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Research article

Application of ISSR analysis for identification of lactic acid bacteria strains isolated from Egyptians milk products

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Abstract

The aim of this study is to isolate and identify probiotic lactic acid bacteria. About four isolates of lactic acid bacteria were isolated from different sources of milk products. Identification of isolates was done by morphological, biochemical and physiological characterizations. Results indicated that all the four isolates were able to tolerate acidic conditions at low pH (3) and survived at different concentration of bile salts (0.5-1.5%) and showed tolerance to 6.5% NaCl concentration. All isolates have antioxidant activity ranged from 63.2% to 83.7% and cholesterol removal ranged from 17% to 27%. The genetic relationships among the isolates were determined by Inter Simple Sequence Repeat (ISSR) technique Polymerase Chain Reaction (PCR), the results demonstrated a distinctive comparison of clusters and their pattern was greatly related to the clustering obtained with the morphological and biochemical identification. The selected lactic acid bacteria exhibited excellent probiotic characteristics and thus can be used as a potential source of probiotic.

Key words: probiotic, ISSR, β-galactosidase, antioxidant.

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1. Introduction

Lactic acid bacteria (LAB) are rod shaped, Gram-positive, non-spore forming and catalase-negative bacteria Lactobacillus produces circular, white and waxy colonies. They are obligate fermenters, fermenting glucose to lactic acid, ethanol and CO2, and can survive at a pH as low as 2.5. According to their morphology, LAB is divided into rods and cocci and according to the mode of glucose fermentation, to homofermentative and heterofermentative [1].

LAB provide several potential health and nutritional benefits, including improving the nutritional value of food, controlling gastrointestinal infections, improving digestion of lactose, controlling serum cholesterol levels, and controlling some types of cancer[2,3]. Reduce cholesterol level [4] alleviate lactose intolerance, stimulate the immune system, and also they may be able to stabilize the micro flora of the gut [5]. These health benefits

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derive from a diverse range of biological activities and mechanisms [6]. These various possible attributes of *lactobacilli* promote beneficial effects to human health. Thus, *lactobacilli* are now a focus of intensive research worldwide and new species are being reported. Probiotic strains of *lactobacilli* are used in different medical and health-related areas including the control of intestinal inflammation; treating infections during pregnancy; management of allergic diseases; control of antibiotic related diarrhea and prevention of urinary tract infections [7].

Microorganisms play an essential role in the food fermentations. LAB has been well known for centuries about their responsible mainly used in food preservation including dairy, meat, vegetables and bakery products due to their fermentative capacities and safety either separately or in combination with other conventional treatment. LAB has been isolated from several foods, including dairy products, meat products, plants, sewage, manure animals and also humans [8].

Conventional phenotypic and biochemical criteria often lead to ambiguous and time-consuming identification of the LAB. Therefore, the main focus for the identification has changed from phenotypic to genotypic methods because of more sensitive and accurate results, as reported for lactic acid bacteria by several authors. Molecular methods, such as protein pattern analysis, hybridization with specific DNA probes, 16s rRNA gene analysis, random amplified polymorphic DNA (RAPD) and Inter simple sequence repeat (ISSR) PCR have been used to replace classical identification methods However rRNA gene sequencing may not have enough accurate distinguish among closely related species[9]**.**

Inter simple sequence repeat (ISSR) technique is a PCR based technique which involves amplification of DNA segments between two identical microsatellite repeat regions 'oriented in opposite direction using primers designed from microsatellite core regions. The technique uses microsatellite primers, usually 16–25bp long, of di-nucleotide, trinucleotide, tetra-nucleotide or penta-nucleotide repeats to target multiple genomic loci. The primers can be either unanchored or more usually anchored at 3' or 5' end with 1 to 4 degenerate bases extended into the flanking sequences[10].

ISSR markers have many advantages over other marker systems. ISSR technique is simple, quick and less costly like the RAPD technique. ISSR markers have high reproducibility than RAPD primers due to the longer primer length. The studies on reproducibility showed that about 92–95% of the scored fragments could be repeated across DNA samples of the same cultivars and across separate PCR runs[11,12]This study aims to isolate and identify of lactic acid bacteria that exhibited excellent probiotic characteristics and thus can be used as a potential source of probiotic.

2. Materials and Methods

Isolation of lactic acid bacteria (LAB)

The LAB was isolated by dilution and plating from 4 different sources collected in different regions of Egypt. Briefly, 1 g of each sample was homogenized in 9 mL of sodium chloride (0.9%). Serial decimal dilutions were obtained and plated on *lactobacilli de Man*, Rogosa, Sharpe (MRS) and M17 agar media (Difco, Sparks, MD) and incubated in anaerobic (Co2 5%) conditions at 37°C for 72 h. Representative colonies of all morphologies were taken randomly and purified on the same media by sub culturing. The isolated bacteria were

examined using gram staining under a light microscope (Olympus BX 50, Japan) with a magnification of 100x.

Identification of the Bacterial Strains

Identification of the probiotic bacterial isolates was performed based on their morphological, physiological, and biochemical characteristics, as described in Bergey's Manual of Systematic Bacteriology [13].

Acid and Bile Salt Tolerance

The procedures of Tambekar D. et al.,[14] were used with some modification, to determine the tolerance of various strains to bile and acid. Isolates were inoculated into a suitable medium (MRS of varying pH, *i.e.*, pH 3, 5 and 7, as well as broth with varying concentrations of bile salt (0.5, 1.0, 1.5 and 2%) and incubated at 37°C for 24h. The culture tubes were observed for the presence or absence of growth in terms of turbidity, as an indication of microbial growth.

Temperature sensitivity

The selected bacterial cultures were grown at varying temperatures, i.e. 15, 37 and 45°C for 48hrs. Then 0.1 ml inoculum was transferred to MRS plates by pouring plate method and incubated at 37°C for 48 hrs. The growth of LAB on MRS agar plates was used to designate isolates as temperature tolerant [15].

NaCl tolerance

Salt tolerance of selected bacterial cultures was assessed after 48hrs of incubation at a concentration of 1-8% NaCl in MRS broth[16,17]the isolates were inoculated in MRS broth having different NaCl concentration (1.0%, 2.0%, 4.0% and 6.5%) and incubated at 37°C for 24h. The culture tubes were observed for the presence or absence of growth in terms of turbidity, as an indication of microbial growth.

β-galactosidase activity

β-galactosidase activity in whole cells was determined according to the method described by Miller J et al.,[18] involving the hydrolysis of substrate o-nitrophenyl- *β*-galacto pyranoside (ONPG) to o-nitrophenol (ONP) was used to measure *β*-galactosidase activity.

Antibiotic susceptibility

The susceptibility to antibiotics, which are commonly used by disc diffusion method [19] the culture (1%) in MRS soft agar (0.8% agar) was overlaid on MRS agar plate. An antibiotic disc was placed on it to allow the diffusion of antibiotics into the medium and then incubated at 37°C for 24 h. The inhibition zone around each antibiotic was measured to check the susceptibility of the culture.

Bile salts de-conjugation

The ability of the strains to de-conjugate bile salts was determined according to the method of Taranto M et al,.[20]. Bile salt plates were prepared by adding 0.5% (w/v) of sodium salts (Sigma) of taurodeoxycholic acid (TDC) to MRS agar autoclaved (121°C, 15 min) and immediately used. The isolates were screened for BSH activity by spotting 10µl aliquots of overnight cultures on MRS agar plates supplemented with 0.5% (w/v) sodium salt of taurodeoxycholic acid (TDCA*;* Sigma, USA)*.* Plates were incubated anaerobically at 37°C for 72h. The precipitation zone surrounding colonies indicated the bile salt hydrolase activity of bacteria*.*

Antioxidant activity

The antioxidant activity was determined by measuring the free radical scavenging

ability of the yogurt water extract using DPPH inhibition assay as described by Shetty S. et al.,[21].

Anti-cholesterol activity

Some natural microorganisms in the intestine are known to be beneficial in terms of lowering serum cholesterol [22].

Inter-simple Sequence Repeat (ISSR) PCR for the four lactic acid bacteria:

DNA extractions

Genomic DNA was extracted from the probiotic strain that grown on MRS broth (MRS, Difico) at 37°C under anaerobic condition Co2 5% for 24 hrs. The overnight culture (freshly prepared) was subjected to Inter-simple Sequence Repeat Approach (ISSR) PCR partial amplification by use protocol of Qiagene genomic DNA purification kit.

Primer sequences

The following is a set of commonly used dinucleotide primers, which work for various groups.

Table 1. List of inter-simple sequence repeat (ISSR) primers

List of the inter-simple sequence repeat (ISSR) primers		
NO	**PRIMER NAME**	**PRIMER SEQUENCES**
8	ISSR-1	5'-(AC)8 G-3'
9	ISSR-2	5'-(AC)8T-3'
10	ISSR-3	5'-(AC)8C-3'
11	ISSR-4	5'-(AG)8C-3'
12	ISSR-5	5'-(CT)8G-3'

Thermal cycling

The following amplification program is the general program used for running ISSRs 94°C for 4 minutes; 35 cycles of: 94°C for 50 seconds, 45°C for 1min., 72°C for 1 mint 72°C for 10 mint and 4°C hold forever. After the PCR assay, the PCR products were run on 1.2% agarose gel (Sigma) in 1x Tris-Borate-EDTA (TBE). About 10µl PCR products were loaded into sample wells and voltage at 100 volt was used for 1 hour. The gel was stained with ethidiumbromide (0.5 µg/ml) solution for 1 min and de-stained in water for 30 min. The gel was visualized under UVtrans-illuminator and photographed by Gel documentation system.

Data analysis

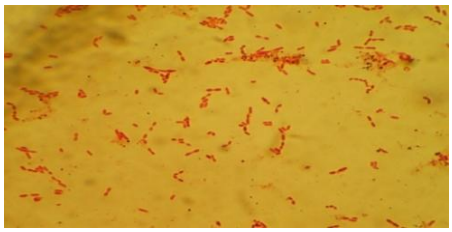
Banding patterns for each isolate, as well as for the reference strain, were recorded in a binary code: 1 (positive) and 0 (negative). Analysis of the binary scores was performed by using the biostatistical program, NTSYS-pc. A similarity matrix was calculated using the simple matching coefficient and cluster analysis of the matrix data was performed using the unweighted pair-group method arithmetic average (UPGMA).

3. Results and Discussion

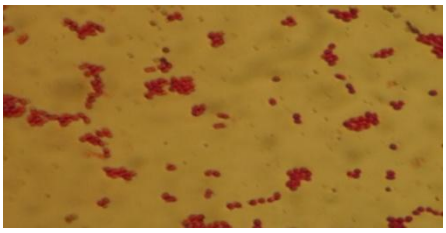
Isolation of lactic acid bacteria (LAB)

A screening procedure was performed to select a potential probiotic strains from milk and milk products. Four bacterial isolates were isolated and purified. The isolates were selected randomly based on their

difference in colony morphology, color, texture and margin. From Figure 1, three isolates were cocci but only one isolate was bacilli.



Bacilli



Cocci

Figure 1. Microscopic examination of the selected probiotic bacteria

The probiotic isolates were identified by colonial, morphological and biochemical characteristics. The colonial, morphological and biochemical characteristics of probiotic isolates are presented in the Table 2.

The characteristics of LAB belonging to genus *Lactobacillus* should be gram positive, rod shaped, non-endospore forming, catalase negative, must be acid producing and gas formation may or may not be there from sugars and moreover probiotic should also be pH, temperature, NaCl, bile salt tolerant and lactic acid producers from sugar. In the present study all the isolated bacterial cultures match the characteristics of the genus *Lactobacillus* as they are Gram positive, mostly rod shaped cells, non endospore forming, catalase negative, acid producing but non gas forming and were able to withstand a varying range of pH, temperature NaCl, bile salt tolerant and lactic acid producers from sugar. So, this was confirmed that isolates resembled the characteristics of the genus *Lactobacillus* as described by Holt J et al.,[23].

Table 2. Morphological and biochemical characterization of the selected isolates

Characteristics	isolateA	isolate B	isolate C	Isolate D
Colony color	Creamish	White	white	Creamish white
Colony shape	Circular and large	Circular	Circular and compact	Circular
Shape and arrangement	Cocci	Cocci	Rods with rounded ends	Cocci, streptococci
Gram stain	G+ ve	G+ ve	G+ ve	G+ ve
Catalas test	-	-	-	-
Oxidase test	-	-	-	-
Spore forming	-	-	-	-
Citrate utilization	-	-	-	-
Nitrate reduction	-	-	-	-
Starch hydrolysis	-	-	+	-
Casein hydrolysis	-	-	+	-
Gas from glucose	+	+	-	+

Different isolates showed different types of sugar utilization patterns including (glucose, fructose, galactose, lactose, sucrose, mannitol, sorbitol and maltose). Table 3 explained that all isolates able to ferment all monosaccharaides such as glucose, fructose and galactose and turned yellow color indicating positive. On the

other hand some selected isolates have the ability to utilize sugar alcohol such as (mannitol and sorbitol) and disaccharides and other not utilized. Where some tubes containing isolates and sugar turned yellow whereas the other remained brown colored, indicating the positive and negative tests, respectively for sugar fermentation.

Table 3. Sugar Fermentation Pattern of the Probiotic Isolates.

Carbohydrate fermentation	Isolate A	Isolate B	Isolate C	Isolate D
Glucose	+	+	+	+
Fructose	+	+	+	+
Galactose	+	+	+	+
Lactose	+	+	-	+
Sucrose	+	+	+	+
Mannitol	+	-	-	-
Sorbitol	-	-	-	-
Maltose	+	+	-	+

Table 4. Physiological characterization of the selected isolates

Characteristics	Growth at Temperature			Effect of NaCl %				Effect of bile salt %				Effect of PH				
	15°C	37°C	45°C	2	4	6.5	8	0.5	1	1.5	2	7	5	4	3	2
Strain A	-	+	+	++	+	-	-	+	+	-	-	++	+	+	-	-
Strain B	+	+	-	++	+	+	-	+	+	+	-	++	+	+	+	-
Strain C	+	+	-	++	+	+	-	+	+	-	-	++	+	+	+	-
Strain D	+	+	+	++	+	+	-	+	-	-	-	+	+	+	-	-

In the present study, the selected LAB isolates were able to grow in pH 7.0, 5.0, 4.0, and 3.0 but were unable to grow at pH 2.0 and were also able to survive in 0.5, 1.0, and 1.5 but were unable to grow at 2.0 % bile salt concentrations. Tambekar D. et al., [14] reported that the three isolated excellent probiotic acid tolerance at pH 3.0 and bile salt tolerance at 2.0%. Tolerance to bile salts is a prerequisite for colonization and metabolic activity of bacteria in the small intestine of the host [24]**.** This will help *Lactobacilli* to reach the small intestine and colon and contribute in balancing the intestinal microflora[14].

As shown in table 4 all the LAB isolates were able to survive at temperature 37°C and but some of them (B and C) were unable to survived at 45°C. All isolate except isolate A were able to grow at low temperature 15°C. The temperature is an important factor, which can dramatically affect the bacterial growth. The reason for choosing this temperature the range was to detect whether the isolated cultures were able to grow within range of normal body temperature or not. As if the isolates were not able to survive within the selected temperature range then they would not have been able to survive in the human gut, which is an essential factor of probiotics to show their effectiveness. The results obtained were positive for growth at chosen temperature. According to Ibourahema C et al., [25] bacterial cells cultured with a high salt concentration could show a loss of turgor pressure, which would then affect their physiology, enzyme activity, water activity and metabolism.

In table 4 all the LAB isolates were able to withstand at NaCl concentration ranging from 2% to 6.5% except isolate A was unable to grow at 6.5% and 8% NaCl concentration but all the LAB isolates were unable to withstand at 8% NaCl concentration [26], the lactic acid bacterial isolates were able to tolerate 1-6.5% NaCl concentration, NaCl is an inhibitory substance, which may inhibit growth of certain types of bacteria. If the lactic acid bacteria were sensitive to NaCl then it would not be able to show its activity in the presence of NaCl so it was essential to test the NaCl tolerance of lactic acid bacterial isolates, whereas Hoque Z et al., [17] observed the NaCl (1-6%) tolerance of their *Lactobacillus* sp. isolated from yoghurts.

As shown in table (5) A, B, C and D isolates have good potential for application in functional foods and health-related products. To augment these results, the present study researched the antioxidant properties of these isolates. To elucidate the antioxidant activity of the 4 isolates, DPPH radical scavenging activities were determined as described in Table 5 shows the DPPH radical scavenging activity of the 4 isolates.

Table 5. Antioxidant activity, cholesterol assimilation and β-galactosidase activity of selected isolates of lactic acid bacteria

Characteristics	β-galactosidase activity u/ml	Antioxidant activity (%)	Cholesterol assimilation (%)
Strain (A)	07.16	66.34	24.30
Strain (B)	19.42	63.17	27.63
Strain (C)	16.71	83.20	18.71
Strain (D)	17.23	73.28	24.98

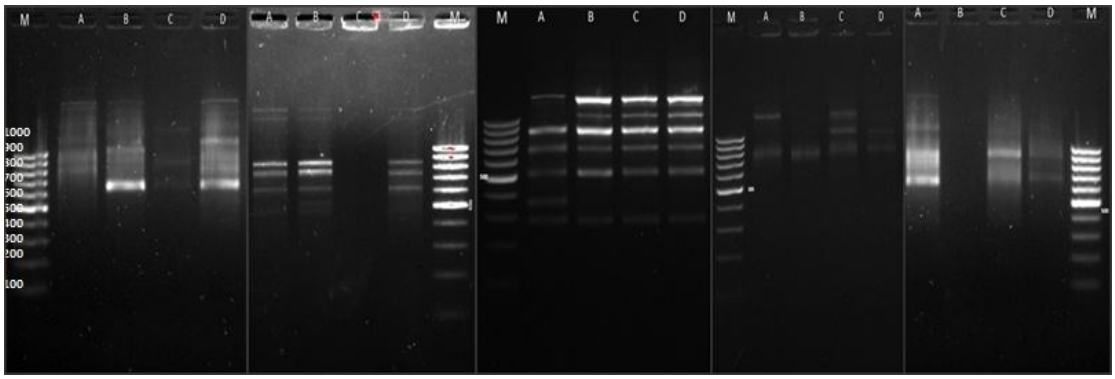
As shown in results in table (5) isolate C was the highest antioxidant activity (83.20%) as compared to all isolates while isolate B was (63.17%) the least antioxidant activity as compared to the control (MRS media without isolate). Bing S et al., [27] suggested that culture supernatant of Lactobacillus acidophilus contains anti-ulcer and anti-oxidative metabolites. Wang J et al., [4] reported that Lactobacillus rhamnosus GG culture supernatant ameliorated acute alcohol-induced intestinal permeability and liver injury [28] found that CFS of *Bifidobacterium* animals exhibited strong antioxidant activities (AAs). Wang J et al., [4] discovered that CFSs from 35 LAB exhibit stronger AAs than bacterial suspensions and cell-free extracts.

Table 6. Antibiotic susceptibility of the selected isolates

Antibiotic	Concentration (µg/disc)	Inhibition zone (mm)			
		A	B	C	D
Ampicillin	10	6	10	8	9
Rifampicin	5	16	R	4	R
Tetracycline	30	15	15	8	13
Chloramphenicol	30	18	12	12	11
Neomycin	10	3	8	13	8
Streptomycin	30	R	R	R	R
Kanamycin	10	R	4	R	R
Amikacine	5	4	R	R	6

All probiotic isolates were resistant to streptomycin and sensitive to tetracycline, chloramphenicol and neomycin. Cueto-vigil M et al., [29] found the sensitivity to tetracycline by most of enterococci samples isolated from cheese, such as the *enterococci* isolated from sheep milk in this work.

Antibiotic susceptibility pattern of selected LAB isolates was observed by using disc diffusion method the results as shown in Table 6. Isolate A was sensitive to tetracycline (15mm), Ampicillin (6mm), Amikacine (4mm) chloramphenicol (18mm), rifamycin (16mm). Streptomycin (12mm) and neomycin (12mm) but resistant to kanamycin. B showed sensitivity for tetracycline (15mm), Ampicillin (10mm), chloramphenicol (12mm), B1 showed resistance to streptomycin and kanamycin but was sensitive to tetracycline (13mm), chloramphenicol (10mm), neomycin (8mm) and Ampicillin (9mm). Such resistance to a wide spectrum of antibiotics indicated that if isolated probiotics induced in patients treated with antibiotic therapy may be helpful in a faster recovery of the patient due to the rapid establishment of desirable microbial flora.



Primer 1 primer 2 primer 3 primer 4 primer 5

Figure 2. Agarose (1.5%) gel electrophoresis of ISSR-PCR products for primers of isolates. Lanes 1: M, 100 bp ladder; lane 2: A, lane 3: B, lane 4: C, lane 5: D,

The ISSR patterns of four isolates A, B, C and D, the obtained with five primers are shown in Figure 2. Each primer resulted in a specific banding profile for all isolates. The number of DNA bands in these profiles ranged from 7 to 17. Although all primers have generated many common bands, sufficient numbers of polymorphic bands were obtained to detect diversity among the test isolates. Cluster analysis of the banding patterns revealed two tightly clustered groups (Figure 3).

The isolates were classified for the dendograme into four pool clusters (figure 3). The clusters(1 and 2) cluster 1 include isolates (A,B and D), cluster 2 include only isolate C, this cluster is so far from cluster 1, they have genetic similarity of approximately 76%. In the contrast, pools A, B and D with very close distance between them, Isolates B and D (about 90%), Isolates A and D about (77%).

Table 7. Similarity percentages between isolates A, B, C and D.

	A	**B**	**C**	**D**
A	**1.00**			
B	0.72	**1.00**		
C	0.56	0.51	**1.00**	
D	0.77	0.90	0.66	**1.00**

Dendrogram produced after numerical analysis of the Inter-simple Sequence Repeat (ISSR)profiles of isolates using the DICE coefficient and unweighted pair group me-thod with the arithmetic averages algorithm (UPGMA) is shown in Figure 3. Nu-merical analysis revealed clearly two distinct clusters at a similarity level of 76% as shown in the dendrogram. Cluster 1 comprised isolates (A, B and D). Cluster 2 included isolate C. The similarity level of members of the cluster 1 changed between 77 and 90%. The isolates of the cluster 2 were clearly separated from cluster 1 by numerical analysis this cluster is so far from cluster 1; they have genetic similarity of approximately 76%.

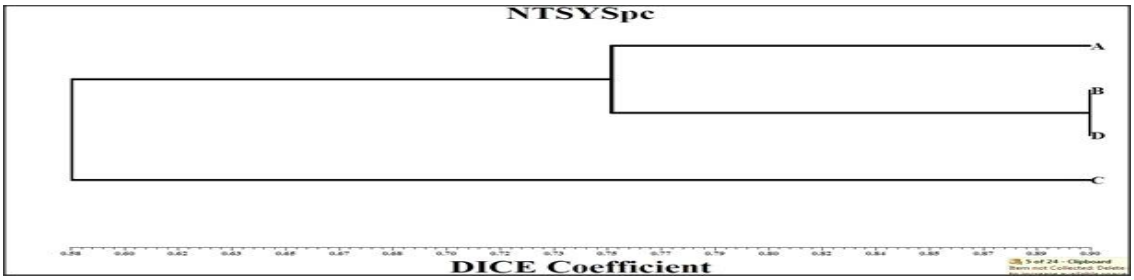


Figure 3. dendrogram based on unweighted pair group method with arithmetic averages algorithm (UPGMA) of Inter-simple Sequence Repeat (ISSR) profiles of isolates.

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