Original Research Article

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Genotypic and phenotypic characterization of lactobacillus acidophilus isolated from milk products.

Doa. A. Abd-Allah [1], Sotohy A. Sotohy [2], Ehsan A. Hassan [3] Abeer A. Mewafy [1]

[1]Department of Microbiology, Faculty of Veterinary Medicine, New Valley University, El-Kharga, Egypt
[2]Department of Animal and Environmental Hygiene, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt
[3]Department of Medical Microbiology and Immunology, Faculty of Medicine, Assiut University, Assiut, Egypt.
*Corresponding author: abeer112004@yahoo.com
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ABSTRACT: This study aimed to isolate and genetically investigate the l. acidophilus. We collected two hundred milk product samples (130 from Assiut and 70 from New Valley) from different sources including yogurt (90), rayeb (80), and milk powder (30). Lactobacillus are isolated on MRS agar and the resulting colonies were purified by repeated subculture and preliminarily identified as Lactobacillus sp. based on Gram staining, morphology and biochemical tests. Complementary studies on aspects like the ability to grow at different temperatures and resistance to inhibitory substances such as bile, NaCl 4% and 5.5% were performed. Then, Genotypic investigation for isolation of L. acidophilus. The occurrence of lactobacillus in New Valley was 22.85% and 25.71% of yogurt and rayeb respectively and the occurrence of Lactobacillus in Assiut was 18.46%,16.15% and 6.92% of yogurt, rayeb and Milk powder respectively. After the genotypic characterization of these isolates, nineteen L. acidophilus have been isolated.

KEYWORDS: *L.acidophilus*, Yogurt, Rayeb, Milk powder.

1. Introduction

Probiotics are live bacteria or yeasts that when given in sufficient proportions provide a health benefit to the host [1]. The representative species of probiotics belong to Lactobacillus and Bifidobacterium. Lactobacilli are naturally present or added in raw milk and dairy products such as yogurts, cheese and fermented milk due to their health benefit to the customer[2]. Lactobacillus species most frequently found in dairy products are L.acidophillus. This is because their therapeutic benefits include improvement in intestinal disorders and lactose intolerance, altered vitamin content of milk, antagonist against various pathogenic organisms and anti-carcinogenic activities. These bacteria are widely used in the production of fermented foods and beverages and contribute to both the sensory qualities of the food and the prevention of spoilage [3].

2. Materials and methods:

2.1. Preparation of samples [4]

Preparation of pasteurized yoghurt, rayeb milk, cereals, and milk powder for isolation of Lactobacillus. For Lactobacillus counting and isolation tenfold serial dilutions up to 1011were prepared by mixing 1gm of the sample with 9 ml peptone physiological saline solution.

2.2. Isolation of Lactobacillus:

2.2.1. Counting and isolation of Lactobacilli [5]

15 - 25 ml sterile (45 - 50°C) De Man Rogosa Sharpe (MRS) agar was poured into sterile Petri dishes containing 1 ml of the diluted test sample. The inoculum was distributed throughout the medium by rotating the plate in one direction and then in the reverse direction. The medium was allowed to solidify on a flat surface for 5 - 15 minutes and incubated at 37°C for 40 - 42 hours in a 10% Co2 incubator. Colonies appear as white smooth and convex with regular edges varying in size 1-5 mm. The colonies were counted, and the number of cfμ/g was determined. The isolated strains were kept in MRS broth

plus 20% glycerol at - 20 °C and sub-cultured every six months, another copy of the strains was kept on MRS agar slants at 4 °C and sub-cultured every 4 weeks (Shruthy et al., 2011). Isolates were examined for general characters of Lactobacilli. The isolates were Gram-positive, catalase-negative, non- motile, non- spore-forming rods and indole negative.

2.3. Identification of the isolates

2.3.1. Morphological identification by Gram stain [4]:

A film was prepared from a pure culture of each isolate, stained by Gram stain and examined microscopically. Lactobacillus are Gram positive, (non) motile and non-spore forming rods.

2.3.2. Motility test [6]

Tubes of semisolid medium were inoculated with a pure culture of suspected isolates by stabbing to a depth of approximately 2 cm with a bacteriological needle. After overnight incubation at a suitable temperature, motility was evident as a haze of growth extending into the agar from the stabbing line. Growth of non-motile organisms is restricted only to the stabbing line.

2.3.3. Biochemical identification and Biotyping:

A-Indole production test [7]

Tryptone water tubes were inoculated with a pure culture of the tested organism. Then were incubated at 35°C for 18-24 hours, and then drops of Kovac's reagent were added down the inner wall of the tubes. The development of a bright red color within seconds after adding the reagent indicated a positive reaction while no change in the broth indicated a negative result. Lactobacillus was indole negative.

B-Catalase test [8]

This test demonstrated the production of catalase, an enzyme that catalysis the release of oxygen from hydrogen peroxide. A few drops of 3% hydrogen peroxide solution were placed on a clean microscopic slide and the growth of the organism was removed and immersed in the hydrogen peroxide solution. Immediate bubbling indicates positive results. Lactobacillus was catalase negative.

C. Oxidase test [8]

The oxidase test was used to assist in the identification of the organisms producing cytochrome oxidase enzyme. The oxidase disc saturated with 1% tetramethyl- p. phenylene diamine dihydrochloride was laid in a Petri dish and moistened with distilled water. The colony to be tested was picked up and smeared over the moist area. A positive reaction was indicated by an intense deep-purple color appearing within 5-10 seconds and a negative reaction was indicated by the absence of coloration or coloration later than 60 seconds. Lactobacillus was oxidase negative.

D. Carbohydrate fermentation for Lactobacillus [9]

Fermentation of carbohydrates was determined in MRS broth containing phenol red (0.01 g per L) as a PH indicator. The medium is autoclaved at 121°C for 15 min. After autoclaving 1ml of different types of sterile sugar solution (10% concentration) arabinose, fructose, lactose, mannose and xylose). Then the sugar media were distributed into the different tubes. Then 200 ul of an overnight bacterial culture was inoculated into broth medium and incubated anaerobically at 37 °C for 24 hours. The appearance of a yellow color indicated sugar fermentation.

2.4. Properties of probiotic isolates:

2.4.1. Growth at Different Temperatures for Lactobacillus [10]

Temperature test media were MRS containing bromocresol purple indicator (0.04 gm per liter). They were prepared and transferred into tubes as 5 ml. Then fiftyμL of overnight incubated cultures were inoculated into tubes and incubated for 7 days at 15 °C and 45 °C. During the incubation time cell growths at any temperatures was observed by the change of the indicator color from purple to yellow.

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2.4.2. *Growth at Different NaCl Concentrations for Lactobacillus [10]*

Isolates were tested for their tolerance for different NaCl concentrations. For this purpose, 4% and 6.5% NaCl concentrations were selected. Test mediums containing bromocresol purple indicator (0.04gm per liter) were prepared according to the appropriate concentrations and transferred into tubes in 5 ml volume. These tubes were inoculated with 1% overnight cultures and then incubated at 37 °C for 7 days. The change of color from purple to yellow was proof of cell growth.

2.4.3. *Hemolytic Activity [11]*

To determine bacterial hemolytic activity, blood hemolysis was evaluated on Columbia blood agar supplemented with 5% sheep blood. Each bacterial suspension was streaked in the blood agar plates. After 24 h incubation at 37°C, the plates were examined for signs of β-hemolysis (clear zones around colonies), α-hemolysis (a green zone around colonies) or γ-hemolysis (no halo around colonies). Lactobacillus was non hemolytic.

2.4.4. *Bile tolerance [12]:*

MRS broth was prepared with different concentrations of bile salts at 0, 0.1, 0.3, 0.5 and 0.7 % and dispensed in 10 ml volume test tubes and sterilized. Freshly prepared cultures (20 µL) were inoculated into MRS broth and incubated at 37°C for 24 hr under anaerobic conditions. Optical densities (OD) were Spectro-photometrically measured at 620 nm against uninoculated broth after 0, 2, 6, and 24 h. So:

$$\text{Percentage of resistance} = \frac{\text{Increment of OD in MRS broth with bile}}{\text{increment of OD in MRS broth without bile}} \times 100$$

Strains showing resistance percentage at the value of 0.3% of bile more than 50% were considered as bile resistance strains.

2.5. *PCR assay for detection of L, acidophilus:*

2.5.1. *Extraction of DNA*

- A few isolated colonies of overnight growth bacteria were suspended thoroughly in 300 µl sterile deionized water.
- The suspension was boiled in a water bath for 10 min.
- It was centrifuged at 4000 rpm for 17 min.
- The supernatant was taken as a template and stored at 20° C.

Table 1: Oligonucleotide primers sequences:

Gene	Sequence	Amplified product	Reference
L. acidophilus 16S-23S rRNA	CCT TTC TAA GGA AGC GAA GGA T ACG CTT GGT ATT CCA AAT CGC	129 bp	Kim *et al.*, 2020

2.5.2. *Preparation of PCR Master Mix :*

Table 2: Preparation of PCR Master Mix according to Emerald Amp GT PCR master mix (Takara)Code No.RR310Akit :

Component	Volume/reaction
Emerald Amp GT PCR master mix (2x premix)	12.5 µL
PCR grade water	5.5 µL
Forward primer(20 pmol)	1 µL
Reverse primer (20 pmol)	1 µL
Template DNA	5 µL
Total	25 µL

2.5.3. *Cycling conditions of the primers during PCR*

Temperature and time conditions of the two primers during PCR are shown in Table (3).

Table 3: Cycling conditions during PCR:

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
16S-23S rRNA	94°C 5 min.	94°C 30 sec.	60°C 30 sec.	72°C 30 sec.	35	72°C 7 min.

2.5.4. *DNA Molecular weight marker*

The ladder was mixed gently by pipetting up and down.10 µL of the required ladder were directly loaded.

2.5.5. *Agarose gel electrophoreses [13] with modification*

Electrophoresis grade agarose (1 g) was prepared in 100 ml TBE buffer in a sterile flask, it was heated in microwave to dissolve all granules with agitation, and allowed to cool at 70°C, then 0.5 µg/ml ethidium bromide was added and mixed thoroughly. The warm agarose was poured directly in gel casting apparatus with desired comb in apposition and left at room temperature for polymerization. The comb was then removed, and the electrophoresis tank was filled with TBE buffer. Twenty µL of each uniplex PCR product, negative and positive control were

loaded to the gel. The power supply was 1-5 volts/cm of the tank length. The run was stopped after about 30 min and the gel was transferred to UV cabinet. The gel was photographed by a gel documentation system and the data was analyzed through computer software.

3. Results

3.1. Isolation:

3.2. Assiut samples:

A) Yogurt samples:

The highest percentage of lactobacillus spp. are obtained from type A (100%) and type D (100%). The lowest percentage is present in type B (0%) and Type E (0%).

Table 4: Frequency of distribution of lactobacilli spp. in the examined pasteurized yogurt samples:

Source of samples	Number of yogurt samples	Positive *lactobacillus* samples	
		No.	%
A	10	10	100%
B	10	0	0%
C	10	4	40%
D	10	10	100%
E	10	0	0%
Total	50	24	48%

B) Rayeb samples:

The highest percentage of lactobacillus spp. is obtained from type A (100%) and type C (100%). The lowest percentage is present in type B (0%) and Type E (0%).

Table 5: Frequency of distribution of lactobacilli spp. in the examined rayeb samples:

Source of samples	Number of rayeb samples	Positive lactobacillus samples	
		No.	%
A	10	10	100%
B	10	0	0%
C	10	3	30%
D	20	8	40%
Total	50	21	42%

C) Milk powder samples:

The highest percentage of lactobacillus spp. is obtained from type B (70%). The lowest percentage is present in type A (30%).

Table 6: Frequency of distribution of lactobacilli spp. in the examined milk powder samples:

Source of samples	Number of milk powder samples	Positive *lactobacillus* samples	
		No.	%
A	10	3	30%
B	10	2	20%
C	10	4	40%
Total	30	9	30%

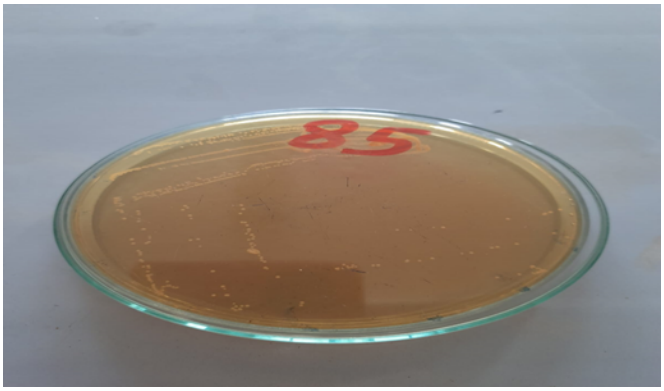

Figure 1: White colonies of Lactobacillus on MRS agar.

3.3. New valley samples:

A-Yogurt samples:

B-Rayeb samples:

3.4. Counting of lactobacillus:

According to the data in table (16): In Assiut: the high average lactobacillus count was found in yogurt samples (2.66x109±2.8x108 cf µLml). But In New Valley, the High average lactobacillus count was found in rayeb (9.20x1012±3.8x1011).

3.5. Identification of the isolates

3.5.1. Morphological identification by Gram stain:

Lactobacillus are Gram-positive, rod shape, (non) motile and non-spore forming.

3.5.2. Motility test:

Lactobacillus are non motile

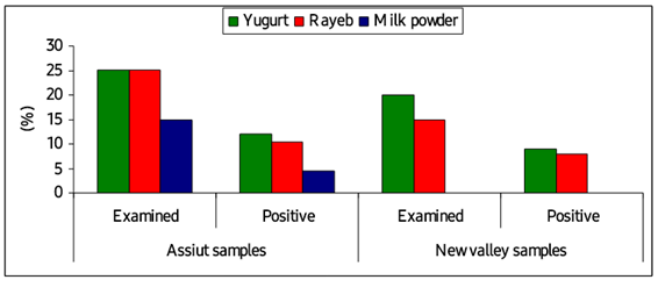

Figure 2: Percentage of all positive samples

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Table 7: Frequency of distribution of lactobacilli spp. in the examined yogurt samples:

Source of samples	Number of yogurt samples	Positive lactobacillus samples	
		No.	%
A	20	10	50%
B	20	8	40%
Total	40	18	45%

Table 8: Frequency of distribution of lactobacilli spp. in the examined rayeb samples on MRS:

Source of samples	Number of yogurt samples	Positive lactobacillus samples	
		No.	%
A	30	16	53.33%
Total	30	16	53.33%

3.5.3. Biochemical identification and Biotyping

3.5.4. a-Indole production test

Lactobacillus are indole negative

3.5.5. b-Catalase test

Lactobacillus are catalase negative

3.5.6. c-oxidase test

Lactobacillus are oxidase negative.

3.5.7. d-Carbohydrate fermentation for Lactobacillus

Lactobacillus acidophilus is positive only for fructose, lactose and mannose

3.6. Properties of probiotic isolates

3.6.1. Growth at Different NaCl Concentrations for Lactobacillus

3.6.2. Growth at Different Temperatures for Lactobacillus

Lactobacillus was non hemolytic.

3.6.3. Bile tolerance

3.7. Genotypic characterization of L.acidophilus species by PCR

A total of 77 positive lactobacillus samples were examined by PCR, out of them 19 L .acidophilus with a percentage of 40.5% as shown in the Table 15.

4. Discussion

Out of 90 yogurt samples, only 42 (46.67%) of samples contained lactobacilli sp.. These results were similar to Bhattacharya and Das (2010) 50%, Shruthy et al., (2011) 50% and Samuel et al., (2016) 36.98%. This is higher than [14] 6.9% and [15] 11%. And lower than [16] 62.30% and [17] A 82.6%. Out of 80 rayeb samples, 36(46.25%) of samples contained lactobacilli spp. The result of the examined rayeb milk samples was higher than [18] 30% and [19]. This lower than [20] 68.1% All isolated lactobacilli have the ability to grow at 4.4% NaCl concentration similar to [21, 22]. About 77.27% had tolerance against 5.5% NaCl concentration and this agreement with [23, 24] The result in table (9) showed that lactobacillus count in Assiut Yogurt was 2.66x109±2.8x108, Rayeb was 2.59x109±2.9x108 and Milk powder was 1.90x10±4.0x10.In New Valley, the lactobacillus count in yogurt was 3.57x1012±2.50x1011and in rayeb was 9.20x1012±3.8x1011. The lactobacillus counts in yogurt

Table 9: Percentage of all positive samples:

Type of sample	Total sample No.	Positive *lactobacillus* samples				P-value for
		Assiut samples		New valley samples		
		No.	%	No.	%	
Yugurt	**90**	**24**	**12.00^{aA}**	**18**	**9.00^{aB}**	**Product = 0.014**
Rayeb	**80**	**21**	**10.50^{aA}**	**16**	**8.00^{aA}**	**Place = 0.031**
Milk powder	**30**	**9**	**4.50^{bA}**	**0**	**0^{bB}**	
Total	**200**	**54**	**27.00**	**34**	**17.00**	

a, b& c: There is no significant difference (P>0.05) between any two means, within the same column have the same superscript letter. A & B: There is no significant difference (P>0.05) between any two means, within the same row have the same superscript letter.

Table 10: Comparison between counting of lactobacillus in Assiut and New Valley

	Assiut			New Valley	
	Yogurt	**Rayeb**	**Milk**	**Yogurt**	**Rayeb**
Mean	$2.66x10^{9}$ $\pm2.8x10^{8}$	$2.59x10^{9}$ $\pm2.9x10^{8}$	1.90x10 ±4.0x10	$3.57x10^{12}$ $\pm2.50x10^{11}$	$9.20x10^{12}$ $\pm3.8x10^{11}$
Minimum	$3.10x10^{9}$	$3.1x10^{9}$	0.6x10	$1.60x10^{12}$	$3.0x10^{12}$
Maximum	$5.72x10^{9}$	$5.28x10^{9}$	3.0x10	$4.56x10^{12}$	$3.76x10^{12}$
Range	$5.41x10^{9}$	$4.97x10^{9}$	2.4x10	$4.54x10^{12}$	$3.76x10^{14}$
Median	$2.80x10^{9}$	$2.53x10^{9}$	2.4x10	$3.150x10^{12}$	$3.0x10^{12}$

Table 11: Log number of total count:

Type of sample	Assiut	New Valley
Yogurt	9.35 ± 0.06^{aB}	13.91 ± 0.15^{aA}
Rayeb	9.33 ± 0.07^{aB}	12.22 ± 0.29^{bA}
Milk	2.48 ± 0.11^{b}	-

a, b & c: There is no significant difference (P>0.05) between any two means, within the same column have the same superscript letter. A & B: There is no significant difference (P>0.05) between any two means, within the same row have the same superscript letter.

Table 12: Percentage of fermentation of different sugar

Strains	Mannose		Fructose		Lactose		Arabinose		Xylose	
Lactobacillus	No.	%	No.	%	No	%	No.	%	No.	%
	87	98.86%	87	97.72%	78	88.63%	1	1.13%	0	0%

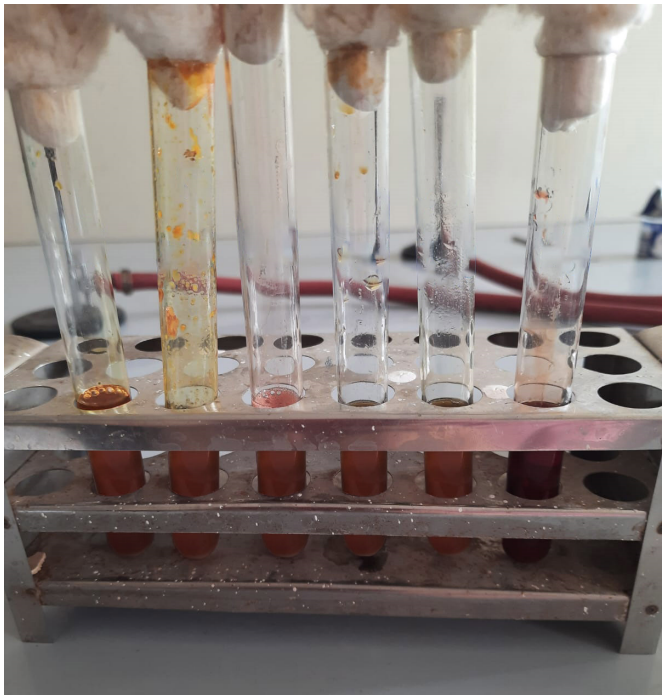

Figure 3: Carbohydrates fermentation test.

Table 13: shows that all strains of lactobacillus can grow in the presence of 4% NaCl, However, only a few strains resist to 5.5% NaCl.

Strains	NaCl%			
	4%		5.5%	
Lactobacillus	No.	%	No.	%
	88	100%	68	77.27

High salt tolerance is a desirable property for organism to be used as probiotics. It is known that NaCl is an inhibitory substance which may inhibit growth of certain types of bacteria.

Table 14: Tolerance of lactobacillus to temperature

Strains	Temperature			
	15°C		45 °C	
Lactobacillus	No.	%	No.	%
	60	68.18%	71	80.68%

Table 15: Effect of different bile salt concentrations on the growth of lactobacillus acidophilus strain:

Strains	Bile salt conc. (mg/dl.)	Time (h)				Surviving (%) after 24 h
		0	2	6	24	
L.acidophilus	0.0	0.101	0.450	1.254	2.810	100%
	0.1	0.137	0.471	1.203	2.737	97.40%
	0.3	0.142	0.367	1.142	2.670	95.01%
	0.5	0.128	0.313	0.940	2.434	86.61%
	0.7	0.112	0.292	0.720	2.101	74.76%

Figure 4: Positive isolates grow at 4% and 5.5 % NaCl.

Table 16: Percentage of L.acidophilus species in the isolated samples:

	Positive PCR (n=77)	
	No.	%
L.acidophilus	19	40.5

and rayeb agreed with [25] which reported that probiotic products should contain 106-107 cfu/g at the time of consumption. But milk powder is considered non-functional according to the number of lactobacilli. Also, our yogurt and rayeb samples agreed with [26] which recommended 109 probiotic bacteria/g in the probiotic to be functional. However lower results were obtained by Ebrahim,2017 who showed that the lactobacillus count in rayeb were 4.16×106, in yogurt were 4.55105× but higher results obtained in milk powder were 4.27 105×. There is a wide variation of growth at 15 and 45 °C. Only 68.18% grew at 15°C and 80.68% grew at 45°C and this result agreed with [21] but [24] said that all the selected LAB isolates were able to survive at temperatures 25, 30 and 37 and 40°C. Temperature is an important factor that can dramatically affect bacterial growth. The reason for choosing this temperature range was to detect whether the isolated cultures could grow within the range of normal body temperature. If the isolates were not able to survive within the selected temperature range, then they would not have survived in the human gut, which is an essential factor of probiotics to show their effectiveness. The growth of microorganisms including bacteria was greatly affected

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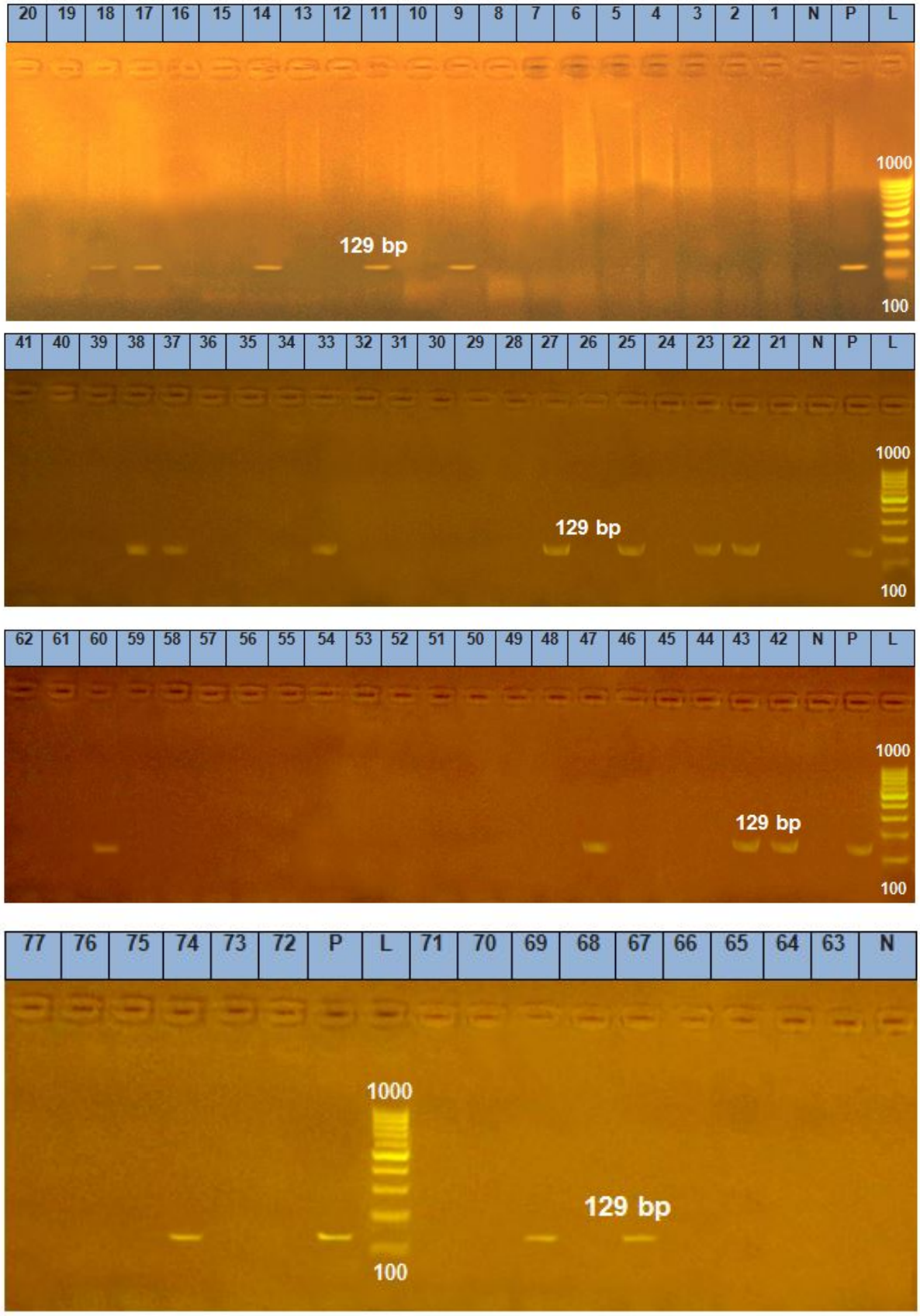

Figure 5: Genotypic characterization of L.*acidophilus* species by PCR

by the temperature of the environment. Lactic acid bacteria are mesophiles, growing at a temperature range of 10 – 45°C, with an optimum temperature of 30 – 40°C. Based on the result, it is suggested that these strains may be potential for use as probiotic organisms because all the isolates were resistant and able to grow in 0.3% bile salt concentration and this agreement with [27]. Also, a similar result was obtained by [28], who said that lactic acid bacteria were also able to survive in 0.5% bile salt concentrations. Similar to our study, other studies have reported that the isolated Lactobacillus strains indicate high bile salt tolerance with 88–92% survival rates [29]. A study by [30, 31] reported that the isolated Lactobacillus strains have a high tolerance in the presence of 0.3% bile salt. In contrast,[32, 32] demonstrated that the bile tolerance was 87.41%, 75.49%and 69.53% for the 0.1%, 0.3% and 0.5% of bile salt concentrations. In addition, Rajoka et al. 2018 demonstrated that the Lactobacillus isolates present a low level of bile salts tolerance with less than 50% survival rate in the presence of bile salts. A total of 77 positive lactobacillus samples were examined by PCR, out of them 19 L .acidophilus with a percentage of 40.5% as shown in the table(). Similar results 39.2 % were obtained by Ebrahim, 2017.But a different result 68.96% obtained by [33].

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