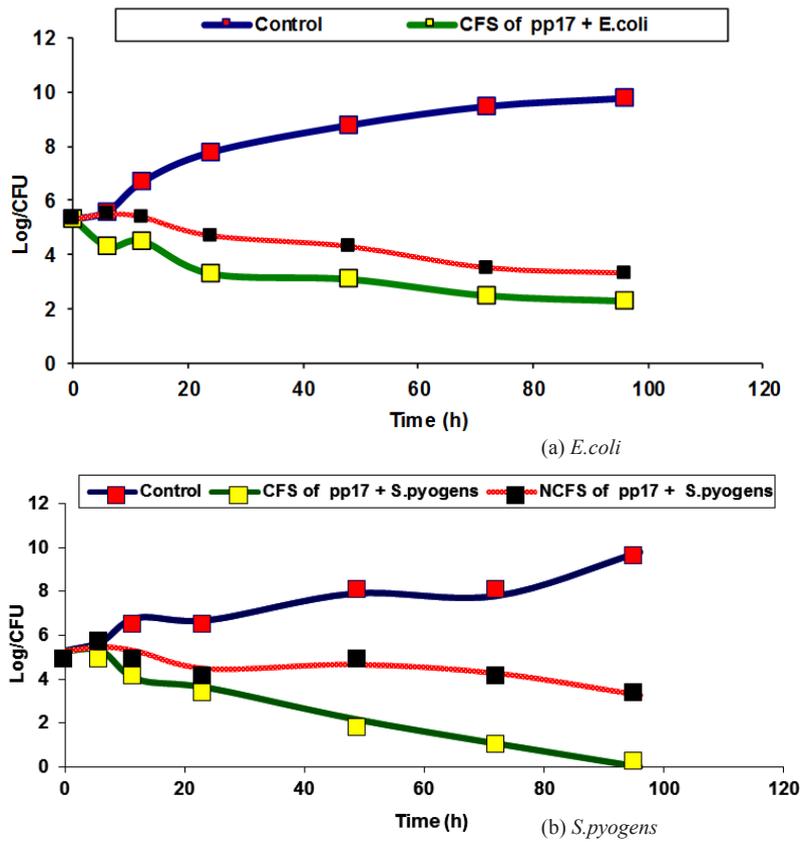


Fig. 5. Inhibitory effect of CFS and NCFS of PP17 against *E.coli* and *S.pyogens*

the antibiotic resistance is important for intestinal microbiota to recover quickly after antibiotic treatment. While this resistance is highly danger in people who suffer from bacterial infections. As LAB may act as a carrier to antibiotic resistance genes similar to those found in human pathogens. So they can transfer resistance genes to pathogenic bacteria⁴³.

From these results, it can be observed that both CK21 and PP17 strains have the best biotechnological properties such as inhibition of some gram positive and gram negative pathogenic bacteria, acidification of medium by production of acids, growing at wide ranges of pH and temperature and tolerance to stress conditions such as NaCl, pancreatin and bile salt. So, this strains could be used as a probiotic bacteria, starter and

protective cultures for food fermentation. These results are similar with Mello et al.⁴¹ who reported that *L.fermentum* can be used as a safe probiotic and food additive. Also, Banwo et al.²⁹ found that the *E.faecium* strains exhibited a good probiotic activity because of their tolerance to low pH, bile salts and their inhibitory effect against food pathogens and spoilage micro-organisms. Due to the importance of this two organisms, studies were further concentrated to characterize their inhibitory substance.

The inhibitory activity of NCFS from either CK21 or PP17 was inactivated after treatment of this CFS by protease enzymes (trypsin, pepsin and α -chemotrypsin), while the activity remained stable after catalase treatment Table 5. This confirming the proteinaceous nature of their

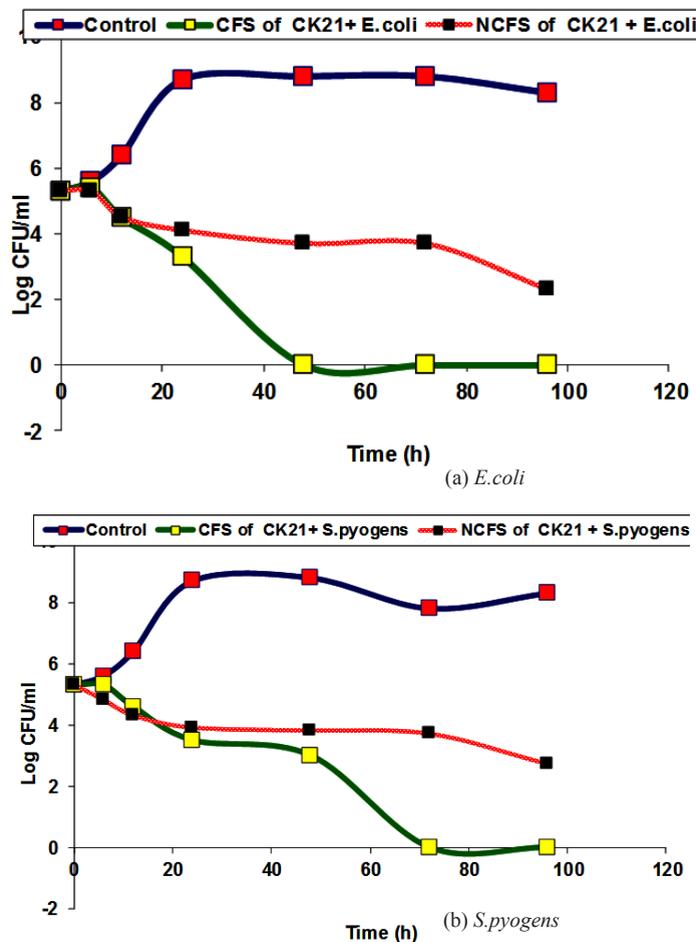


Fig. 6. Inhibition of *E. coli* and *S. pyogenes* by NCFS and CFS from CK21 strain

antimicrobial substance as similar with Priscilia et al.⁴⁴. To study the stability of the inhibitory substance (s) of both CK21 and PP17, CFS of both strains were adjusted at different pH values (2, 4, 6, 8, and 9) for 12 h and then readjusted at pH7. The activity of inhibitory substances of both strains was pH dependent with maximal activity at pH 4-6, and lower activity at slightly alkaline and lower acidic pH values. This showed that the inhibitory substance was stable at pH range 2-8. So, CK21 and PP17 strains can be used as starter and protective culture for food fermentations at acidic pH level, such acidic pH level will not effect on the stability of their bacteriocin produced. The results are similar with Ohenhen et al.⁴⁵ who reported that the bacteriocin activity of some LAB was high at acidic level 2, 4, 6 and decreased at alkaline level by testing their antimicrobial activity against *E.coli* and *S.aures*

The inhibitory substances of both CK21 and PP17 strains were tested for it is thermal stability. The results showed that these substances were heat stable up to 100°C for 30 min in both strains Table (5). While, the antimicrobial activity lost in both strains after exposure to 121°C for 15 min. So, the antimicrobial compound of both strains could be classified under heat stable bacteriocin like substance. Similarly, Park et al.⁴⁶ reported that enterocins produced by *Enterococcus faecium* JCM 5804 was heat stable up to 100°C for 30 min but the inhibitory activity was lost at 121°C for 15 min. Also, Maria and Janakiraman⁴⁷ revealed that the bacteriocin of *L. acidophilus* NCIM5426 was heat stable up to 100°C while part of activity was lost at 121°C for 15 min. Thermal stability of inhibitory substance of LAB is a typical characteristic for bacteriocin and bacteriocin like substance. Temperature stability is important if the bacteriocin are used as a food preservative, because many procedures of food preparation involve a heating step.

The inhibitory substances of both CK21 and PP17 strains were treated by different organic solvents but their antimicrobial activity did not affected Table 5. The fact that the bacteriocins were stable with organic solvents indicated that they were soluble in them. These results similar with the work of Ghanbari et al.⁴⁸ who found that lactocin RN78 and plantracin LC74 bacteriocins

which were produced by different *lactobacillus* spp. kept their antimicrobial activity after treatment by organic solvents such as butanol, ethanol and methanol.

Inhibition of some pathogenic bacteria as *E.coli* and *S.pyogenes* by CFS and NCFS of CK21 and PP17 strains were studied, *In vitro*. The results showed that there was a reduction in the viable cell count of pathogenic bacteria after treatment with CFS and NCFS of CK21 and PP17 strain Fig (5a, b). The growth of pathogenic bacteria decreased distinctively, reaching almost 5-7 log cycles (growth reduction) within 72h in case in PP17 strain. While CFS of CK21 prevented growth of *E.coli* and *S.pyogens* respectively Fig (6a, b). Also NCFS of this strain inhibited both selected pathogenic bacteria vigorously and difference in colony counts among control and treated samples were almost 5-6 log cycles. In general CFS obtained from both probiotic strains (CK21 and PP17) was more active than NCFS in inhibition of both *E.coli* and *S.pyogens*. This due to CFS can contain in addition to bacteriocin, organic acids such as lactic acids, diacetyl, ethanol and acetaldehyde³¹. These results are similar with that obtained by Hor and Liang⁴⁹ who found that the growth inhibition of *S.aureus* by CFSs of five selected LAB higher than that obtained by NCFS which make reduction in growth inhibition by 50% in the percentage of inhibition than the initial level. Bajpai et al.⁵⁰ showed that there is a remarkable decline in CFU counts of *S. aureus* KCTC-1621 and *E.coli* O157:H7 when exposed to the 289 CFS of *P. pentosaceus* 4I1.

The two lactic acid bacterial strains; CK21 and PP17 that were isolated from cheese and pickles respectively were selected due to their qualified properties as these strains are able to make medium acidification, production of some enzymes, grew well at wide pH and temperature range and make inhibition to some gram positive and gram negative foodborne pathogens *in vitro*. Such properties are good features for lactic acid bacterial strains to be used as starter and protective culture for food fermentations. Addition of these strains to human diet by consumption with foods fermented with them lead to improvement of many human nutritional functions due to their probiotics characters. However, further work will

be necessary in the future to study the inhibition of pathogenic bacteria by these strains *in situ* and to study their probiotic capabilities *in vivo*.

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