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Study of Morphological and Genetic Characteristics of Probiotic Bacteria isolated from Local Fermented Milk

Nada Aqeel Karam Al-Khafaji[1], Hussein L. Algboory[2*]

[1] College of Food Science, Al-Qasim Green University, Babylon, Iraq
Email: nada.p100919@uoqasim.edu.iq
[2] College of Agriculture, Al-Qasim Green University, Babylon, Iraq
Email: hblcf@yahoo.com

***Correspondence author:** Hussein L. Algboory (hblcf@yahoo.com)

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Abstract

This study included the isolation and identification of probiotic bacteria from fermented milk (labneh).The samples of fermented milk were collected from different shops .Six isolates were obtained through morphological microscopy and biochemical tests. Bacterial isolates were also identified using 16srRNA.The results demonstrated that the six isolates belonged to Lactobacillus helveticus PT001, Lb. helveticus bcpca-qj 10, Lb. helveticus SJ, Lb. acidophilus F, Lb. johnsonii MS1, Lb. jensenii H31. The biological properties of bacterial isolates were studied in terms of their resistance to pH and bile salts, and it was found that all isolates were resistant to pH (1.5%) and bile salts (1, 3%), and had the ability to adhere to ,it was resistant to some antibiotics, It has the inhibitory action of some pathogenic bacteria such as Salmonella typhimurium and Staphylococcus aureus ATCC 8625.

Keyword

Labneh, Prebiotic bacteria, Lactobacillus.

Probiotic bacteria are defined by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) as live microorganisms that, when taken in sufficient quantities, confer health benefits on the host. They include the Lactobacillus bacteria, Bifidobacterium, and Saccharomyces boulardii, which are preferably taken from human sources (Cheng, 2015).

Probiotic bacteria are a means of defending human health and enhancing the immunity of the human body. These organisms have a healthy effect on the host's body, especially when consumed in the correct scientific methods. They work to curb disease infections of the digestive system because they have anti-microbial properties, in addition to their role in improving lactose metabolism, reducing total cholesterol in the blood, reducing the incidence of cancer, and the success of using them in controlling various cases of diarrhea, especially in children and others (Ahmed, 2011).

Lactobacillus is the most widely used type of probiotic bacteria. It brings health benefits to the host, significantly within the digestive system (Vitali et al. 2012).

Its therapeutic role is not limited to the digestive system exclusively, but it also includes the respiratory system and the prevention of infectious diseases, especially children and other age groups. It also has the ability to reduce the incidence of irritable bowel syndrome, reduce the level of ammonia in the blood, produce vitamin B, Improve the body's ability to absorb minerals and Production of nutritionally important peptides (Shi et al. 2013; Algboory and Muhialdin 2021)

When the eaten food containing probiotic bacteria enters the digestive system, it will be exposed to extreme gastrointestinal conditions, including an extreme acidic stomach environment with low pH 2-1.5, followed by exposure to an environment containing bile salts, which is an antimicrobial agent. Thus, the vitality of probiotics will be affected and their numbers are significantly reduced, which may explain resorting to eating high

numbers of them and good quantities of food (Del Piano et al. 2011).

Because of the importance of these bacteria and their role in influencing human health, the aim of this study is to obtain isolates of probiotic bacteria from local fermented milk (labneh)and study their characteristics.

Materials and working methods

Isolation and identification of Probiotics

Several samples were collected from buttermilk sold in local markets in Babil Governorate. Decimal dilutions were made and 1 ml was taken and cultured on MRS agar medium by decantation method. The plates were incubated under anaerobic conditions using anaerobic jar at 37°C for 48 hours (Buck and Gilliland, 1995). The isolates were diagnosed according to the methods adopted in (Axelsson, 2004), by studying biochemical tests such as the enzyme catalase test, growth at temperatures of 45 °C, clotting test, the ability of bacteria to grow in Nacl at a concentration of 6.5%, acid production. Lactic using MRS-caco3 (Change et al., 2013).

Genetic diagnosis of bacterial isolates

DNA extraction of bacterial isolates: DNA of bacterial isolates was extracted according to the manufacturer's protocol using TM Total DNA kits Favorgen (Taiwan) used as a template for PCR.

Polymerase Chain Reaction (PCR):- PCR technology was used to amplify DNA using primers 27F 5'-AGAGTTTGATCCTGGCTCA-3'and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Loy et al., 2002). The PCR prepared from (USA-Bio-Rad) consists of three successive steps (denaturation, annealing, and elongation) of repeated cycles to obtain the PCR product.

Agarose gel electrophoresis

The method of agarose gel electrophoresis was used to detect DNA according to what was mentioned by (ZMR et al., 2016). Using 1.5% agarose gel and 100µl of 1XTBE and heated until all gel particles dissolved then 1.3µl of ethidium bromide dye was added. The specific sequences of the gene nitrogen bases of bacterial isolates obtained from fermented milk (Laban local) were compared with the sequences in a bank NCBI Genes.

Study of the properties of probiotics for bacterial isolates:-

Acidity and bile salt resistance: Bacterial isolates were activated in liquid MRS medium at 37 °C for 24 h then 1 ml was transferred to tubes containing MRS medium pH 1.5 (pH regulated by 0.1N HCl), and 1 ml was transferred from the culture The bacteria were transferred to MRS liquid medium containing 1.3 % (w/v) of oxgall bile salts (Difco, USA) and then incubated at (0,1.5, 3) hours at 37° C. Bacterial growth was observed through viable colonies growing on solid MRS medium and incubated at 37°C for 48 hours (Yadav et al., 2016). Calculating the Survivability bacteria:- Survivability (%) The equation mentioned by was used (Liong M & Shah N, 2005).

Antibiotic sensitivity:- The method mentioned by (Singh et al., 2012) was used, where the disc diffusion method was used using the antibiotics Ciprofloxacin, Penicillin G, Azithromycin, Vancomycin.

Evaluation of Antibacterial Activity:-

The method mentioned by (Gupta et al., 1996) was used. The inhibitory activity of cell free extracts (CFE) was estimated for cultures of Bacterial isolates against test bacteria by will diffusion method, where the dishes were planted. Containing Muller-Hinton agar (MHA) with 0.1 ml of the test bacteria, the dishes were left for two hours, and then incubated at 37°C for 18 hours.

Study of the adhesion of bacteria: -

The method mentioned by (Schentiz et al., 1993) was used using the "sub culturing" process for more than 10 times, with one transfusion per 1-2 days, with the growth nature recorded.

Results and discussion

Isolation and initial identification of probiotic bacteria

Probiotic bacteria were isolated from curd samples. 6 isolates were diagnosed based on microscopic and biochemical characteristics. As shown in Table (1), the results of the present study revealed that all bacterial isolates are positive for long or short Gram-stain, non-forming spores, and non-motile. They test negative for catalase due to inability to produce the enzyme peroxidase, which works to convert hydrogen peroxide into oxygen and water, ability to produce lactic acid, growth at a temperature of 45 degrees Celsius, growth in a medium containing 6.5% of (NaCl) salt, and ability to coagulate milk. These results are consistent with what was found by (Ahmed and Kanwal, 2004) with evidence from (Garrity Bergey's et al. 2004).

Table (1) Bio diagnostic tests for bacterial isolates

Isolation	Cram dye	Catalis	Acid production	Coagulation	(NaCl) 6.5	Growth at 45 AD
A	+	-	+	+	+	+
B	+	-	+	+	+	+
C	+	-	+	+	+	+
D	+	-	+	+	+	+
E	+	-	+	+	+	+
F	+	-	+	+	+	+

(Positive: +) (Negative: -)

Genetic diagnosis of bacterial isolates by 16SrRNA

The gene (16SrRNA) was amplified for bacterial isolates using PCR technique. Figure (1) shows the products of electrophoresis of bacterial isolates using PCR technique. The nitrogen base sequences analysis of the six bacterial isolates showed that they belong to four species: 3 Lb.helveticus isolates, one Lb.acidophilus, one Lb.johnsonii, and one Lb.jensenii. The number of diagnosed isolates, the percentage of matches, and the corresponding bacterial species found in the genebank are shown in (Table 2). Figure (2) shows the recording of the isolate Lactobacillus.jenseniiH31 (F) in NCBI GenBank (OL587496).

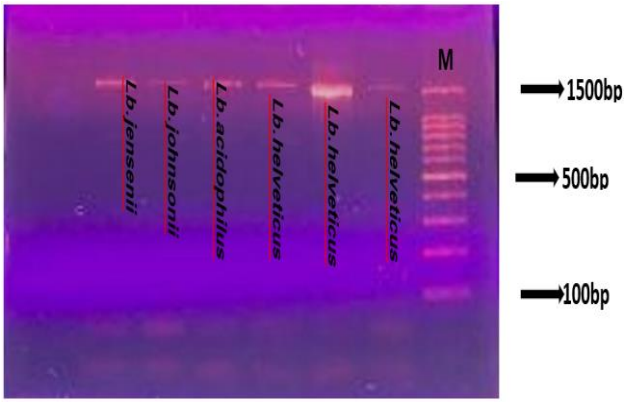
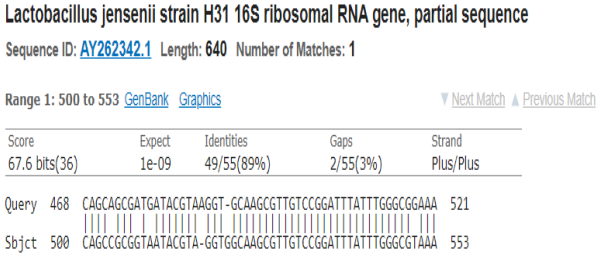


Figure (1) shows electrophoresis of PCR products, amplification of 16SrRNA 1500 gene of bacterial isolates on 1% agarose gel.

Table (2) The number of diagnosed isolates, the percentage of matches, and the corresponding bacterial species.

Percentage Identity	Identity	NCBI accession No.	Strain ID
83%	Lactobacillus.helveticus strain PT001	KX247766.1	A
95%	Lactobacillus.helveticus strain bcpca-qj 10	KX247766.1	B
99%	Lactobacillus.helveticus strain SJ	LC377274.1	C
90%	Lactobacillus.acidophilus strain F	MT645504.1	D
89%	Lactobacillus.johnsonii strain MS1	OK147904.1	E
89%	Lactobacillus.jensenii strain H31	AY262342.1	F


Lactobacillus jensenii strain H31 16S ribosomal RNA gene, partial sequence

Sequence ID: AY262342.1 Length: 640 Number of Matches: 1

Range 1: 500 to 553 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
67.6 bits(36)	1e-09	49/55(89%)	2/55(3%)	Plus/Plus

Query 468 CAGCAGCGATGATACGTAAGGT-GCAAGCGTTGTCCGGATTTATTTGGGCGGAAA 521
Sbjct 500 CAGCCGCGGTAATACGTA-GGTGGCAAGCGTTGTCCGGATTTATTTGGGCGTAAA 553

Figure 2. Recording of the Lactobacillus.jensenii H31 isolate in the NCBI GenBank

Studying the properties of probiotics for bacterial strains

The resistance of bacteria to pH

The resistance of probiotic bacteria to the severe decrease in the pH close to what is found in the stomach has been studied as it is one of the important characteristics that are considered with interest when selecting bacterial strains to be used in the manufacture of therapeutic dairy products. The conditions of the experiment were chosen to be close to what is found in The human stomach with an exposure period of one to three hours, which is the estimated time for food to remain in the stomach (Schillinger et al. 2005). The results shown in Table (3) revealed the resistance of bacterial isolates to pH 1.5% compared to pH 7.0% in periods of 1.5 and 3 hours. None of the isolates showed inability to tolerate acidity. The best isolates resistant to pH 1.5% compared to pH 7.0% were Lb. helveticus bcpca-qj 10 at time of 1.5 hours, and Lb.acidophilus F at time of 3 hours. These results are consistent with what was mentioned by

(Harutoshi et al. 2007), which showed the ability of Lb. helveticus and Lb. acidophilus to grow in medium with a pH of 2 and 3% for periods of 1 and 3 hours. Studies showed that the reason for resistance of probiotic bacteria to low levels of acidity may be due to the physiological state of the cell cytoplasm, which regulates the pH between outside and inside cells. It causes enzymes to inactivate or may damage proteins and nucleic acids when it is low (intracellular) (Angmo, 2014). The reason for the high resistance to acidity can be attributed to the high activity of the enzyme ATPase, which is possessed by strains of Lactobacillus bacteria. This enzyme increases the bacteria's tolerance to acidic conditions by generating the driving force of the proton used as a source of energy transfer across the cell membrane. Therefore, the difference in acid tolerance is related to the difference in the activity of this enzyme (Lebeer et al. 2008).

Table (3) The resistance of bacterial isolates to pH.

Lb.jensenii H31	Lb.johnsonii MS1	Lb.acidophilus F	Lb.helveticus SJ	Lb.helveticus bcpca-qj 10	Lb.helveticus PT001	Condition
6.68 6.68	6.72 6.72	6.78 6.78	6.54 6.54	6.61 6.61	6.74 6.74	(0h) pH 1.5 (0h) pH 7.0
--	--	--	--	--	--	Survivability (%)
6.55 6.83	6.61 6.94	6.69 6.94	6.43 6.68	6.50 6.71	6.68 6.92	(1.5h)pH 1.5 (1.5h)pH 7.0
95.9	95.2	96.3	96.2	96.8	96.5	Survivability (%)
6.25 6.94	6.43 6.98	6.44 6.96	6.14 6.83	6.25 6.87	6.41 6.96	(3h) pH 1.5 (3h) pH 7.0
90	92.1	92.5	89.8	90.9	92	Survivability (%)

Bacterial resistance to Bile salts

The results shown in Table (4) reveal the resistance of bacterial isolates to bile salts at a concentration of 1.3%, compared to the control at 1.5,3 hours. None of the isolates showed intolerance to high concentrations of bile salts. These results are close to what was demonstrated by (Cho et al. 2013) who found that probiotic bacteria are resistant to bile salts at concentrations ranging from 1 to 3%. The studies showed the importance of resistance of probiotic bacteria to the bile salts secreted by the duodenum from the small intestine for the survival of bacteria because the cell membrane contains fatty acids that can be destroyed by salts bile (Yadav, 2016). So, probiotic bacteria have several mechanisms of resistance to bile salts, including the bacteria's possession of the enzyme bile salt hydrolase that breaks down bile salts (breaks down and reduces the digestive capacity of bile salts) and thus reduces the toxic effect of bile salt on the bacteria. Its susceptibility to bile salts is enhanced (Erkkiд and Petдjд, 2000). Hydrogen bonds also connect surface layer proteins to other layers of the wall etc. Some elements in the cell wall maintain its integrity, increase the activity of metabolic processes, and increase the efficiency of amino acid transport and fatty acid biosynthesis (Taranto et al. 2006).

Table (4) The resistance of bacterial isolates to Bile salts.

Lb.jensenii H31	Lb.johnsonii MS1	Lb.acidophilus F	Lb.helveticus SJ	Lb.helveticus bcpca-qj 10	Lb.helveticus PT001	Condition
6.83 6.83	6.64 6.64	6.79 6.79	6.69 6.69	6.81 6.81	6.60 6.60	(0h)1%oxgall (0h) control
--	--	--	--	--	--	Survivability (%)
6.71 6.95	6.55 6.75	6.53 6.86	6.62 6.89	6.65 6.89	6.57 6.85	(1.5h)1%oxgall (1.5h) control
96.5	97	95.1	96	96.5	95.9	Survivability (%)
6.47 6.98	6.25 6.93	6.30 6.96	6.39 6.97	6.43 6.97	6.46 6.94	(3h)1%oxgall (3h) control
92.6	90.1	90.5	91.6	92.2	93	Survivability (%)
6.83 6.83	6.64 6.64	6.79 6.79	6.69 6.69	6.81 6.81	6.60 6.60	(0h)3%oxgall (0h) control
--	--	--	--	--	--	Survivability (%)
6.39 6.95	6.25 6.75	6.34 6.86	6.34 6.89	6.34 6.89	6.30 6.85	(1.5h)3%oxgall (1.5h) control
91.9	92.5	92.4	92	92	91.9	Survivability (%)
6.07 6.98	6.04 6.93	6.11 6.96	6.11 6.97	6.17 6.97	6.11 6.94	(3h)3%oxgall (3h) control
86.9	87.1	87.7	87.6	88.5	88	Survivability (%)

Antibiotics

Antibiotic sensitivity check is one of the main characteristics of probiotics, which means that antibiotics cannot destroy bacteria. Because probiotic bacteria are powerful nutritional supplements that help rebuild the balance of beneficial microorganisms in the human gut, it is therefore important to carefully evaluate for the safety and efficacy of all strains of microorganisms before incorporating them into food products (Konika, 2015). The results shown in Table (5) reveal the sensitivity of the bacterial isolates under study to antibiotics that usually kill bacteria. The results also revealed that bacterial isolates Lb.helveticus PT001, Lb.helveticus bcpca-qj 10, Lb. helveticus SJ, Lb.acidophilus F, and Lb.johnsonii MS1, possess high sensitivity to Azithromycin and PenicillinG antibiotics. They are less sensitive to Ciprofloxacin and not sensitive to Vancomycin antibiotic except for Lb.helveticus PT001, which was sensitive to all antibiotics used in the present study. Whereas Lb. jenseniiH31 isolate was resistant to all antibiotics used. These results are consistent with (Temmerman et al. 2003) who indicated that Lb.acidophilus is sensitive to the antibiotics Ciprofloxacin and Vancomycin. The antibiotic resistance of bacteria is due to the fact that they carry antibiotic resistance genes, which may be carried by plasmid or resulting from gene transfer from one bacterium to another. There are several mechanisms by which bacteria must resist antibiotics, including switching in the enzyme or switching membrane permeability (Sharma et al. 2016). Having D-Ala-D-Lactate in the peptidoglycan instead of the normal dipeptide D-Ala- D-Ala is the goal of the antibiotic (Tulumoglu et al. 2013).

Table (5) The resistance of bacterial isolates to antibiotics.

Antibiotic Susceptibility						Symbol	Antibiotics
Lb.jensenii H31	Lb.johnsonii MS1	Lb.acidophilus F	Lb.helveticus SJ	Lb.helveticus bcpca-qj10	Lb.helveticus PT001		
R	S	S	S	S	S	AZM	Azithromycin
R	S	S	S	S	S	CIP	Ciprofloxacin
R	R	R	R	R	S	VA	Vancomycin
R	S	S	S	S	S	P	Penicillin G

(R Resistant -: S Sensitive)

Inhibitory Efficacy

Another health benefit of Lactobacillus probiotics is their inhibitory effect on the growth of pathogenic bacteria. The inhibitory effects of Lactobacillus on pathogenic microorganisms could be due to many factors such as the production of H2O2 and organic acids (Millette, et al. 2007; Nakai and Siebert, 2003). The health benefit of probiotics could be due to the bacterial production of bacteriocins and substances other metabolic. The results shown in Table (6) reveal a clear inhibitory activity against pathogenic bacteria strains. It is clear that inhibitory areas were formed around the pits as shown in Figure (3). It was found in this study that all isolates have the ability to inhibit pathogenic bacteria. These results are close to what was found by (Kalus et al. 2017) who found that Lb. acidophilus has good inhibitory activity against pathogenic bacteria.

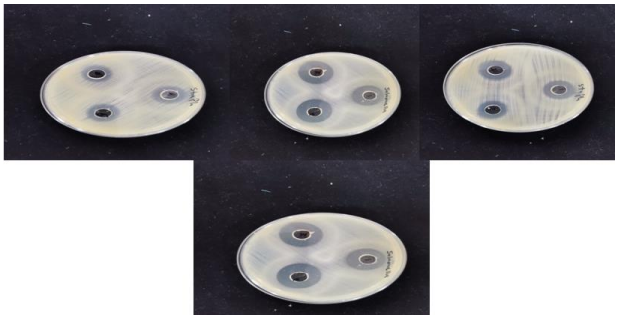


Fig. (3) Demonstrates the inhibition of bacterial isolates against Salmonella typhimurium and. Staphylococcus aureus ATCC8625.

Table (6) The inhibitory activity of bacterial isolates against Salmonella typhimurium and. Staphylococcus aureus ATCC8625.

bacterial filtrate	damping rate (mm) *	
	Sal. typhimurium	Staphylococcus aureus
Lb.helveticus PT001	15	11
Lb.helveticus bcpca-qj 10	15	10
Lb.helveticus SJ	15	11
Lb.acidophilus F	13	10
Lb.johnsonii MS1	11	11
Lb.jensenii H31	11	19

*The result is subtracted from the diameter of the hole 7 mm

Adhesion

Figure (4) shows the results of the transfections of the bacterial isolates used in the study. The results showed the appearance of a precipitate and the absence of a floating substance and turbidity of the medium after the bacteria were transferred more than 10 times, with one transfusion every 1-2 days. The results showed the ability of the bacterial isolates to stick to the walls of stomach. These results are consistent with what Schentiz et al. (1993) mentioned, who referred to the growth of probiotic bacteria on the MRS medium during the isolation phase and other stages. It was constantly observed that its growth in the liquid medium was accompanied by sediments at the bottom of the development tubes. There is turbidity. The appearance of Aggregate deposits at Bacterial growth on MRS Broth medium is an evidence of bacterial adhesion. The loss of sedimentation ability and plankontic growth gives an indication that bacteria are not possessed or lost by rapid translocation.

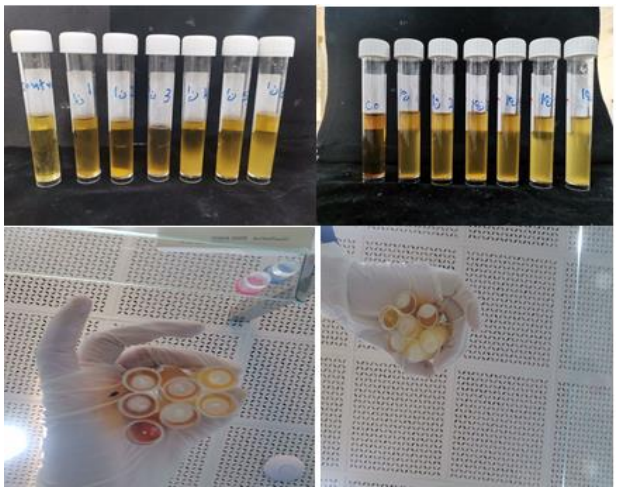


Figure (4) The suction property of bacterial isolation.

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