Isolation, characterization, and molecular identification of probiotics showing promising hypoglycemia operating activities

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Received: 22 September 2022
Revised: 22 October 2022
Accepted: 22 October 2022
Published: 21 February 2023

Egyptian Pharmaceutical Journal 2023, 22:105–110

Background
Probiotics are the most useful microorganisms for animal and human health; they are used in the pharmaceutical and food industries for many products that enhance digestion and immunity.
Objective
The objective of our study was to isolate, characterize, and identify a probiotic bacterial strain and determine its hypoglycemia operating parameters.
Materials and methods
Our research was carried out through the isolation of probiotic colonies from milk samples on MRS medium. Bacterial isolates were characterized both morphologically and biochemically. The collected bacterial isolates were tested for their low pH tolerance on phosphate buffer pH 2.0 and bile salt tolerance in MRS-THIO liquid medium. Glucose assimilation activity was tested by measuring the residual glucose concentration on MRS liquid medium at 37°C after 24 and 48 h by GOD-PAP enzymatic colorimetric method. The initial glucose concentration was 500 mg/dl. The most potent isolate was identified by methods of 16S-rDNA sequencing.
Results and conclusion
Twenty-one bacterial isolates were isolated and characterized. Bacterial isolates showed the highest resistance to acidic pH 2.0 and they were bile-tolerant. Results of glucose assimilation showed that there was a marked increase in sugar consumption rate after 48 h more than 24 h in most of the bacterial isolates. The top 10 isolates were selected for the testing of the rest of the parameters. Results show that no noticeable differences were observed in the consumption of glucose in the low-glucose concentration, but with more glucose concentration more glucose consumption rate differences were recorded among organisms. The top two organisms that have the ability to reproduce and consume glucose even in high-glucose concentrations were Ab 9 and Ab 2 with results of glucose residual concentration of 108 and 124 mg/dl, respectively. The phylogenetic tree showed that the most potent isolate Ab 2 was identified as *Lactobacillus brevis*.

Keywords:
16S-rDNA, acid and bile tolerance, glucose assimilation, probiotics

Egypt Pharmaceut J 22:105–110

1687-4315

Introduction

Probiotics are defined as 'living microorganisms which, when consumed in adequate numbers, can provide health benefits to the host [1].' In general, the health benefits of probiotics in treating various diseases have been reported in many scientific kinds of research. Those diseases include inflammatory bowel disease, irritable bowel syndrome, constipation, and antibiotic-associated disorders. Also, they have a strong impact on treating diseases like allergy-related conditions, hypertension, diabetes, and diarrhea [2].

Commercially available probiotic bacteria are from the *Bifidobacterium*, *Streptococcus*, *Lactobacillus*, and *Enterococcus* genera. Probiotics are strains that possess several properties, such as tolerance to gastrointestinal conditions (gastric, intestinal, and low bile low concentration, and pH conditions); they can adhere to epithelial cells and have antimicrobial properties. They can also assimilate cholesterol in the human intestine, are capable of bile salt hydrolysis, and are completely safe to be used for humans. On the other hand, they can tolerate the fermentation process and long storage periods [3,4]. However, potential probiotics do not have to possess all the above characteristics. Industrial characteristics such as tolerance to heat treatment, particularly spray drying, are also preferable [5].

The balance of the gut flora in humans plays an important role in the expression of overweight as a symptom associated with insulin resistance (type 2 diabetes) as reported by Ley *et al.* [6]. The

imbalance of the gut flora causes body weight gain and increases insulin resistance as proven by many data from the reported research on humans and laboratory mice [7]. Probiotics are used in food and food supplement industries to adjust the percentage of beneficial gut flora organisms as the assumed mechanism to prevent obesity and diabetes. An example of commercial probiotics used for this purpose is probiotic-supplemented fermented milk 'Dahi' (yogurt) that can protect from streptozotocin-induced diabetes in animal models and also suppress diet-induced insulin resistance. It was also observed that the probiotic found in Dahi enhanced the antioxidant properties of the food product to prevent more diabetes progression and complications [8]. The present study aimed to isolate, screen, and identify a potential probiotic with hypoglycemia activity and the effect of some operating parameters on its hypoglycemia activity.

Materials and methods

Isolation and screening of probiotic bacteria

A total of five samples from two origins of cow and sheep milk were collected from a rural area of Giza, Egypt. Five milliliters of each milk sample was suspended in 20 ml sodium citrate solution (pH 7.0) and homogenized for 2 min. Then, 1 ml of each of the samples was added to 10 ml of MRS broth and incubated for 24 h in aerobic conditions at 37°C. Finally, 0.02 ml of those diluted solutions was spread for 48 h on MRS agar media [9]. Single colonies produced on the growth agar plates were selected and transferred to 15 ml of broth culture medium and incubated for 24 h at 37°C. The pure isolates were characterized using Gram stain, cell morphology, and catalase reaction according to standard procedures [10]. Gram-positive and catalase-negative isolates were selected and stored in 25% (w/v) glycerol at −70°C for further assessments.

Acidic pH tolerance

Screening of isolated bacteria was done according to Conway *et al.* [11] for selecting the low pH-tolerant isolates. For this purpose, 50 µl of the respective stock cultures of the probiotic was incubated in 5 ml MRS broth at 37°C for 24 h. Each isolate culture medium was centrifuged at 5000 rpm for 15 min, the supernatants were removed, and the bacterial cells were resuspended for 3 h in 1 ml PBS (pH 2.0) at 37°C. Each sample was added to MRS agar medium Petri dishes by the pour plate method and then incubated for 24 h. Results were recorded, and the number of produced colonies was compared with the control sample incubated in normal MRS broth (pH 7.4) for 3 h, the bacterial survival rates were calculated using the following equation:

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

The total viable counts of bacterial isolates in MRS agar medium after treatment with the acids are represented as N1, while N0 represents the total viable counts of isolates before incubation in low pH conditions.

Bile salt tolerance

The tolerance of bacteria to high bile concentration, described by Walker and Gilliland [12] was used. Fifty microliters stock cultures of the probiotic bacterial isolates were incubated in a 5 ml MRS growth medium at 37°C for 24 h. Then, the respective bacterial isolates (50 µl) were resuspended in 5 ml MRS-THIO broth containing MRS supplemented with 0.3% (w/v) sodium thioglycollate for 3 h at 37°C. The bacteria survival rate of the treated cells was valuated using the pour plate technique on MRS agar at 37°C after 24 h incubation. The survival rate for bile resistance was calculated using the following equation:

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

Total colonies after treatment with bile salts are represented as N1 while N0 represents the total colonies before incubating in bile salt conditions.

Screening for selection of the promising probiotic-lowering glucose

Twenty-one organisms were cultured on MRS broth media, each organism was cultured separately in 10 ml of MRS broth media in a closure cap test tube, and incubated at the optimal incubational temperature of 37°C for 24 h, then glucose consumption was evaluated from each organism by calculating the residual glucose concentration in the broth medium of each organism compared with the control. The control was 10 ml of sterile MRS broth medium adapted and incubated at the same conditions as the bacterial isolate test tubes.

Glucose assay using GOD-PAP enzymatic colorimetric method

Glucose was determined after enzymatic oxidation in the presence of glucose oxidase. The formed hydrogen peroxide reacts under the catalysis of peroxidase (PAP) with phenol and 4-aminoantipyrine to form a red violet quinoneimine dye as an indicator. Supernatants of cell-free culture mix were incubated for 10 min at 37°C or 20 min at 15–25°C. Measure absorbance of the

specimen (A specimen) and standard (A standard) against reagent blank within after minutes [13] according to the equation:

$$\text{Glucose concentration(mg/dl)} = \text{A specimen/A standard} \times 100$$

Molecular identification

LAB identification was carried out using 16S-rDNA sequence analysis of selected strains, which was amplified by the PCR procedure described by Ayyash *et al.* [14]. PCR primers 27 F (5′-AGA GTT TGA TCC TGG CTCAG-3′) and 1492 R (5′-TAC GGY TAC CTT GTT ACGACTT-3′) were used for amplification.

Results and discussion

Isolation and characterization of probiotic bacteria

In the present work, milk samples were selected to isolate probiotic bacteria. The samples were gathered from different sources to get a wide diversity of bacterial strains.

Cow and sheep milk were used for probiotic screening and isolation. The selection of bacterial isolates was based on the typical appearance in the morphology of the produced colonies. For identification, biochemical characterization and molecular techniques were used.

All 21 isolates grown at 37°C in MRS agar under aerobic conditions produced colonies that were hemispherical white or achromatic. Each colony was separately propagated for further assessment.

The 21 isolates were Gram-positive, bacilli in shape, and negative for catalase; these isolates were subjected to further examination. These preliminary characteristics agreed with probiotics isolated by Axelsson [15] and Arasu *et al.* [16].

Table 1 shows the acid-tolerance study for 21 isolates after exposure for 3 h to pH 2.0. Strain Ab1 was the most resistant to the acid environment among the isolates. All the isolates showed better tolerance to acidic pH of 2.0, as compared with normal conditions; isolate H 21 showed the lowest survival rate at pH 2.0 which was 55% survival rate.

The survival under the environmental conditions of the gastrointestinal tract is an important characteristic feature of the isolate for potential use as a probiotic. Thus, the probiotic properties such as bile resistance and acid tolerance were assessed to examine their ability to survive the stomach and upper intestine acidic conditions [17]. Our isolates showed the ability to pass the basic criteria for probiotic features as they can tolerate pH 2 and growth in 0.3% bile salts. Bile-salt tolerance varies significantly among the probiotic species even between strains themselves. Probiotics' resistance to bile salt can be attributed to the activity of the enzyme BSH, which is responsible for the degradation of bile salts lowering its toxic effect [18].

Table 1 Isolation and characterization of probiotic bacteria

Origin	Isolates code	Low-pH survival (%)	Bile salt survival (%)
Cow milk	Ab 1	86	94
Cow milk	Ab 2	82	88
Cow milk	Ab 3	78	91
Cow milk	Ab 4	65	85
Cow milk	Ab 5	70	79
Caw milk	Ab 6	66	81
Cow milk	Ab 7	73	92
Cow milk	Ab 8	65	87
Cow milk	Ab 9	71	94
Cow milk	Ab 10	65	79
Cow milk	Ab 11	69	85
Cow milk	Ab 12	42	67
Cow milk	Ab 13	59	74
Cow milk	Ab 14	76	83
Sheep milk	H 15	73	90
Sheep milk	H 16	71	69
Sheep milk	H 17	47	83
Sheep milk	H 18	65	87
Sheep milk	H 19	78	91
Sheep milk	H 20	71	89
Sheep milk	H 21	55	75

In our work, data revealed that most of the strains could survive in the presence of different concentrations of bile salts ranging from 0.3 to 1.0% after 3 h of incubation in addition to their ability to hydrolyze the bile acids. The isolates proved their ability to grow at different pH values ranging from 2.0 to 7.0. The results obtained are in agreement with Soliman *et al.* [19] using 21 isolates of Lactobacilli from Egyptian milk and showed that they can tolerate acid and bile salts.

The ability of the probiotic strains to tolerate salt concentration is commonly used in the selection of potential probiotic candidates. Our data agree with that reported by Adnan and Tan [20].

As evident from Table 1, the survival of selected isolates was also examined by the difference in viable cell counts after 3 h of incubation in MRS containing 0.2% bile salts. As given in Table 1, isolates Ab1 and Ab 9 showed the best survival rate (94% at 0.2% bile salt). Also, in other isolates, Ab 12 is the lowest survival rate (67% at 0.2% bile salt).

Screening to select the most potent isolates lowering glucose and final pH

The aim of the present experiment was to establish the capacities of 21 isolates originating from sheep and cow milk microflora to hypoglycemia activity. Results given in Table 2 revealed that all tested strains were efficient hypoglycemia isolates. After screening for glucose removal activity, the most potent (Ab2, Ab4, Ab 9, Ab 13, and Ab 14) were selected; *Lactobacillus* isolates can remove glucose. The high assimilation results are listed for Ab 9 isolate.

Hypoglycemia operating parameters

Effect of incubation period on glucose reduction using bacterial isolates

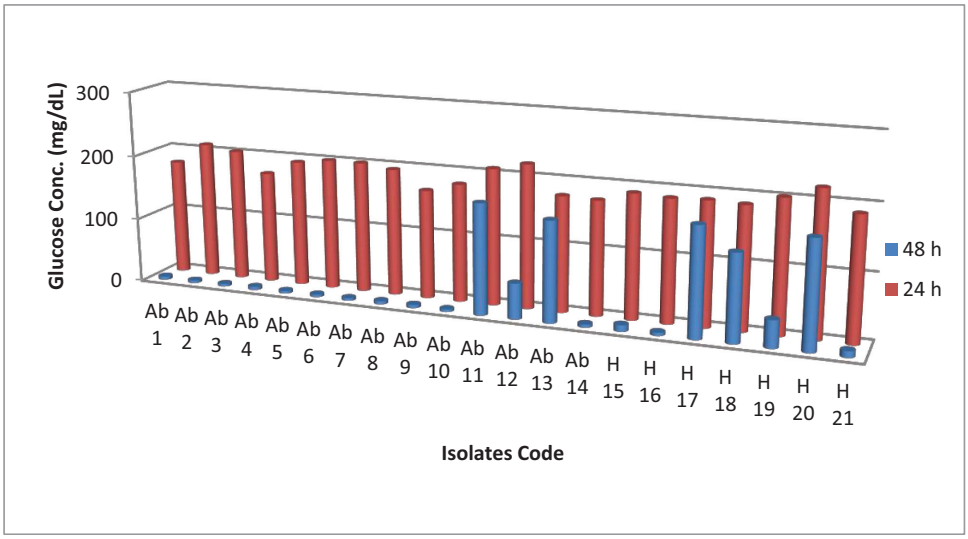
The results in Fig. 1 show that after 24 h of probiotics incubation it was observed that some organisms were much faster than others in adaptation to consumption of glucose in the MRS medium, which may act as a good factor in our screening according to the fast response to glucose assimilation.

Results show that after 24 h of probiotic isolate incubation, differences in glucose consumption were observed. The best organism in consuming glucose was Ab 9 after 24 h of incubation where the glucose concentration reached 167 mg/dl compared with 212 mg/dl in the control sample. Results after 24 h of incubation show that there is no noticeable difference among most of the organisms in glucose consumption. Results of glucose assimilation after 48 h show that there was marked difference between glucose consumption in most of the organisms on one hand and between the sugar consumption rate between 24 and 48 h.

Table 2 Screening to select the most potent isolates for lowering glucose

No.	Isolates code	Residual glucose concentration (mg/dl)	Final pH
Standard control		212	6.5
1	Ab 1	210	5
2	Ab 2	178	4
3	Ab 3	203	5
4	Ab 4	172	5
5	Ab 5	194	5
6	Ab 6	201	5
7	Ab 7	201	5
8	Ab 8	195	6
9	Ab 9	167	5
10	Ab 10	181	5
11	Ab 11	209	4
12	Ab 12	220	5
13	Ab 13	177	5
14	Ab 14	175	5
15	H 15	190	5
16	H 16	187	5
17	H 17	189	4
18	H 18	187	5
19	H 19	202	5
20	H 20	220	5
21	H 21	188	5

Figure 1



Effect of incubation period on glucose reduction by bacterial isolates.

Effect of glucose concentration on lowering the power of bacterial isolates

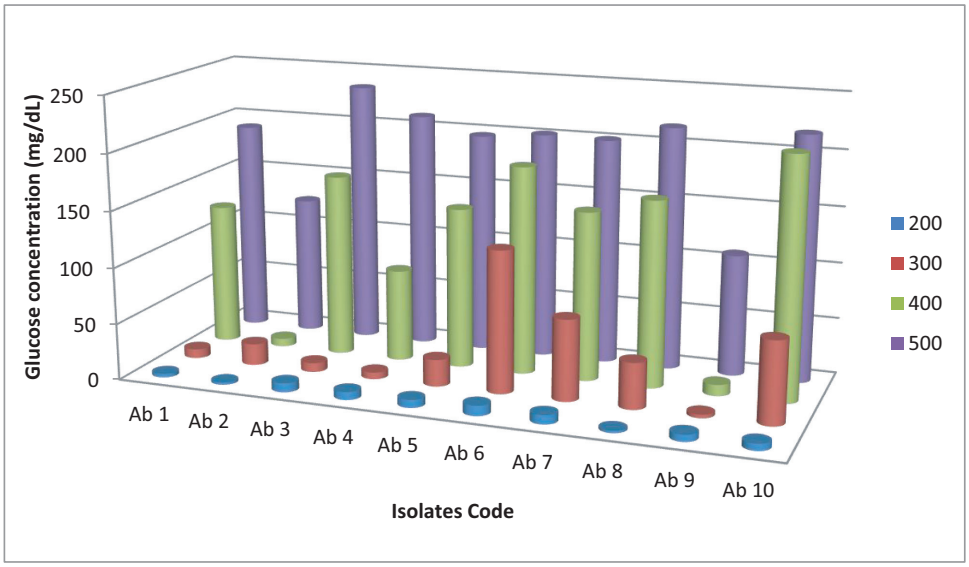
Results presented in Fig. 2 show that there are no big differences observed in the consumption of glucose in the low glucose concentration (200 mg/dl), but with more glucose concentration, glucose consumption increased as well, which indicates that the top two organisms having the ability to reproduce and consume glucose even in high glucose concentrations were Ab 9 and Ab 2 with results of glucose residual concentration 108 and 124 mg/dl, respectively.

The operating parameters of hypoglycemia activity are carried out based on the incubation period and glucose concentration. In our study, probiotic isolates control the glucose level (decrease) and the linear relationship appeared with the increase of incubation period and glucose concentration. The most potent isolate coded Ab 9 identified as *Lactobacillus brevis* assimilated as 79.6% glucose from the growth medium after 48 h.

Molecular identification of the promising probiotic isolate

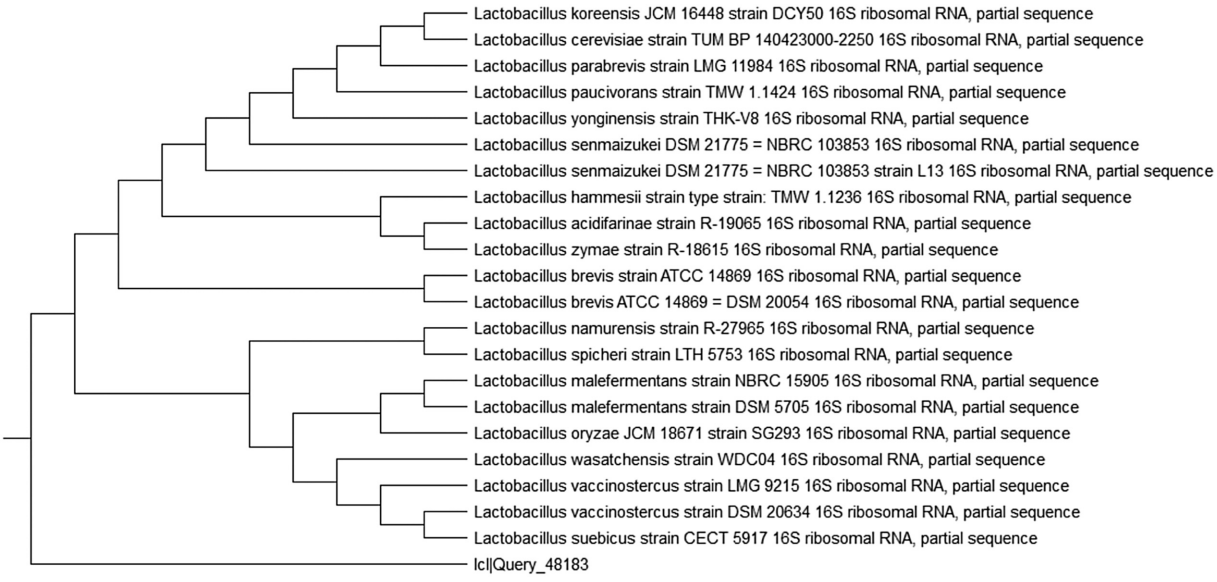
By comparing the results of the biochemical characterization of bacterial isolates with Bergey's manual of systematic bacteriology, the isolates were identified as *Lactobacillus spp*. Moreover, according to the blast results of the most potent bacterial isolate with more than 99% similarities, isolate Ab 9 was identified

Figure 2



Effect of glucose concentration on glucose reduction bacterial isolates.

Figure 3



Phylogenetic tree for molecular identification of the probiotic isolate Ab 9.

as *L. brevis*. The partial sequences were submitted to the GenBank at the NCBI database. Its phylogenetic tree is shown in Fig. 3.

The isolates were identified by 16S-rDNA sequencing, such as *L. casei*, and these results are in harmony with Saeedi *et al.* [21] and Cibik *et al.* [22] as they showed that 16S-rDNA gene sequencing can be considered a potent approach that enables both tracing phylogenetic relationships between bacteria and identification of bacterial strains isolated from various environmental sources and fermentation specimens.

Probiotics were recorded as health supporters in several studies at a different position. El-Waseif *et al.* [23] suggested that *Lactobacillus casei* is a promising probiotic functional component for reducing serum cholesterol concentration. El-Waseif *et al.* [24] recorded that *Lactobacillus paracasei* can use cholesterol assimilation and Taguchi design as an experimental factorial design used to optimize cholesterol assimilation conditions. The model reported that 93% is the maximum cholesterol assimilation at optimal conditions for assimilation at a cholesterol concentration of 120 μg/ml, incubation time of 96 h, bile salt concentration of 0.1%, probiotic dose of 200 μl, and initial pH of 6.5. El-Waseif *et al.* [25] focused on the possibility of using exopolysaccharide and its nano form from *L. brevis* for human pharmaceutical use as it enhanced anti-colon cancer.

Conclusion

The probiotic *L. brevis* was isolated from cow's milk and tested for its probiotic properties. Results demonstrated that it has an inhibitory effect against both Gram-positive and Gram-negative bacteria and can be potentially used for further research on animal models for applications in the treatment of diabetes paving the way for the treatment of type 2 diabetes in humans.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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