Cumhuriyet Sci. J., 44(1) (2023) 1-6
DOI: https://doi.org/10.17776/csj.1128138

Cumhuriyet Science Journal

| csj.cumhuriyet.edu.tr | Founded: 2002 ISSN: 2587-2680 e-ISSN: 2587-246X Publisher: Sivas Cumhuriyet University

Screening of Probiotic Properties of *Bacillus Licheniformis* Isolated from Yoghurt

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Research Article

History
Received: 09/06/2022
Accepted: 27/12/2022

ABSTRACT

Probiotic microorganisms are an indispensable part of a healthy life. The usage areas of the probiotic *Bacillus licheniformis* bacteria are quite wide, from contributing to the development of the immune system to reducing cholesterol, which is an important cause of cardiovascular diseases. In this study, *Bacillus licheniformis* (*isolate 29)* was isolated from traditional yoghurt sample obtained from town of Hatipli/ Tokat/ Türkiye. And it's genotypic and probiotic characterizations were performed. Our results showed that the isolated *Bacillus licheniformis* grew better at pH 5. The survival rate of the bacteria in artificial gastric and intestinal fluid was determined as 47.51% and 65.29%, respectively. The isolate degraded all tested sodium salts, including Sodium glycocholate hydrate, Sodium taurodeoxy Cholate, Sodium taurocholic acid, Sodium tauroglyco Cholate, Sodium thioglycolate. Its surface hydrophobicity was found to be moderately 30.31 ± 0.009% in *n*-Hexadecane. Although RA5 antibiotic had the largest zone diameter (10 mm), antibiotic susceptibility of *Bacillus licheniformis* was not among the antibiotic discs tested. These findings indicated that the isolate can be a good probiotics candidate to lower the cholesterol levels.

Keywords: Cholesterol, *Bacillus licheniformis*, Probiotic, Sodium salts, Yoghurt

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Introduction

Probiotic organisms are defined by the World Health Organization/Food and Agriculture Organization of the United Nations as living organisms that, when consumed in sufficient quantities, make some adjustments in the large intestine of the consuming person and contribute positively to host health [1, 2]. Although there are many different classes of probiotic microorganisms, lactic acid bacteria (LAB) are one of the largest groups including *Lactobacillus*, *Lactococcus*, *Carnobacterium*, *Enterococcus*, *Streptococcus*, *Pediococcus*, *Propionibacterium*, and *Leuconostoc* [3, 4]. The use of LAB bacteria in the production of dairy products such as cheese and yogurt has a long history [5, 6].

Although the first study on *Bacillus licheniformis* was made in 1945, it has found the expected interest in the last 20 years. The number of studies over the last 20 years resembles a bacterial logarithmic growth curve [7]. *Bacillus* and its metabolites have been used in biotechnological applications such as enzymes, amino acids, antibiotic production, preparation of fermented foods, and insect control production for many years [7]. *Bacillus* species gram positive rod shape [8] may have advantages over different probiotic bacteria species due to their ability to form endospores. For example, in the preparation of high temperature fermented products, the survival potential of the bacteria is high. *B. subtilis, B. licheniformis, B. clausii, B. coagulans, B. cereus, B. pumilus*, and *B. laterosporus* are some species which used human consumption as probiotics [9, 10]. Biosporine® is a probiotic product consisting of a mixture of *Bacillus subtilis* and *Bacillus licheniformis*, commercially available in Russia and Ukraine today [8, 9, 11]. Primal Defense™ (USA), MegaSporeBiotic™ (USA), Prescript-Assist® SBO Probiotic (USA), Body biotics (UK), Zhengchangsheng®(Korea) are other commercial products including *Bacillus licheniformis* for human consumption [7].

Probiotic bacteria are important for human health because of killing pathological microorganisms, increase the immune system, and regulate the cholesterol level in the blood vessel. Cholesterol in the blood increases the risk of cardiovascular disease by causing vascular occlusion. This disease is known as hypercholesterolemia. It is estimated that a 1% decrease in serum cholesterol level will cause a 2% to 3% reduction in cardiovascular occlusion [12]. Cardiovascular disease is one of the leading health issues with high mortality rates. By 2030, the disease course is predicted to nearly triple [13]. Some probiotic bacteria could reduce cholesterol levels by digesting bile salts, which are the precursors of cholesterol. Mechanisms of cholesterol reduction by probiotic bacteria are hypothesized in the literature [14]. In the mouse experiment, it was observed that the body fat accumulation decreased and the lipid metabolism rate decreased in mice fed with yoghurt containing *Bacillus licheniformis.* These bacteria have bile salt hydrolysis enzyme activity [7, 15, 16]. Other potential way can be decreasing of the cholesterol solubility to prevent the uptake from gut [12]. In today's study on a mouse model, it is seen that *Bacillus licheniformis* bacteria inhibit obesity by regulating intestinal flora [17].

In this study, *Bacillus licheniformis* were purified as a new isolate from the yoghurt sample of the town of Hatipli/Türkiye and some probiotic properties such as pH stability, surface hydrophobicity, antibiotic sensitivity, and degradation of sodium salts were determined.

Material Methods

Bacillus licheniformis was isolated from single yoghurt sample from Hatipli/Tokat/Türkiye (40.547117, 37.228158). LB (Luria−Bertani) broth (CONDA), Agar Fluka (05039) (sigma). Pepsin (sigma), trypsin from beef pancreas (thermo), Sodium glycocholate hydrate, Sodium taurodeoxy Cholate, Sodium taurocholic acid, Sodium tauroglyco Cholate (*Chemcruz* D1317), Sodium thioglycolate (Sigma 101851207), antibiotic discs (Bioanalyse), n-Hexadecane (Merck 596470), Xylene (sigma16446), used in our experiments. The others used chemicals are analytical grade. The all experiments were made as triplicate.

Isolation and Identification of Bacillus Licheniformis

Bacillus licheniformis was isolated from traditional yoghurt sample and kept at +4 °C. An overnight cell culture was obtained by inoculation an aliquot of the sample into 10 ml LB broth (pH 7.2), and incubated at 37 °C. Single bacterial colonies were grown from the overnight culture by using the pour-plate method. Some of the colonies were then transferred onto LB-agar plates. The pure cultures were stored at −80 °C. For the molecular characterization, we bought service from BM Company at Ankara/Türkiye [18]. DNA was prepared and used for the amplification of 16S rRNA gene as general steps. The sequences obtained from an automatic DNA sequencer were subjected to BLAST analysis and similarities were determined using the National Center of Biotechnology Information databases (http://www.ncbi.nlm.nih.gov) [19]. An accession number for the sequence was also obtained (as ON496990).

Survivals of Bacterial Strain in Low pH

Bacillus licheniformis species were growth in 10 ml sterile LB broth pH 7.2 with shaking at 110 rpm at 37 °C during overnight [20]. Ten milliliters of the bacteria were centrifuged for 10 min at 4,000 rpm at +4°C. The pellet was suspended in fresh LB broth (pH 7.2, pH 5, pH 4, pH 3, and pH 2). Bacterial survival in pH 7.2, pH 5, pH 4, pH 3, and pH 2 was determined. Their growing absorbance values were recorded versus time. Cell growth rates were presented as growth curves with standard deviation. The experiment was made triplicate.

Survival in Simulated Gastric Juice

The survival rate of the bacteria in the simulated gastric juice was determined. For this, 10 ml sterile and fresh 1^x PBS pH 2 solution with 3 g/L concentration of pepsin was prepared [19]. The solution was passed through a 0.45 µm filter. Concentrated bacterial culture supernatant was poured out. 10 ml of prepared artificial gastric juice was added to it. Serial dilution was made in 4.5 ml sterile saline (0.85%) by taking 0.5 ml sample. 100 µl were cultivated from various dilute tubes. After spreading the sample on the LB agar plate by smear method, it was kept at 37 °C every other day. The exposed colonies were counted. After the bacteria were waited for 5 hours at 37 °C in the artificial stomach environment, the sample count was done again. The survival rate of the bacteria was calculated with the following formula [21].

$$\text{Survival rate \%} = (N1 / N0) \times 100 \quad (1)$$

Here (N0= Total bacterial number at first time in simulated gastric juice and N1 = Total bacterial number after 5 hours in simulated gastric juice).

Survival in Simulated Intestinal Juice

The survival rate of the bacteria was determined in the simulated intestinal juice. For this, 10 ml sterile and fresh 1^x PBS pH 8 solution with a concentration of 1 g/L of trypsin was prepared [19]. The solution was passed through a 0.45 µm filter. Concentrated bacterial culture supernatant was poured out. 10 ml of prepared artificial intestine juice was added to it. Serial dilution was made in 4.5 ml sterile saline (0.85%) by taking 0.5 ml sample. 100 µl were cultivated from various dilute tubes. After spreading the sample on the LB agar plate by smear method, it was kept at 37 °C every other day. The exposed colonies were counted. After the bacteria were waited at 37 °C for 24 hours in the artificial intestinal environment, the sample count was repeated. The survival rate of the bacteria was calculated with the formula above (simulated gastric juice).

Antibiotic Sensitivity

Disc diffusion method was used according to our previously published article [22]. Fresh LB agar (1.5%) medium was prepared and 100 µl bacterial cultures was spread on the agar plates. Antibiotic discs included kanamycin (K30), ampicillin (AM10), streptomycin (S10), tetracycline (TE30), gentamicin (CN30), chloramphenicol (C30), penicillin (P2 units), erythromycin (E15), rifampin (RA5), neomycin (N30), vancomycin (VA30), and empty disc (00). The antibiotic discs were placed on the agar surface. After overnight incubation under the optimum growth conditions, dimeters of inhibition zones were measured with a ruler and the measurements were recorded.

Surface Hydrophobicity

Surface hydrophobicity of bacteria was investigated using reference articles in the literature [23, 24]. The bacterial sample was freshly propagated and the culture aliquoted in 3 ml aliquots in sterile falcon tubes. Cells were centrifuged (10 minutes at 4000 rpm at +4°C). The supernatant was decanted and 10 ml of phosphate urea magnesium sulfate buffer (pH 6.5) was added. After the pellet was dissolved, centrifugation was performed under the same conditions. This process was repeated 3 times.

Initial cell densities were determined at 450 nm, and then 0.6 ml of n-Hexadecane, n-Hexane and xylene were slowly added to the cell suspensions (3 ml). The mixed solution was placed in a 37°C water bath and incubated for 15 minutes. The samples were then vortexed for 15 seconds at 2 minute intervals and left on the bench for 25 minutes. Absorbance values were recorded at 450 nm. The percent hydrophobicity was calculated using the following formula:

$$\text{Hydrophobicity}\,\% = ((OD_{450nm}\,N0 - OD_{450nm}\,N1)/OD_{450nm}\,N0) \times 100 \quad (1)$$

(OD_{450nm} N1: the absorbance value for final bacteria concentration, after the experiment, OD_{450nm} N0: the absorbance value for initial bacteria concentration before the experiment).

Degradation of Sodium Salts

The isolate was grown overnight on LB agar at 37 °C. Sodium salts, 0.005 g/ml, (Sodium glycocholate hydrate, Sodium taurodeoxy Cholate, Sodium taurocholic acid, Sodium tauroglyco Cholate, Sodium thioglycolate), MRS (MAN, ROGOSA and SHARPE) agar were prepared at pH 6.3. The colonies formed were immersed in sodium salt solid medium. Incubated under optimum conditions. The activity of the *Bacillus licheniformis* against sodium salts was checked. The white transparent structure formed around the colony was determined as positive [14, 22, 23].

Results

Genomic Characterization

Genotypic characterization studies of pure culture isolated from yoghurt culture were carried out. *Bacillus licheniformis* was determined as a result of NCBI BLAST analysis (Figure 1).

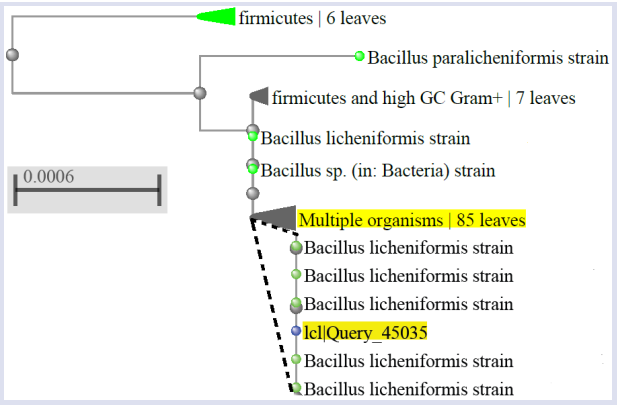

Figure 1. The dendrogram result of *Bacillus licheniformis*.

Tolerance to low pH

The growth graph of bacteria in low pH environments is indicated in figure 2. No growth OD increase was observed in the bacterial pH 2 medium. It is seen that bacterial growth is better in the pH 5, 4, 3 than pH 7.2 medium.

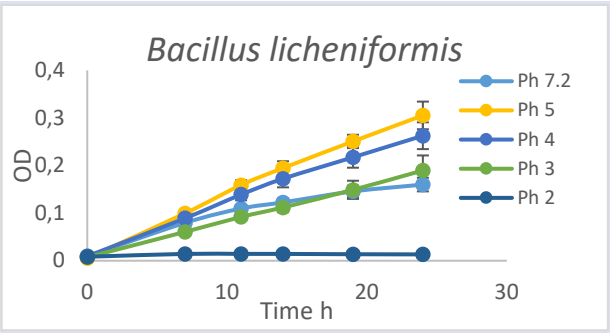

Figure 2. Bacterial growth in different pH including 7.2, 5, 4, 3, and 2. The absorbance values were recorded by the spectrophotometer at different intervals for 24 hours.

Survival Rate in Simulated Gastric and Intestinal Juice

The survival rate of bacteria in artificial gastric and intestinal fluid was determined as 47.51% and 65.29%, respectively (Table 1 and Figure 3).

Bacillus licheniformis	Initial bacterial number (log(CFU/ml))	Final bacterial number (log(CFU/ml))	Survival rate
Simulated gastric juice	21.91 ± 1.14	10.41 ± 0.31	47.51%
Simulated intestinal juice	14.55 ± 1.22	9.5 ± 0.78	65.29%

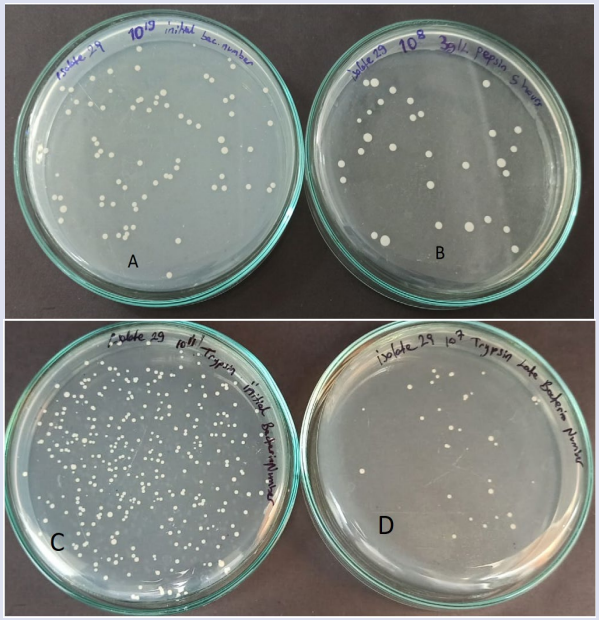

Figure 3. The test results of the survival of bacteria in the artificial stomach and intestinal environment. A) The number of initial colonies in the artificial stomach medium, B) The number of bacteria cultivated after 5 hours in the artificial stomach medium, C) The initial number of bacteria in the artificial intestinal medium, D) The number of bacteria cultivated after 24 hours in the artificial intestinal medium.

Antibiotic Sensitivity

The bacteria were found to be resistant to the tested antibiotics (figure 4). The best inhibition zone was seen with RA5 antibiotic (10 mm).

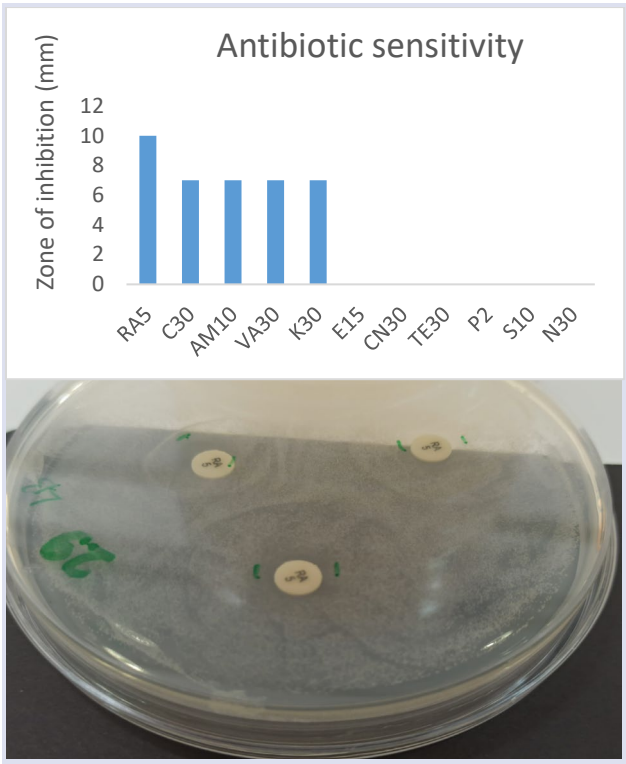

Figure 4. The antibiotic susceptibility test results of *Bacillus licheniformis* (above). Indicates the zone diameter measured on the LB agar plate (below).

3.4 Surface Hydrophobicity

Among the tested hydrophobic solvents, the surface hydrophobicity values of the bacteria were 31.75 ± 0.014%, 30.31 ± 0.009%, 34.87 ± 0.008% n-Hexane, n-Hexadecane, Xylene, respectively (Table 2).

Table 2: Bacterial surface hydrophobicity

Hydrophobic solvent	n-Hexane	n-Hexadecane	Xylene
Surface hydrophobicity %	31.75±0.014	30.31 ± 0.009	34.87±0.008

Degradation of Sodium Salts

Table 3: Degradation ability of sodium salts by *Bacillus licheniformis*

Sodium Salts	Sodium glycocholate hydrate	Sodium taurodeoxy Cholate	Sodium taurocholic acid	Sodium tauroglyco	Sodium thioglycolate
Bacillus licheniformis	+	+	+	+	+

Considering the result of the bacteria in terms of its ability to break down sodium salts (Table 3 and Figure 5), it was seen that it could destroy all the tested salts.

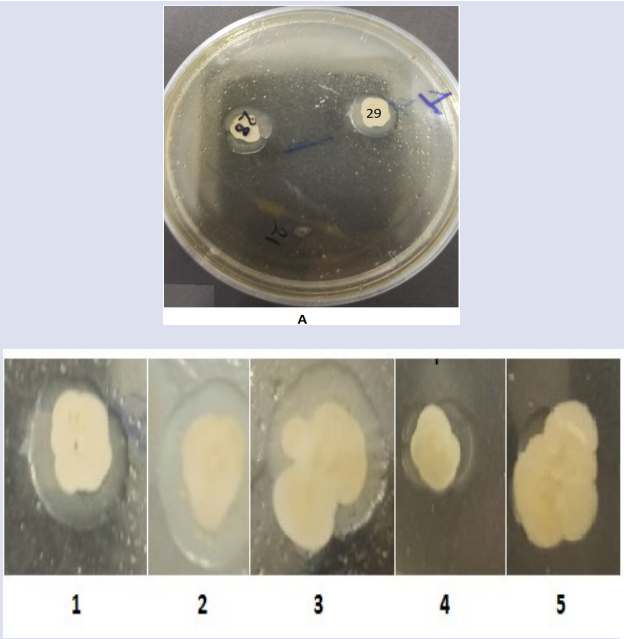

Figure 5. The degradation of sodium salts by the bacteria. A) Shows the colony structure and transparent zone formed by the bacteria grown on MRS agar with specific sodium glycocholate hydrate medium. 1) Sodium glycocholate hydrate, 2) Sodium taurodeoxy Cholate, 3) Sodium taurocholic acid, 4) Sodium tauroglyco Cholate, 5) Sodium thioglycolate.

Discussion

As a result of the genotypic characterization of the bacterial strain obtained from the yoghurt culture, it was determined that it belonged to the *Bacillus licheniformis* bacterium. The bacteria, which are probiotic strains, have been used in previous studies. In addition, there are *Bacillus licheniformis* probiotic bacteria obtained from different traditional foods [9, 25].

A good probiotic bacteria must be able to survive or tolerate acidic stress conditions and the presence of bile salts [26]. When the growth curve of the bacteria is examined in extreme environments, it is seen that it performs the best growth at pH 5 among the tests. In pH 4 and pH 3 environments, the growth curve of the bacteria is better than pH 7.2. These data show that the bacteria can multiply in an acidic environment. For the pH 2, there was no increase in bacterial absorbance during the tested time.

When the logarithmic survival results of the bacteria in the artificial stomach and intestinal environment were evaluated, it was observed that their survival rates were 47.51% and 65.29% respectively. In the literature, it was observed that no *Bacillus licheniformis* after incubation for 90 minutes [27] and above 90% at 3 h incubation in simulated gastric juice at pH 2 [28]. According to Niu, K. M et al., it has been shown to fully survive after 3 hours in a

pH 2.5 acid environment [29], it can be said that the isolate is better than various isolates in terms of survival in the artificial stomach environment. This is the desired property of probiotic bacteria.

The bacteria was found to be resistant to all antibiotics tested. Although the rifampin antibiotic has the most effective zone diameter among the antibiotics tested, the bacteria are still resistant. In a similar study performed by R. Sharma et al., it was concluded that *Bacillus licheniformis* was resistant to 16 tested antibiotics [27]. Patients receiving antibiotic therapy against *Bacillus licheniformis* show antibiotic resistance, which is an important probiotic feature for the survival of *Bacillus licheniformis [30]*.

Hydrophobicity studies are important for understanding the adhesion of bacterial cells to surface epithelial cells. Adhesion to surface epithelial cells results from the interaction between bacterial cell and epithelial cell. This is also associated with hydrophobicity and autoaggregation properties. The interaction between bacteria and epithelial cells may be caused by proteins and exopolysaccharide molecules reflecting the auto-aggregation feature. Stronger binding may be due to hydrophobic interaction [31]. As a result of surface hydrophobicity, the bacteria appears to be moderately hydrophobic. Similar results were obtained by P. Shobharani et al. [32] and Khan, Md Idrish Raja, et al. [33]. However K. Ragul et al., obtained better results in terms of surface hydrophobicity [26].

The bacteria are very effective against sodium salts. It can break down the precursors of cholesterol [34]. This means that it can contribute to the reduction of cholesterol level. It was observed that lipid metabolism accelerated and body fat accumulation decreased in the rat model fed with yoghurt sample containing *Bacillus licheniformis* bacteria [35]. It was concluded that the G.T. Cao et al., study's decreased the final body weight in mice, increased glucose intolerance and decreased hepatic fat accumulation [17]. It has been observed by Mahdi et al., that *Bacillus licheniformis* bacteria have a significant effect on lowering the cholesterol level [36].

Conclusion

The *Bacillus licheniformis* grew in acidic environments. The survival rate of bacteria in artificial gastric and intestinal fluid was good. It degraded all tested sodium salts, including sodium glycocholate hydrate, sodium taurodeoxycholate, sodium taurocholic acid, sodium thioglycolate. These results can meaning as cholesterol lowering bacteria. Its surface hydrophobicity was found to be moderately. As a result, it can be stated that the isolated bacteria is a good probiotic organism according to the tested parameters.

Acknowledgement

Thanks to my mommy because of preparation of traditional dairy yoghurt.

Conflict interests

The author(s) declared no potential conflicts of interest.

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