

## ISOLATION AND CHARACTERIZATION OF A POTENTIALLY PROBIOTIC *LACTOBACILLUS DELBRUEKII* SSP. *LACTIS* FROM QARISH CHEESE

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### ABSTRACT

Probiotics are non- pathogen living microorganisms which express beneficial effects on host, when are administered in adequate amounts. The present study was undertaken to isolate and characterize certain lactic acid bacteria (LAB) isolate which could be used as a probiotic

LAB was isolated by culturing the samples on MRS agar. The isolates were screened for their probiotic potential using *in-vitro* tests, including tolerance to acid and bile, survival in simulated gastrointestinal tract conditions, and hemolytic activity as well as antibiotic susceptibility. In addition, antibacterial activity of the isolated strains against *E. coli O157* and *Salmonella enterica* was determined.

*Lactobacillus delbruekii* ssp. *lactis* showed the highest tolerance to low pH, bile and simulated gastrointestinal tract conditions. This strain exhibited resistance to Nalidixic acid, Cloxacillin, Aztreonam and Colistin as well as effective antibacterial activity against two Gram negative pathogens, lacking hemolytic activity. Finally, the strain was identified as *Lactobacillus delbruekii* ssp. *lactis* using biochemical characterization and *16S rRNA* sequencing assay.

**Discussion and conclusion:** In the present work, a potentially probiotic was *Lactobacillus delbruekii* ssp. *lactis* isolated from dairy product (Qarish Cheese). The bacterium displayed good probiotic properties.

**Key words:** Lactic acid bacteria, *Lactobacillus delbruekii* ssp. *lactis*, Probiotics, dairy product (Qarish Cheese).

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## Introduction

Probiotics are non-pathogenic microorganisms that when ingested in adequate amounts, exert a positive influence on their host's health (Schlundt, 2012). A variety of microorganisms typically food grade has been evaluated for their probiotic potential and are applied as adjunct cultures in various types of food products or in therapeutic preparations (Rodgers, 2008).

Probiotics are defined as live microorganisms that are believed to provide health benefits when consumed (Hill et al., 2014). The term probiotic is currently used to name ingested microorganisms associated with benefits for humans and animals (Magdalena, et al., 2006).

Probiotics are considered to be generally safe, but they may cause bacteria-host interactions and unwanted side effects in rare cases (Singhi and Kumar, 2016).

In addition, probiotics should be non-pathogenic, stable in acid and bile, able to adhere to and colonize human gut mucosa. They should be scientifically demonstrated to have beneficial physiological effects and safety so that they can be used to improve microbial balance and to confer health benefit, especially of LAB strains (Singhi and Baranwal, 2008). Cheese have been considered as good sources of LAB and ideal vehicles to deliver probiotic bacteria to human gastrointestinal tract (Karimi et al., 2012).

The aim of the present study was to isolate LAB from Qarish Cheese and to investigate their probiotic potential. In addition, inhibitory activity of the isolates against *E. coli* O157 and *Salmonella enterica* has also been studied.

## Materials and Methods

**Sample collection and isolation of LAB:** Samples were collected aseptically from ten different markets in the Sharqia Governorate, Egypt (Qarish Cheese). Stored at 4°C and transferred immediately to the laboratory. The homogenized suspension was serially diluted in 0.1% peptone water and 1 mL of appropriate dilution ( $10^{-1}$ ,  $10^{-2}$  and  $10^{-3}$ ) was plated on to MRS agar (Thongaram, 2016). pH 6.2 and pH5.5, The plates were incubated at 37 °C for 48 h. the Gram positive, catalase negative, non-motile bacterial isolates were purified using streak culture technique and stored at 4°C.

**Acid and bile tolerance:** Tolerance to acid was determined according to the method described earlier (Yu et al., 2013), as the following: isolated strains were



grown in MRS broth at 37°C for 48 h, and sub-cultured in fresh MRS broth adjusted to pH 2.0 with hydrochloric acid. The initial bacterial concentration was adjusted to 10<sup>7</sup> CFU/mL and their survival was determined after 60 and 120 min at pH 2.0. Bile tolerance test was conducted using the method described by **Yu and Tsen, (1993)**.

The Isolated bacteria were grown in MRS broth with and without 3% (w/v) bile. The cultures were incubated at 37°C and the growth was monitored by measuring their optical densities (OD) at 620 nm. The bile tolerance was estimated by comparing cell growth of organisms.

**Resistance to lysozyme:** The tolerance for the isolated bacteria against lysozyme was determined by the method of **Zago et al, (2011)**. The culture was grown in 10 mL MRS medium at 25°C and 180 rpm for 10-12 h. The cells were centrifuged at 10,000 rpm for 15 min. The cell pellet was washed with 5 mL of phosphate buffer saline (pH 7.0) two times and resuspended in 2 mL of Ringer solution, 0.5 mL suspension of the isolated bacteria was inoculated in 5 mL sterile electrolyte solution (SES; 0.22 g/l CaCl<sub>2</sub>, 6.2 g/l NaCl, 2.2 g/l KCl, 1.2 g/l NaHCO<sub>3</sub>) containing 100 mg/l of lysozyme (Titan Biotech L.TD. Rajasthan, India) and incubated at 37°C for 2 h. The bacterial suspension in sterile electrolyte solution without lysozyme was taken as a control. The aliquot (100 µl) from sample was spread on to MRS agar plates and incubated at 25°C for 48 h. The microbial colonies grown on plates were counted and their survival was calculated as percent of CFU/mL (colony forming unit per mL) obtained from lysozyme treatment with respect to the control (without lysozyme treatment).

**Tolerance against gastric juice:** The tolerance of washed cell suspensions for the isolated bacteria to simulate gastric (pepsin, 3 g / l in sterile saline (0.5%) and adjusting the simulated gastric juices pH to 2.0 with concentrated HCl) was determined as described by **Charteris et al, (1998)**. To 0.2 mL of washed cell suspension in a 2.0 mL capacity screw-cap microfuge were mixed to 1.0 mL of simulated gastric (pH 2.0) and 0.3 mL NaCl (0.5% w/v). The materials were vortexed at setting 5 for 10 second and incubated at 37 °C. When assaying gastric transit tolerance, aliquots of 0.1 mL were removed after 0 and 180 min for determination of total viable count.

**Tolerance against simulated pancreatic juices:** The tolerance of washed cell suspensions for the isolated bacteria to small intestinal transit was determined as described by **Charteris et al, (1998)**. To 0.2 mL of washed cell suspension in a 2.0 mL capacity screw-cap microfuge tube were admixed 1.0 mL of pancreatic juice (panceratin 1 g / l) in sterile saline (0.5%) and adjusting the pH to 8.0 with



concentrated 0.1 M NaOH. The materials were vortexed at setting 5 for 10 second and incubated at 37 °C. When assaying transit tolerance, aliquots of 0.1 mL were removed after 0 and 240 min for determination of total viable count when assaying for small intestinal transit tolerance.

**Antibiotic resistance:** Antibiotic resistance was examined by the disc diffusion method described by **Thirabunyanon and Boonprasom, (2009)**. The isolates to be tested were grown overnight in fresh MRS broth of pH 6.4 at 37 °C. About 100 µl of the suspension was spread onto the surface of the MRS agar plates. The antibiotic disks were inserted aseptically onto the agar surface. The plates were then incubated under anaerobic conditions for 24 h at 37 °C. The antibiotic resistance of the isolates was determined by calculating the diameter of the zones of inhibition around the antibiotic disks. The susceptibility was expressed in terms of resistance (R), intermediate susceptibility (I), and susceptibility (S) based on data from the National Committee for Clinical Laboratory Standards NCCLS, (2002).

In order to evaluate hemolytic activity of the isolates, fresh bacterial cultures were plated on Columbia agar base plates containing 5% v/v human blood. The plates were examined for signs of β- hemolysis (clear zones around colonies), α- hemolysis (green-hued zones around colonies) or γ- hemolysis (no zones around colonies) following 48 h incubation at 37°C. (**Mohamed et al., 2015**).

**Antimicrobial activity against Gram negative pathogens:** Antimicrobial activity was carried out according to agar well diffusion assay as described previously (**Rammelsberg and Radler, 1990**). *Escherichia coli* O157:H7, *Salmonella enterica*, (strain were kindly provided from bacterially laboratory, faculty of medicine, zagazig university) were cultured in brain heart infusion (BHI) broth for 24 h., then microbial density was adjusted to 10<sup>7</sup> CFU/mL and cultured on nutrient agar. *Lactobacillus delbruekii* ssp. *lactis* was grown in MRS broth under anaerobic condition at 37 °C for 20 h. Cell free culture supernatants (CFCS) were obtained by centrifuging the MRS broth at 10000 rpm. for 15 min. 100 µl of the CFCS was placed into the wells of the nutrition agar, there the plates were incubated at 37 °C for 15 h., The diameter of the clear zones around each well was measured. Strains with clear zones less than 11 mm, 11-16 mm, 17-22 mm and more than 23 mm, were grouped as negative ( - ), mild ( + ), strong ( ++ ), and very strong ( +++ ) inhibitor , respectively. Sterile MRS broth was used as negative control. Two major mechanisms of antimicrobial activity are production of organic acids, which reduce the pH and the production of hydrogen peroxide and bacteriocins replicates.



**Biochemical and molecular identification of the isolated strain:** Based on the results of the assays described above, one strain was identified using physiological and biochemical tests according to Bergey's Manual of Systematic Bacteriology vol.2 (**Holt and Krieg, 1984**). In order to confirm the result from biochemical characterization, *16S rRNA* sequencing was performed. Briefly, total DNA from isolated strain was extracted using (GeneJet genomic DNA purification Kit Thermo K0721 USA) according to the manufacturer's manual. Then, *16S rRNA* gene was amplified using the F (5' **AGA GTT TGA TCC TGG CTC AG** 3') and R (5' **GGT TAC CTT GTT ACG ACT T** 3') primers (**Eden et al., 1991**). The thermal cycler was programmed as follows: 10 min at 95°C; 37 cycles of 1 min at 95°C, 1 min at 65°C, 1 min at 72°C and 10 min at 72°C.

## Results

**Isolation and identification of potent probiotic strain:** the bacterial strain (*Lactobacillus delbrueckii* ssp. *lactis*) isolated from Qarish cheese was Gram positive, Catalase negative, non-motile rod was regarded as LAB and further characterized as potent probiotic strains. Identification of this strain using physiological, biochemical and molecular assays was performed.

**Acid and bile tolerance:** Acid tolerance assay showed that *Lactobacillus delbrueckii* ssp. *lactis* tolerated pH 2 after 120 min incubation with survival percentage of 80% compared to the control. The bacterial isolate treated with 0.3% bile salt, showed high resistant with survival rates 100% compared to bacteria treated without bile salt. the increment of O.D. for MRS broth with bile was equal to the increment of O.D. for MRS broth without bile.

**Resistance to condition simulating GI tract:** *Lactobacillus delbrueckii* ssp. *lactis* showed resistance to 100 µg/mL of lysozyme with 80% ( $2.03 \times 10^6$  CFU/mL) survival after 2 h. as compared to control having  $2.51 \times 10^6$  CFU/mL. While, the percent survival of *Lactobacillus delbrueckii* ssp. *lactis* showed resistance to 100 µg/mL of simulated gastric with 93% ( $1.61 \times 10^6$  CFU/mL) survival after 3 h. as compared to total viable count in control ( $1.72 \times 10^6$  CFU/mL) after 0 h. Our results revealed that the isolate *Lactobacillus delbrueckii* ssp. *Lactis* was highly susceptible to Panceratin solution (pH 8.0) while, the solution had less effect on survival rate of isolate *Lactobacillus delbrueckii* ssp. *lactis*. Isolate showed survival percentage of 31% ( $1.00 \times 10^6$  CFU/mL) survival after 4 h as compared to total viable count in control ( $3.10 \times 10^6$  CFU/mL) after 0 h, (**Diagram, 1**).

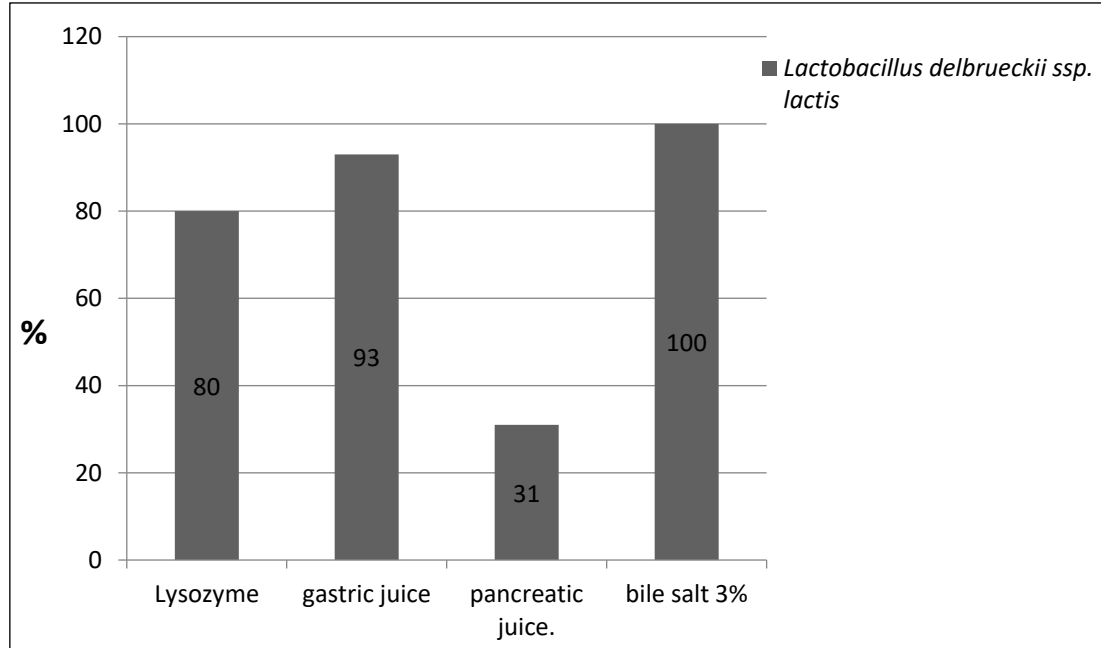


Diagram (1). Effect of lysozyme, gastric juice, pancreatic juice, bile salt 3% on survival rate of *Lactobacillus delbrueckii ssp. lactis*

**Antibiotic susceptibility and hemolytic activity:** The *Lactobacillus delbrueckii ssp. lactis* showed resistance to majority of antibiotics used in this study, it's showed 100% resistant to Nalidixic acid, Cloxacillin, Aztreonam and Colistin. **Table (1).** In addition, none of isolates exhibited hemolytic activity.

**Antimicrobial activity against Gram negative pathogens:** Antibacterial activity is an important feature of the probiotic strain. The isolate was checked for their antibacterial activity against two gastrointestinal pathogens, *E. coli O157* and *S. enterica*. The results revealed that *Lactobacillus delbrueckii ssp. lactis*. Inhibited both tested strain (*Escherichia coli O157:H7*, *Salmonella enterica*) with zone diameter more than 15 mm.



Table (1): Showed the *Lactobacillus delbrueckii* ssp. *lactis* resistant to antibiotic. R = Resistant, S = Sensitive, I = Intermediate Resistant.

Types of antibiotic	<i>Lactobacillus delbrueckii</i> ssp. <i>lactis</i>
Cefoperazone	I
Nalidixic acid	R
Penicillin G	S
Levofloxacin	I
Cloxacillin	R
Cefadroxil	I
Cefoperazone / sulbactam	S
Aztreonam	R
Gatifloxacin	I
Ampicillin	I
Rifampin	S
Colistin	R
Cefaclor	S
Flucloxacillin	R
Cefamandole	S
Erythromycin	S
Chloramphenicol	S
Clindamycin	S
Doxycycline	S
Carbenicillin	I
Streptomycin	R
Oxacillin	I
Amoxicillin / clavulanic acid	I
Imipenem	S
Amikacin	R
Netilmicin	I
Ceftazidime	I
Tobramycin	R
Gentamicin	I
Cefoxitin	R

### **Discussion and conclusion**

In the current work, Qarish cheese was examined in order to isolate LAB and some established *in-vitro* tests were applied to evaluate the probiotic potential of the isolates. The isolate was found to display potentially probiotic characteristics.



Identification of this strain using biochemical and molecular assays showed the presence of the *Lactobacillus delbrueckii ssp. lactis* as a potent probiotic strain in Qarish cheese. For a probiotic strain, survival under gastrointestinal environment condition is important criteria to be fulfilled which depends on tolerance to low pH and high bile concentration as well as resistance to gastrointestinal enzymes. Tolerance to extremely acidic condition is an important feature of a probiotic strain (**Guo et al., 2009**). *Lactobacillus delbrueckii ssp. lactis* isolated from Qarish cheese showed good tolerance to low pH showing its ability to survive under acidic environment of stomach. This result agreement with **Ashraf and Smith, (2016)**. The physiological concentration of bile in the small intestinal

appear able to grow in 0.3% bile salt during the third to the 4 hours after incubation and the OD620 were observed through this time to monitored viable colonies growth. Similar finding was reported by **Yavuzdurmaz, (2007)** who studied the tolerance of different strains of Lactic acid species and found that all of the probiotic bacteria isolates are resistant to 0.3% bile salt during 4 hours as they survive. The CFU values and OD620 were observed.

The present study showed that overall the 7 probiotic bacteria isolates (*Lactobacillus delbrueckii sp. lactis* resistance to lysozyme 100 µg/mL after 48 h. of incubation. This result similar with **Riaz, et al, (2015)** they found the LAB strains from dairy origin were to be resistant at all concentration of lysozymes. the lysozyme was used in different concentrations i.e. 200 µg/mL, 300µg/mL. The indigenous LAB strains were found to have growth in up to 300µg/mL concentration of lysozyme. Also, the current result showed that the *Lactobacillus delbrueckii ssp. lactis*, isolate tolerance to simulated gastric juice (pH 2) after 3 hours incubation. This result agreed with **Ashraf and Smith, (2016)** where they found that all tested strains exhibited survival during simulated gastric transit at pH 2 and pH 3 for 3 hours. An essential element in their selection is their ability to reach, survive and persist in the environment in which they are proposed to act.

where viability of these cultures is affected mainly by gastric acid present in the stomach. the strain exhibited survival during simulated gastric transit at pH 3 for 3 h.; however, viability was affected at pH 2. demonstrated survival rate more than 50% and retained viability during 3 hours exposure to pepsin pH 2 and are considered intrinsically tolerant to gastric transit.

The tolerance against simulated pancreatic juices (small intestinal transit), probiotic bacteria isolate in this study appear tolerance against pancreatic juices [ 1.0 mL of pancreatic (pH 8.0) juice and 0.3 mL NaCl (0.5% w/v)] after 4 hours





incubation. This result is similar with **Zamani et al, (2016)** who recorded that not dramatically decrease survivors of probiotic bacteria strains even after 4 hours incubation.

Frequent antibiotic administration causes gut microbiota imbalance and an increased susceptibility to infection was caused by opportunistic microorganisms (**Le Blanc et al., 2010**) Probiotic strains which are resistant to antibiotics can proliferate in gut and maintain microbial balance and reduce opportunistic microorganisms (**Guo et al., 2009**). The isolated of probiotic bacteria showed resistance to multiple antibiotics. It's showed 100% resistant to Nalidixic acid, Cloxacillin, Aztreonam and Colistin, this result agreement with **Ashraf and Shah, (2011) and Radulović et al, (2012)**, the result showed that no blood hemolysis was observed, indicative that they were non- pathogenic, this agreement with **Maragkoudakis et al, (2006)**.

During the present study *Lactobacillus delbrueckii* ssp. *lactis* which showed good tolerance when investigated for antimicrobial activity against the enteropathogenic bacteria (*Escherichia coli* O157:H7, *Salmonella enterica*), appeared clear inhibition zone around the each well. The diameter of the inhibition area was proportional to the antimicrobial activity of probiotic bacteria. The inhibition of *Lactobacillus* against these pathogenic bacteria had been reported in previous studies (**Ammor et al., 2006; Tulumoglu et al., 2013; Asurmendi et al., 2015**).

The use of bacteriocin- producing LAB could play a role in controlling colonization by pathogenic and spoilage bacteria in food processing facilities, they may lead to inhibition or killing the pathogenic bacteria.

*Lactobacillus delbrueckii* ssp. *lactis* as a potential probiotic strain was isolated from Qarish Cheese, its probiotic features were characterized. This isolate showed tolerance to high bile concentration, low pH and survived under condition simulating human gastrointestinal tract. Thus, it could be predicted that the isolate would be able to pass stomach and reach intestine in adequate amounts. In addition, this strain displayed a good antibacterial activity against two Gram negative food-borne pathogens. Thus, *L. delbrueckii* ssp. *lactis* could be considered as a good probiotic candidate. However, further investigations including in-vivo experiments as well as molecular analysis would be helpful to elucidate its potential health benefits.

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## الخلاصة

البروبيوتيك هي كائنات حية دقيقة غير الممرضة ذات اثر مفيد، عندما يتم استهلاكها بكميات كافية . وتعتبر منتجات الألبان التقليدية مصادر جيدة للبروبيوتيك. تهدف الدراسة الحالية إلى عزل بكتيريا حمض اللاكتيك من منتجات الألبان (جبنه قريش) في محافظة الشرقية بمصر وتقييم إمكاناتها الحيوية .

تم عزل اللاكتوباسيلس عن طريق زراعة العينات على بيئة (MRS). تم فحص العزلات لإمكانية اظهارها كبروبيوتيك باستخدام الاختبارات في المختبر، بما في ذلك المقاومة مع الحمض والصفراء، والبقاء على قيد الحياة في ظروف الجهاز الهضمي، والنشاط الانحلالي وكذلك الحساسية للمضادات الحيوية. وبالإضافة إلى ذلك تم تحديد النشاط المضاد للبكتيريا من سلالات معزولة ضد الايشريشيا القولونية O157:H7 والسالمونيلا المعوية .

اظهرت لاكتوباسيلس ديلبروكي تحت جنس لاكتس اعلى درجة من المقاومة لدرجات الحموضة المنخفضة، والصفراء وظروف الجهاز الهضمي . أظهرت هذه السلالة مقاومة ضد المضادات الحيوية , حمض ناليديكسيك، كلوكساسيلين، أز تريونام وكوليستين , وكذلك فعالية النشاط المضاد للبكتيريا ضد اثنين من مسببات الأمراض السلبية غرام والتي هي محللة للدم. وأخيرا ، تم تعريف سلالة لاكتوباسيلس ديلبروكي تحت جنس لاكتس. باستخدام الطرق البيوكيميائية والجزئية.

في الدراسة الحالية، عزلت بكتيريا لاكتوباسيلس ديلبروكي تحت جنس لاكتس من منتجات الألبان (جبنه قريش). حيث أظهرت البكتيريا خصائص بروبيوتيك جيدة.