Isolation, Characterization and Identification of Lactic Acid Bacteria as Probiotic

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Abstract

Lactic acid bacteria that grow as the adventitious microflora of foods or that are added to foods as cultures are generally considered to be harmless or even an advantage for human health. The objective of this study was to isolate, screen and identify lactic acid bacteria from different sources. As well, evaluate the more potent lactic acid bacteria isolates for their probiotic characteristics. Ninety-six bacterial isolates were isolated from different 18 sources, 51 (53%) isolates were able to hydrolyze 0.5% bile salts while 28 isolates were able to hydrolyze 1% bile salts. Also, only 11 isolates were able to tolerate 2% bile salts after 6h incubation periods. On the other hand, lactic acid bacteria isolates were able to survive at pH 1.5 and 3.0 as well 8.0. Also, these isolates were potent for cholesterol assimilation, NaCl tolerance, exopolysaccharides production, antioxidant activity. Lactic acid bacteria isolate MS87 that exhibited excellent probiotic characteristics was identified using 16S rRNA sequence as *Pediococcus pentosaceus*. This study led to suggest that environmental-derived lactic acid bacteria-strains could be used as a potential source of probiotic.

Keywords: Lactic acid bacteria, probiotics, bile salts tolerance, antibiotic sensitivity, cholesterol assimilation.

Introduction

Lactic acid bacteria (LAB) have been exploited for centuries as probiotics to promote good human and animal health **(Bhattacharyya, 2009)**. They play important role in food fermentation, primarily by causing the characteristic flavor changes and contributing a preservative effect on the fermented product. These bacteria are widely used as starter cultures in most fermented foods such as dairy products **(Antara *et al*., 2009)**. However, some strains of LAB could be used as probiotic **(Verdenelli *et al*., 2009)**.

LAB used for centuries by man to preserve food, produce antagonistic compounds, including lactic acid, hydrogen peroxide and bacteriocins. Bacteriocins are antimicrobial peptides that are bactericidal toward bacteria taxonomically close to the producer. The specific actions of some bacteriocins toward foodborne pathogenic bacteria and undesirable flora like *Listeria* spp. and *Clostridium* spp. have increased the interest in these compounds **(Daba and Saidi, 2015).** LAB are safe microorganisms with abilities to produce different inhibitory compounds such as bacteriocins, organic acids, hydrogen peroxide, diacetyl and carbon dioxide. LAB can inhibit harmful microorganisms through competitive exclusion mechanism based on competition for binding sites and nutrients **(Vieco-Saiz *et al*., 2019).**

Probiotics are live non-pathogenic microorganisms which give benefits to human and animal health, such as induction of the immune system **(Hirayama and Rafter, 2000)**, prevention of pathogens infection and colonization in the gastrointestinal system **(Lawrence *et al*., 2005)** and modulation of the blood cholesterol level **(Martin *et al*., 2008)**. Several LAB have been used as probiotics in humans and animals **(Morelli, 2000)**. The probiotics criteria include the biosafety of the strains, ability to adhere to and colonize the epithelial cell surface of the gastrointestinal tract of the host and inhibitory activity against enteric pathogens **(Collins *et al*., 1998)**. The acid and bile tolerance as well are two fundamental properties that indicate the ability of probiotic microorganism for survival during passage through the gastrointestinal tract **(Erkkila and Petaja, 2000)**. **Somashekaraiah *et al*. (2019)** reported that LAB exhibited promising probiotic properties and seem favorable for use in functional fermented foods as preservatives. The aim of this study was to isolate, screen and identify LAB from different sources as well as evaluate the more potent probiotic LAB isolates for their probiotic characteristics.

Materials and methods

Collection of samples

Different fresh and preserved food products were collected from local markets in Qaluyobia Governorate, Egypt namely: milk (fresh, Nedo and Hero), rayeb (Johyena and Almarai); yogurt (Balady, Johyena, Danon, Lactel, Healthy, Activia, Nestle); ice cream; Salamy; Boza, Keshk, Serelak and baby feces during 2017. Samples were aseptically transported to the laboratory of Agric. Microbiology Dept., Fac. of Agric., Benha University.

Enrichment and isolation of LAB

Five grams of each sample was mixed with 100 ml of de Man Rogosa Sharp (MRS) broth medium **(de Man *et al*., 1960)** which containing (g/l): 10.0 proteose peptone No.3,10.0 beef extract, 5.0 yeast extract, 20.0 dextrose, 1.0 polysorbate 80, 2.0 ammonium citrate, 5.0 sodium acetate, 0.1

magnesium sulfate, 0.05 manganese sulfate, 2.0 dipotassium phosphate, 20.0 agar. Then, incubated at 37°C for 48 h. After that, 1.0 ml of each mixture was diluted to 10^7, then 0.1 ml of the suitable dilutions were spread onto the surface of MRS agar medium **(Jimenez *et al*., 2008)**. After incubation at 37°C, colonies with different morphologies were collected and purified on the same medium and stored at 4°C.

Survival under gastrointestinal tract conditions

Bile salt hydrolase activity

Two methods were used for determination of bile salts hydrolase activity. The first method by **Toit *et al*. (2003)** was as follows: 24 h old bacterial cultures were streaked on MRS agar supplemented with 0.5 % w/v bile salts (Ox Bile, HIMEDIA, India) and incubated for 48 h at 37°C. The hydrolysis effect was evaluated by partial hydrolysis in comparison to the control MRS dishes and recorded as + and – for positive and negative results.

The second method described by **Sedláčkovám *et al*. (2015)** was as follows: soft MRS agar (pH 5.6), bile salts (1.0 % w/v), and $CaCl_2$ (0.37 5 g/l), was used. Petri dishes were incubated at 37°C for 48 h. 10 µl of each isolate were inoculated on MRS soft agar by puncturing into the agar. Subsequent cultivation of the MRS agar media containing bacterial isolates was carried out at 37°C for 72 h. Visible halos indicate the positive bile salt hydrolase activity of the isolates.

Bile salt tolerance

The method of **Oloyede and Afolabi (2013)** was used to determine bile salt tolerance. MRS broth containing 0, 1.5 and 2.0 % (w/v) bile salts (HIMEDIA, India) was inoculated with 10^7 cfu/ml of each LAB isolate from their respective overnight growth culture after they were centrifuged at 10000 g for 5 min. and washed three times, then incubated for different intervals viz. 0, 2, 4 and 6 h at 37°C. One ml of each isolate was diluted to 10^{-7} and the number of cells were counted using pour plate method for resistance to bile salts. Survival rate (%) = log cfu/ml of treatment / log cfu/ml of control × 100.

Acid tolerance

The modified method of **Erkkila and Petaja (2000)** was applied in this study. Bacterial isolates grown in MRS broth at 37°C for 24 h. were collected by centrifugation at 5000 ×g for 15 min. Cell pellet was washed twice and resuspended into 10 ml of phosphate-buffered saline (PBS) with the pH values 1.5, 3.0 and 8.0 (adjusted using 5 *M* HCl) to achieve 10^3 CFU/ml. The tubes were incubated at 37ºC and the viable cells were counted after exposure to acidic condition for 2, 3, 4 and 5 h. on MRS agar incubated at 37ºC for 48 h. Survival rate (%) = log cfu/ml of treatment / log cfu/ml of control × 100.

Preliminary identification of isolates

Phenotypic characteristics (morphological, physiological and biochemical tests) were performed to identify the most potent isolates according to criteria of Bergy's Manual of Determinative Bacteriology. Shape, motility, Gram staining, catalase activity, growth at different temperature and pH, salt tolerance, sugar fermentation, citrate utilization, H_2S production, VR test, MR test, oxidase test and urease test were determined according to **(Holt *et al*., 1994).**

Probiotic characterizations

NaCl tolerance

For determination of NaCl tolerance of all tested isolates, four test tubes containing MRS broth were adjusted with different concentration (1.5-3-5-7%) of NaCl for each isolate with three replicates. After sterilization, MRS medium was inoculated individually with 1% (v/v) overnight culture (10^7 cfu/ml) and incubated at 37°C for 24 h. After that, the growth was determined by observing culture medium turbidity and recorded as slightly (+), moderately (++) and heavy growth (+++) **(Hoque *et al*., 2010)**.

Antioxidant activity

Preparation of cell free extract

MRS broth medium (pH 6.5±0.2) was individually inoculated with 1 % (v/v) of the over-night grown cultures and incubated at 37°C±0.2 for 24 h in shaking incubator (150 rpm). Cell free extract (CFE) was obtained by centrifugation at 10,000 rpm for 5 min at 4°C and kept at 4°C for non-enzymatic antioxidant assay.

DPPH free radical scavenging assay

2,2-DiPhenyl-2-Picryl hydrazyl hydrate (DPPH) free radical scavenging assay was measured as non-enzymatic assay using the method described by **Heo *et al*. (2006).** 500 µL of CFE, 3000 µl of a freshly prepared solution of DPPH at a concentration of 5 mg/100 ml ethanol was added. Control was prepared using 500 µl of ethanol added to 3000 µL DPPH solution, mixed and incubated for 30 min. in dark. Absorbance (As) was measured at 517 nm after 30 min. Ascorbic acid was used as a standard. The percentage of radical scavenging activity was calculated according to the following equations:

$$\%\ \text{Residual of DPPH after 30 min} = \frac{\text{As517 control} - \text{As517 sample}}{\text{As517 control}} \text{x } 100$$

$$\%\ \text{Inhibited of DPPH after 30 min} = \%\ \text{Residual of DPPH} - 100$$

Hemolytic activity

For hemolytic activity, the overnight grown MRS broth culture of each isolate was streaked on blood agar plate (Hi-Media, India) and incubated at 37°C for 48 h; after that, all plates were checked for the formation of any hemolytic zones viz. clear (β-

hemolysis) or greenish (α-hemolysis), or no hemolytic zone (λ-hemolysis) **(Borah *et al.*, 2016)**.

Antibiotic susceptibility assay

Antibiotic resistance/susceptibility of all isolates were estimated by disc diffusion method as described by **Kacem and Karam (2006).** Seven antibiotics *viz*. penicillin G (10 µg), neomycin (10µg), tetracycline (30µg), erythromycin (15µg), chloramphenicol (30µg), vancomycin (30µg) and gentamycin (10µg) were used in this experiment. MRS medium was individually inoculated with 200 µl of 24 h old cultures (10^7 cfu/ml) and allowed to stand at room temperature for 15 min and then the antibiotic discs were dispensed onto agar using disc dispenser under aseptic conditions, then incubated at 37°C for 24 h. Inhibition zone was measured (mm). Results were expressed as sensitive (S) or resistant (R) according to **Oloyede and Afolabi (2013)**.

Cholesterol assimilation

Cholesterol assimilation was done by the method described by **Searcy and Bergusst (1960)** as follows: bacterial isolates were grown in MRS broth supplemented with 0.3% bile salt (sodium thioglycolate, SRL). Further, 0.1g of cholesterol dissolved in 2.5 mL of ethanol was added to 10 mL of MRS broth with bile salt. The cultures were grown for 24 h at 37ºC. Cells were harvested by centrifugation at 8000 ×g for 10 min. Supernatant was collected and used for cholesterol assay. The uninoculated MRS broth was considered as a control. The concentration of cholesterol was determined using cholesterol standard graph as described by **Tomaro-Duchesneau *et al.* (2014)**. A standard curve of absorbance versus cholesterol concentrations was generated using the cholesterol concentrations: 0, 3.91, 7.81,15.63, 31.25, 62.5, 125, 250, and 500 μg/mL cholesterol in MRS (R^2 = 0.9928). The cholesterol assimilated by LAB isolates was determined using the following equations:

$$\text{Cholesterol assimilated } (\mu g/ml) = [\text{cholesterol } (\mu g/ml)]\ 0h - [\text{cholesterol } (\mu g/ml)]24h$$

$$\%\ \text{cholesterol assimilated} = \frac{\text{cholesterol assimilated } (\mu g/ml)}{\text{cholesterol } (\mu g/ml)\ 0\ h} x100\%$$

Genotypic identification of the potent probiotic isolates

The most potent isolates were completely identified using 16S rRNA sequence technique. The isolates were grown in MRS broth on a rotary shaker (120 rpm) at 30°C for 24 hours. Bacterial Gene Jet genomic DNA purification Kit (Thermo K0721) was used to extract DNA according to SIGMA company instructions. The universal 16S primers used were; forward primer: AGAGTT TGATCCTGGCTCAG and reverse primer: GGT TACCTTGTTACGACT T. The sequencing was performed in two direction using the previously described primers **(Doi *et al.*, 2013).**

Results and discussion

Isolation of LAB

Totally of 96 bacterial isolates were isolated from different sources and coded as MS (1-96). The largest number (12 isolates) were obtained from fresh milk, while the lowest number (2 isolates) obtained from Rayeb milk (Almarai) and yoghurt (Johyena). Same trend of results was recorded by **Weese *et al.* (2004)** who reported that LAB have adapted to grow under widely different environmental conditions, and they are widespread in nature. Apart from food sources, LAB are commonly found in the gastrointestinal tract of various animals and humans **(Tannock, 1995)**. Also, **Oloyede and Afolabi (2013)** isolated fifty LAB isolates from goat milks.

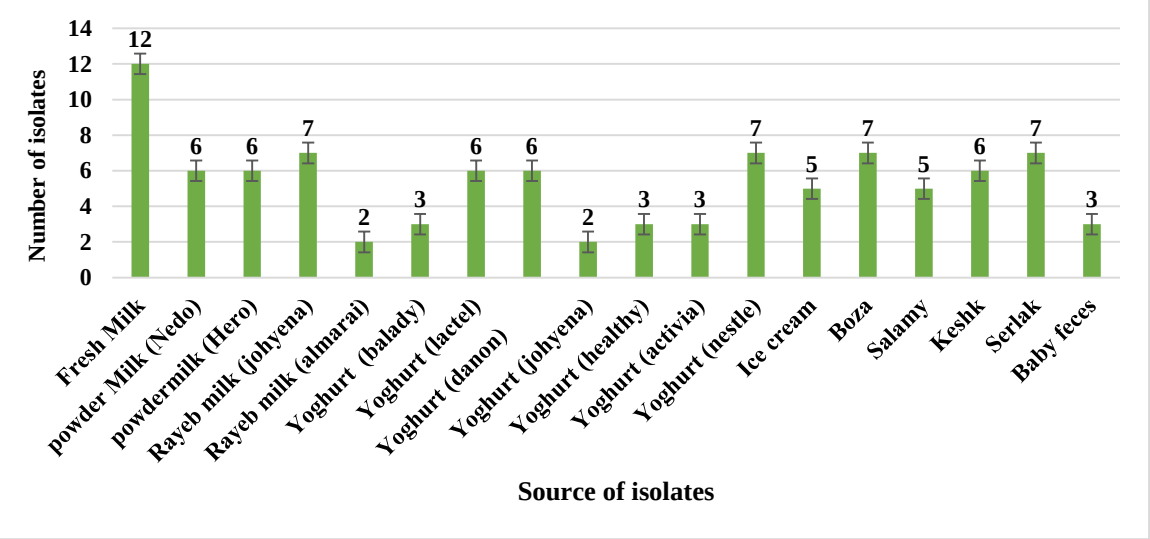

Fig. 1. Number of bacterial isolates from different sources

Survival under GIT conditions

Bile salts hydrolase activity

The ability of the isolated bacteria to hydrolase bile salts was done in two successive steps. In the first step, 51 of 96 (53%) isolates were able to hydrolase 0.5% bile salts while 45 isolates (47%) were not able to hydrolase bile salts at the same concentration **(Fig 2)**. On the other hand, the isolates which gave positive results in the first step were examined in the second step for hydrolase 1.0 % bile salts. Results in **Table (1)** and **Fig (2)** indicated that only 28 from 51 isolates (55%) were able to hydrolase 1% bile salts while, 23 isolates (45%) don't have this ability. The halo zone of hydrolysis was ranged from 1.0 to 5.5 cm. The highest zone was recorded by the isolate MS13 while, the lowest zone was recorded by the isolates MS47, MS55, MS68 and MS84. The bile salt hydrolase, which described in several LAB as probiotic organisms, are produced in the intracellular space. **(Negm El-Dein, *et al.*, 2017).**

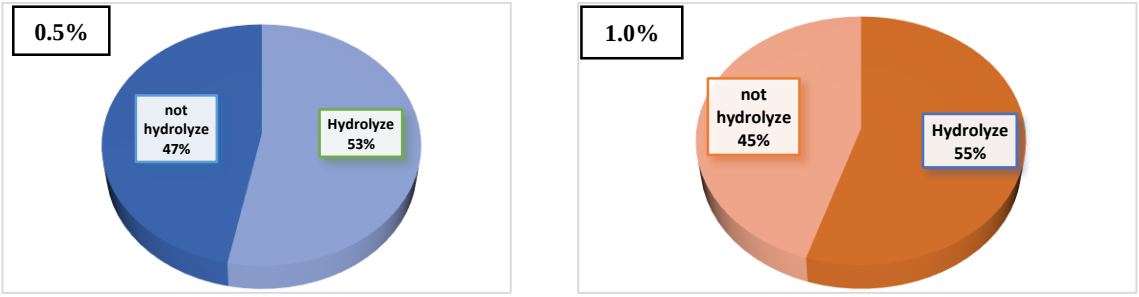

Fig. 2. Bile salts hydrolysis by bacterial isolates

Table 1. Bile salt hydrolase activity at 1%.

Zone (mm) of bile salt hydrolase activity	LAB isolates
< 1.0	MS (5, 9, 10, 14, 16, 20, 21, 32, 35, 39, 40, 41, 43, 45, 51, 56, 58, 69, 73, 74, 91,92,93)
1.0 - 2.5	MS (47 ,24 ,25 ,37 ,46 ,47 ,54, 55, 60, 62, 64, 65, 68, 90)
2.6 – 4.0	MS (8,18, 57 ,67)
4.0 - 5.5	MS (12, 13, 19, 44, 84, 71, 85, 86)
> 5.5	MS87, MS88

Bile salts tolerance

In this experiment two consecutive steps were done, the first was designed to examine the ability of LAB to tolerate 1.5% bile salts while, the second step was to examine the ability of 1.5% bile salt tolerated isolates to tolerate 2.0 % bile salts. Regarding the ability of the isolates to tolerate 1.5% bile salts, data presented in **Table (2)** indicated that the number of the viable cells were decreased with the increasing of the incubation period. Generally, the lowest number of viable cells of all isolates was recorded after 6 hours of incubation.

Table 2. Tolerance of the isolated bacteria for 1.5% bile salt.

LAB isolates	Survival rate (%) after incubation (h.)				LAB isolates	Survival rate (%) after incubation (h.)			
	Zero time	2	4	6		Zero time	2	4	6
MS 8	99.58	98.19	97.39	97.10	**MS 57**	92.79	0.0	0.0	0.0
MS 12	99.85	99.59	99.59	99.05	**MS 60**	96.48	93.63	0.0	0.0
MS 13	99.91	98.48	98.03	95.66	**MS 62**	99.16	99.89	99.47	99.05
MS 17	99.96	0.0	0.0	0.0	**MS 64**	99.41	98.21	98.21	98.21
MS 18	99.81	99.34	98.45	97.93	**MS 65**	99.88	99.80	98.67	98.28
MS 19	100.0	99.97	97.93	95.23	**MS 67**	99.57	99.57	99.55	99.64
MS 24	99.92	99.28	98.84	97.51	**MS 68**	88.97	0.0	0.0	0.0
MS 25	99.84	99.55	99.30	98.45	**MS 71**	99.77	99.46	98.96	96.77
MS 37	99.95	99.05	94.19	92.64	**MS 84**	99.14	99.05	98.63	97.62
MS 44	99.95	99.45	99.06	98.23	**MS 85**	100.0	98.42	97.45	96.22
MS 46	99.88	99.52	98.75	98.32	**MS 86**	99.98	98.92	98.72	97.82
MS 47	99.79	99.69	99.53	99.40	**MS 87**	99.83	99.34	99.00	98.08
MS 54	99.15	99.31	99.15	98.98	**MS 88**	98.86	98.51	98.42	98.18
MS 55	99.70	99.11	90.09	0.0	**MS 90**	100.0	99.49	97.53	94.19

Also, the isolates MS17, MS57 and MS68 didn't able to survive at 1.5% bile salts more than 2 hours. Additionally, the highest survival rate after 6 h. of incubation was recorded by the isolate MS12 followed by the isolate MS54. Similar results were showed by **Fungsin *et al*. (2010)** who proved that the number of survival strains at 4 h. had relatively high tolerance to bile salts up to1.5% (w/v).

Concerning the ability of the isolates to tolerate 2% bile salts, data in **Table (3)** showed that only 11 isolates were able to tolerate 2% bile salts after 6h incubation periods. Moreover, 6 isolates didn't able to tolerate 2 % bile salts at zero time. On the other hand, the highest values of survival rate were recorded by the isolate no. MS88 followed by MS24 then MS19. Generally, survival rate was decreased with the increasing of the incubation period. The selected 11 isolates were chosen to the further experiments. Bile salt tolerance is one of the prerequisites for any species of LAB when it is used as probiotics (**Badi and Bhat, 2017).**

Table 3. Tolerance of bacterial isolates for 2.0% bile salt.

LAB isolates	Survival rate (%) after incubation (h.)				LAB isolates	Survival rate (%) after incubation (h.)			
	Zero time	2	4	6		Zero time	2	4	6
MS 8	85.29	0.0	0.0	0.0	**MS 55**	91.61	90.39	0.0	0.0
MS 12	93.36	92.44	91.18	81.81	**MS 62**	0.0	0.0	0.0	0.0
MS 13	97.81	95.36	88.84	85.20	**MS 64**	0.0	0.0	0.0	0.0
MS 18	91.94	87.63	85.72	81.09	**MS 65**	0.0	0.0	0.0	0.0
MS 19	91.35	92.27	92.94	86.78	**MS 67**	0.0	0.0	0.0	0.0
MS 24	94.18	95.53	94.90	88.31	**MS 71**	88.95	85.77	93.54	73.99
MS 25	0.0	0.0	0.0	0.0	**MS 84**	88.39	86.93	80.83	75.67
MS 37	0.0	0.0	0.0	0.0	**MS 85**	80.79	0.0	0.0	0.0
MS 44	86.69	81.57	81. 57	81.57	**MS 86**	91.12	90.64	88.16	81.56
MS 46	89.49	0.0	0.0	0.0	**MS 87**	91.12	86.61	83.72	79.21
MS 47	89.44	85.82	77.92	74.70	**MS 88**	97.68	95.24	96.07	88.87
MS 54	90.51	86.41	0.0	0.0	**MS 90**	86.87	0.0	0.0	0.0

Tolerance of pH by bacterial isolates

As probiotics are usually administered orally, they must have the ability to survive passage through the stomach and small intestine. Therefore, resistance to the low pH of the gastric juice in the stomach and the small intestine is one of the major important properties of probiotic bacteria **(Yin and Zheng, 2005).** Results of preliminary screening of pH tolerance presented in **Table (4)** showed that most of the isolates have the ability to survive at all tested pH. At pH 1.5 only 20 isolates were able to survive after 5 h. while at the same time, 27 isolates and 24 isolates were able to survive at pH 3.0 and 8.0, respectively. Twenty and twenty-eight strains were characterized for their ability to grow under acidic conditions to ensure their survival through the upper digestive tract to reach the large intestine when they were exposed to pH 1.5 and3.0, respectively for 2, 3, and 5 h. It is known that the resistance of probiotics to low pH greatly varies depending on the species and strain. In general, these results are in agreement with others researchers, who showed that species of lactic acid bacteria are high acid resistant at pH 2.0 and 3.0 (The pH level of the gastric juice) and this resistance could be decreased on very low pH value (**Chang *et al*., 2012**). The pH of gastric juice can be increased in the presence of food components such as cheddar cheese and yoghurt **(Gardiner *et al*., 1999)**.

This protective effect of food components to probiotic bacteria may facilitate the passage of acid-sensitive probiotic strains from the stomach into the small intestine. Resistance to low pH is one of the major selections for probiotic strains**.** LAB are indigenous habitants of human gastro intestinal tract and thought to among the dominant colonies of the small intestine**.** To reach the small intestine, they have to pass through stomach. For selection of strain resistant to low pH 3.0 was used. The time that takes during digestion in stomach is 3 hours, so isolates were tested for resistance to pH 3.0 during 2-5 h.

Preliminary identification of lactic acid bacteria isolates

Eleven isolates that exhibited potential growth at low pH and bile salt 2% were preliminary identified based on their morphological, physiological and biochemical characterization (**Table 5**). Biochemical characteristics were compared with Bergey`s Manual of Determinative Bacteriology **(Holt *et al*., 1994).** All LAB isolates were Gram positive; seven isolates were cocci while four isolates were rods with long and rounded ends. No bubble was observed indicating that the isolated bacteria are catalase negative and could not mediate the decomposition of H_2O_2 to produce oxygen. All the isolates were not able to use citrate as the sole source of carbon and energy, negative for H_2S production, positive for methyl red test and negative for VP test except isolates MS12, MS24, MS44 and MS71. These are the common characteristics of LAB species. These findings are also similar to those reported by **Khagwal1, *et al*. (2019).**

Table 4. Tolerance of pH by bacterial isolates.

Isolate No.	Survival rate % at pH 1.5 after incubation (h)				Survival rate % at pH 3 after incubation (h)				Survival rate % at pH 8 after incubation (h)			
	2	3	4	5	2	3	4	5	2	3	4	5
MS 8	96.4	84.5	0.0	0.0	74.4	52	0.0	0.0	0.0	0.0	0.0	0.0
MS 12	99.7	99.7	99.7	99.6	99.7	99.6	99.6	99.6	99.8	99.7	99.6	99.6
MS 13	101.7	102.2	103.0	107.7	100.4	100.7	101	101.2	98.5	97.9	97.7	97.7
MS 17	98.7	94.3	0.0	0.0	98.9	98.0	97.3	83.0	99.3	94.0	92.9	60.0
MS 18	92.9	91.6	83.7	67.6	100.6	101.6	101.9	102.3	105.6	105.9	106	106
MS 19	99.4	99.4	98.4	98.2	100	100.9	100.9	101.9	100.3	100.6	100.9	102.3
MS 24	93.8	89.2	81.5	76.5	100.2	100.5	100.6	100.6	100.1	100.2	100.4	100.5
MS 25	100.8	101.4	101.7	101.9	100.4	101.1	101.4	101.5	100.0	100.0	100.1	100.1
MS 37	95.0	90.1	82.3	0.0	100.4	101.4	101.8	101.9	100.5	101.1	101.3	101.5
MS 44	100.7	101.1	101.5	101.8	100.0	100.0	100.2	100.3	100.2	100.3	100.4	100.5
MS 46	100.0	100.0	99.9	99.9	100.0	99.8	99.8	99.8	100.0	100.1	100.3	100.1
MS 47	100.3	100.5	100.6	100.8	99.9	99.9	99.9	99.9	98.9	98.9	97.8	97.1
MS 54	97.0	81.5	0.0	0.0	99.4	96.9	94.1	84.2	0.0	0.0	0.0	0.0
MS 55	99.4	98.9	98.9	98.9	100.4	100.7	100.9	101	100.4	100.9	101.4	102.8
MS 57	0.0	0.0	0.0	0.0	100.5	100.7	101.2	101.7	0.0	0.0	0.0	0.0
MS 60	91.7	93. 5	95.3	95.9	100.6	101.1	102.4	102.5	100.3	100.5	101	101.4
MS 62	90.9	82.1	0.0	0.0	100.4	99.5	100.0	100.2	99.9	100.0	100.0	9.99
MS 64	96.7	94.5	94.2	93.7	100.1	100.3	100.5	100.5	83.6	84.6	85.1	86.1
MS 65	101.8	102.4	102.5	103.0	100.1	100.1	100.4	100.5	99.3	97.6	96.0	93.3
MS 67	102.6	102.9	103.4	103.5	100.4	101.0	101.7	102.4	100.8	101.7	102.7	104.2
MS 68	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
MS 71	100.9	101.8	102.3	104.2	100.8	101.6	102.5	102.9	99.6	99.4	99.3	99.3
MS 84	102.8	104.3	107.2	107.7	100.9	102.6	103.7	105.4	101.7	106.2	107.2	109.8
MS 85	99.8	99.7	99.7	99.7	100.4	100.6	100.7	100.8	100.3	100.4	100.6	100.6
MS 86	99.3	98.5	97.9	97.5	100.9	102.8	104.7	105.6	100.0	100.0	100.1	100.1
MS 87	99.8	99.6	99.5	99.4	100.3	101.2	101.5	101.7	100.1	100.4	100.5	100.5
MS 88	99.9	99.8	99.7	99.5	100.0	100.0	100.0	100.0	100.1	100.5	100.8	100.9
MS 90	0.0	0.0	0.0	0.0	100.3	100.8	100.9	101.0	100.4	100.9	100.9	101.0

Table 5. Preliminary identification of lactic acid bacteria isolates.

Properties	MS 12	MS 13	MS 18	MS 19	MS 24	MS 44	MS 71	MS 84	MS 86	MS 87	MS 88
Gram reaction	G	G	G	G	G	G	G	G	G	G	G
Morphology	cocci	cocci	rods	cocci	cocci	cocci	cocci	rods	rods	cocci	rods
Catalase	-	-	-	-	-	-	-	-	-	-	-
Citrate	-	-	-	-	-	-	-	-	-	-	-
Methyl red	+	+	+	+	+	+	+	+	+	+	+
VP-test	+	-	-	-	+	+	+	-	-	-	-
H_2S Production	-	-	-	-	-	-	-	-	-	-	-
Acid from:											
-Galactose	++	+	+	++	+	-	-	-	+	+	+
-Xylose	++	++	+	+	-	-	+	-	-	+	+
-Arabinose	+	-	-	++	-	-	-	-	-	++	-
-Sucrose	++	++	++	+	+	+	-	-	+	-	+
-Glucose	+	+	+	+	+	+	+	+	+	+	+
-Sorbose	++	-	-	+	++	-	-	-	+	+	-
-Lactose	+	+	+	+	+	-	-	+	+	+	+
-Mannitol	+	+	+	+	+	+	+	+	+	+	+
-Sorbitol	+	+	+	+	-	+	+	-	-	+	+
Growth at:											
pH 4.5	+	+	+	+	+	+	+	+	+	+	+
pH 7.6	+	+	+	+	+	+	+	+	+	+	+
pH 9.6	+	+	+	-	+	+	+	+	+	+	+

NaCl tolerance

Salt tolerance was done in **Table (6).** The current results showed that LAB isolates were able to tolerate all tested concentrations of NaCl and good growth was observed at 1.5 – 3.0 % NaCl with some differences as the growth was decreased when concentration of NaCl was increased from 3.0 to 7.0%. On the contrary, at 5.0 % NaCl, only 4 isolates (MS84, MS86, MS87 and MS88) showed a highly growth. LAB group need of salt for growth at concentrations of moderately and extremely halophilic (5-30%). Each genus of LAB has different tolerances to grow on media with different concentrations of NaCl salt (**Vos, *et al*., 2009)**. NaCl is an inhibitory substance which may inhibit growth of certain types of bacteria. The obtained results have the similarities with the findings of **Reale, *et al*. (2015).** They reported that lactobacilli isolated from gastrointestinal tract of swine were tolerable to 4-8% NaCl.

Antioxidant activity

DPPH, a relatively stable organic radical has been widely used in the determination of antioxidant activities of cell free extracts of bacteria. To elucidate the antioxidant activity of the 11 strains, the scavenging ability of the cell free extracts of LAB isolates were compared with the standard antioxidant ascorbic acid .The DPPH radical scavenging activities of culture filtrate of the 11 LAB isolates were near and over 50% **(Table 6).** The maximum antioxidant activity was observed with MS88 showing 75.74 % followed by MS13 with 75.69%. On the other hand, MS12 and MS24 showed antioxidant activity of 74.94 and 72.33 %, respectively. Additionally, the culture supernatant had 50% antioxidant activity as non-purified solution, demonstrating potential as a natural antioxidant. Increase of the antioxidant capacity by optimizing the environmental factors makes it possible to obtain useful industrial materials. **Yang *et al*. (2014)** reported that there is a significant positive correlation among the antioxidant activities and the metabolite of LAB. It is considered that purification of the filtrate could exhibit a higher activity. This study provides support for the formulation of novel probiotic foods or supplements that can play a role in the prevention of oxidative stress and related diseases.

Table 6. NaCl tolerance, and antioxidant activity by the LAB isolates

LAB isolates	NaCl tolerance at concentration (%)				DPPH %	
	1.5	3	5	7	Inhibited	Residual
MS 12	++	++	+	+	74.94	25.06
MS 13	++	++	+	+	75.69	24.31
MS 18	++	++	+	+	52.82	47.18
MS 19	++	++	+	+	49.15	50.85
MS 24	++	++	+	+	72.33	27.67
MS 44	++	++	+	+	46.37	53.63
MS 71	++	++	+	+	64.12	35.88
MS 84	++	++	++	+	58.47	41.53
MS 86	++	++	++	+	50.31	49.69
MS 87	++	++	++	+	47.92	52.08
MS 88	++	++	++	+	75.74	24.26

Hemolytic activity

The eleven isolates with the best results were tested for their non-pathogenic character by streaking them on blood agar plates **(Table7**). Tested strains showed no haemolysis (γ-haemolysis). Evaluation of haemolytic activity is an important safety requirement frequently used to assess potential probiotic strains. Usually no haemolysis by the test strain is the indicative of its safety for human use **(Owusu-Kwarteng *et al.*, 2015).** Absence of haemolytic activity is considered as safety criterion for the selection of a probiotic strain. In our study, none of the selected examined strains exhibited α- or β-haemolytic activity, when grown in Columbia blood agar, whereas all strains were γ-haemolytic (no haemolysis). These results are similar with previous observations where all of the tested strains **(Pisano, *et al.*, 2014)** or most of them **(Argyri, *et al.*, 2013)** are γ-haemolytic.

Table 7. Hemolytic activity of the probiotic isolated LAB.

Isolates code	Hemolytic activity types			Isolates code	Hemolytic activity types		
	α	β	ϒ		α	β	ϒ
MS12	-	-	+	**MS71**	-	-	+
MS13	-	-	+	**MS84**	-	-	+
MS18	-	-	+	**MS86**	-	-	+
MS19	-	-	+	**MS87**	-	-	+
MS24	-	-	+	**MS88**	-	-	+
MS44	-	-	+				

Susceptibility of LAB to antibiotics

The LAB antibiotic susceptibility to different antibiotics is shown in **Table (8)**. Most of the LAB isolates are resistant to many antibiotics. Isolates MS (12, 18, 44, 84, 86, 87) were resistant to all tested antibiotics while strains MS (13, 24, 88) were resistant to 5 antibiotics, also, strains MS (19, 71) were resistant to 4 antibiotics. Among antibiotic resistances, vancomycin resistance is of major concern because vancomycin is one of the antibiotics broadly efficacious against clinical infections caused by multi drug resistant pathogens. Some LAB including strains MS (12, 18, 44,71, 84, 86, 87,88) were found to be resistant to vancomycin. Such resistance is usually intrinsic, that is, chromosomally encoded and non-transmissible **(Zhou *et al*., 2000).** In addition, results indicated that LAB is resistant to most of antibiotics tested and low multiple susceptible were observed. This result is in accordance with various reports that LAB strains are normally resistant to the principal antibiotics such as chloramphenicol, penicillin G and vancomycin **(Hoque *et al*., 2010 and Marroki *et al*., 2011).** Innate resistance of probiotics to some antibiotics suggests their use for preventive and therapeutic purposes for controlling intestinal infections especially when co-administered with the therapeutic use of antibiotics **(Negm El-Dein, Asmaa *et al*., 2017).**

Table 8. LAB isolates susceptibility to different antibiotics.

Isolate code	PE (10 µg)	NEin (10 µg)	ER (15 µg)	TC (30 µg)	V (30 µg)	CP (30 µg)	G (10 µg)
MS 12	R	R	R	R	R	R	R
MS 13	R	R	S	R	R	R	S
MS 18	R	R	R	R	R	R	R
MS 19	R	R	R	R	S	R	R
MS 24	R	R	R	R	S	R	R
MS 44	R	R	R	R	R	R	R
MS 71	R	R	S	R	R	R	S
MS 84	R	R	R	R	R	R	R
MS 86	R	R	R	R	R	R	R
MS 87	R	R	R	R	R	R	R
MS 88	S	R	R	R	R	R	R

PE: Pencillin G, NE: Neofloxin, ER:Erthromycin, TC:Tetracycline, V: Vancomycin, CP: Chloramphincol, G:Gentamycin. R: resistance, S: sensitive according to **Oloyede and Afolabi (2013)**

Cholesterol assimilation

Some of the organism are capable of reducing the cholesterol levels naturally and shows anticholesterol activity. **Table (9**) shows the results of cholesterol reduction by LAB isolates in the presence of 0.3% bile salt. The cholesterol assimilation ranged from 9.79 -93.4%. The strains MS (12,18, 19, 44, and 71) had assimilated greater than 70%. Elevated level of certain blood lipids is a greater risk for cardiovascular disease. A few research reports describe the use of LAB to decrease the serum cholesterol levels in human and animals **(Lee *et al*., 1992).**

Table 9. Cholesterol assimilation by LAB isolates.

LAB isolates	Cholesterol assimilated (µg/ml)	Cholesterol assimilated (%)
MS 12	783.06	93.4
MS 13	82.06	9.79
MS 18	773.86	92.3
MS 19	728.23	86.9
MS 24	407.72	48.6
MS 44	780.85	93.2
MS 71	604.96	72.2
MS 84	551.23	65.8
MS 86	442.86	52.8
MS 87	426.86	50.9
MS 88	567.98	67.8

Cholesterol assimilated (µg/ml) at zero time = 838.07

There are reports indicated that lactic acid bacteria can reduce the serum cholesterol level up to 50% in presence of bile salt in 48 h **(Guslandi *et al.*, 2003).** It has been reported that the ability of the microorganism to reduce the cholesterol level was due to assimilation of cholesterol within bacterial cell and increased excretion of bile salts due to deconjugation by the bile salt hydrolase **(Salminen *et al.*, 2002)**. Probiotic bacteria are advantageous as they are naturally found in foods such as yoghurt, are inexpensive and are generally regarded as safe (GRAS). Of interest are the recent results demonstrating that probiotic bacteria have significant cholesterol-lowering properties (**Tomaro-Duchesneau *et al.*, 2014**). The hypocholesterolemic effects of probiotic bacteria have been linked to intrinsic bile salt hydrolase activity cholesterol assimilation and incorporation in cellular membranes and the production of compounds such as fatty acids, , that can inhibit the activity of enzymes, including HMG-CoA reductase **(Kim, *et al.*,2003).**

16S rRNA Sequence Analyses

After the 16S rRNA sequences of the potent LAB (MS87) was edited, consensus sequence obtained was blasted in GenBank of NCBI and showed similarity of 100% in the GenBank. Accordingly, the phylogenetic tree made from sequenced 16S rRNA region of the bacterial isolate indicated that MS87 isolate was identified and belonging to *Pediococcus pentosaceus*. (Fig.3).

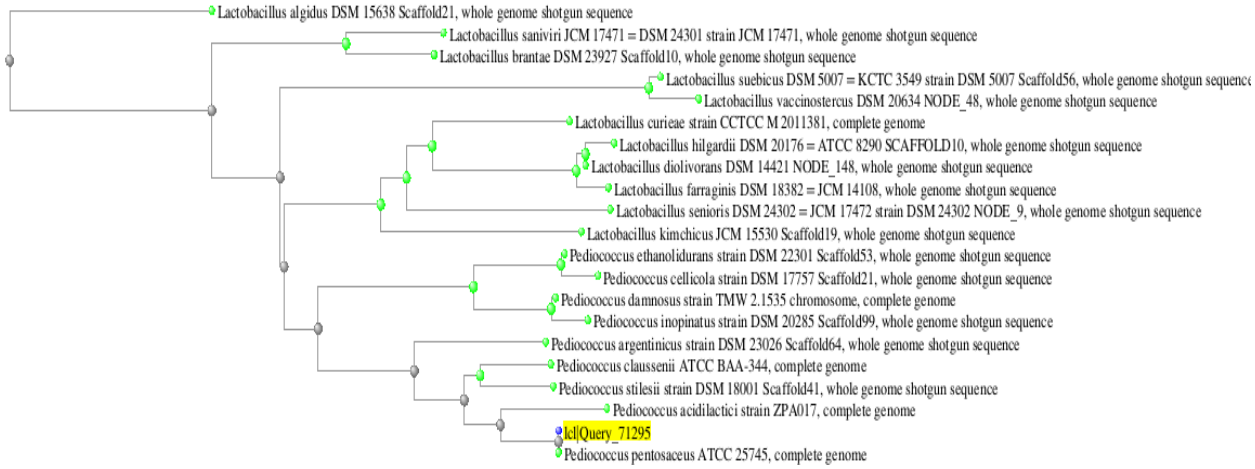

Fig. 3: Phylogenetic tree of nucleotide sequence of LAB isolate MS87

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