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Screening of isolated potential probiotic lactic acid bacteria from Sichuan pickle for cholesterol lowering property and triglycerides lowering activity

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Abstract

The aim of this study was to isolate lactic acid bacteria (LAB) from Sichuan pickle and to assess their probiotic potential. Total of 66 LAB were isolated from 2 types of Sichuan pickle based on their morphological, physiological, biochemical, and genotypic characteristics. Then, 6 strains (BH6, BH9, BH18, BH46, BH48, BH59) were selected as candidates for further studies. The in vitro experiment showed that the cholesterol degradation rate of 4 strains BH6, BH9, BH59 exceeded 60%. And the triglyceride degradation rate of 3 strains BH6, BH9, BH18 was more than 17%. Out of 6 tested LAB strains, most of them (BH6,BH9,BH18,BH48,BH59) exhibited moderate to high survivability (59%-90%) under high concentration of bile salt (3%) and their survival rate was 72%-95% (BH6, BH18, BH46, BH48, BH59) at low pH conditions. The in vitro experiment showed that all LAB strains inhibited the growth of tested pathogenic microorganisms. Based on the results of presently in vitro experiments, it was concluded that the BH6 isolate had better cholesterol lowering ability (65.36%), triglyceride degradation ability (18.97%), bile salt tolerance and bacteriostatic ability. The potential LAB strain, BH6 was identified as *Lactobacillus plantarum* by 16S rRNA sequencing. In conclusion, the strain BH6 screened from Sichuan pickle had good ability to degrade cholesterol and triglyceride; and it had better tolerance to acid and bile salt; which also had good bacteriostatic ability. Therefor the strain BH6 has the potential to be used as edible probiotics.

Keywords: Sichuan pickle; lactic acid bacteria; cholesterol lowering property; triglyceride lowering performance.

Practical Application: *Lactobacillus plantarum* BH6 was isolated from Sichuan pickle, which could be used for pickle fermentation as probiotics. The strain BH6 had good ability to degrade cholesterol, triglyceride and better tolerance to acid and bile salt, which also had good bacteriostatic ability. The research provided useful information for the potential use of this strain in foods.

1 Introduction

At present, the incidence of cardiovascular disease continues to increase worldwide, and dyslipidemia is an important factor in the occurrence of cardiovascular disease and the main components of blood lipids are triglycerides and cholesterol (Dehghan et al., 2018; Babu & Shantharajah, 2019; Ravnskov et al., 2019). Lactic acid bacteria (LAB), as the most important probiotics in human body, have been proved to significantly reduce the level of blood lipids in serum and cause long-term beneficial changes in the intestinal system (Swain et al., 2014; Tokatlı et al., 2015). Mann & Spoerry (1974) were the first to prove that the hypocholesterolemia effect produced by the diet containing LAB strain has the potential to reduce human serum lipids. Li (2018) found the cholesterol removal rate of probiotics isolated from fermented milk was as high as 61.40% in vitro, and in vivo experiments in mice confirmed the potential effect of *Lactobacillus parasitum* Jlus66 on non-alcoholic fatty liver. Jitpakdee et al. (2021) screened two strains of lactic acid bacteria from fermented foods, which were *Pediococcus pentosaceus ENM104 and Lactobacillus plantarum SPS109.* ENM104 reduced cholesterol at 7.53 ± 1.78 in basal medium containing 100 μg/mL cholesterol, while SPS109 released γ-aminobutyric acid (GABA) at 1157.01 ± 4.76 μg/mL in MRS containing 5 mg/mL monosodium glutamate (MSG), respectively.

Traditional Sichuan pickle (TSP) are common fermented food with a variety of microorganisms. Wei (2013) analyzed the bacterial diversity in traditional Sichuan pickles, and the results showed that *Lactobacillus* accounted for 88.4%. Ao et al. (2011) used denaturing gradient gel electrophoresis to analyze the microbial diversity in Sichuan pickles, and found that *Lactobacillus* was the dominant bacteria, so it can be known that traditional Sichuan fermented kimchi is a good source of lactic acid probiotics (Rao et al., 2019). Till now, there are few studies on the resources and functions of bacteria in Sichuan pickles. The aim of the present study was to isolate, identify and screen for potential probiotic LAB with degrade cholesterol and triglycerides capacity, acid tolerance, bile salt tolerance and antibacterial activity. It provides a certain reference for the development of functional probiotics.

2 Materials and methods

2.1 Collection of samples

Two different Paocai samples were collected from local homes in Yibin in Sichuan, all of them were made from Vegetables. The samples were collected aseptically in 250 mL sterile plastic bottles and stored in the laboratory at a temperature of 4 °C.

2.2 Isolation of LAB

Take 1mL Pickle juice from each sample and add 9 mL 0.9% (w/w) sodium chloride aseptic solution to make the initial dilution

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(10^{-1}). Appropriate serial dilutions (10^{-5}, 10^{-6} and 10^{-7}) were made, and 200 µL diluted Pickle samples taken and transferred to de Man, Rogosa and Sharpe (MRS Agar, Himedia, India) agar plates (Man et al., 1960). The plates were incubated at 37 °C for 48 h in anaerobic conditions as described by Etyemez & Balcazar (2016). Then the strains with different shape, size and color were selected from the culture medium. A single colony was picked to the plate of MRS medium by plate scribing method and incubated at 37°C for 24 h. After 24 h, a single colony was picked again to another plate and incubated at 37°C for 24 h. These operations were repeated three times according to this method to obtain purified strains. The purified strains were inoculated in MRS slope medium, incubated at 37 °C for 24 h, and then stored in refrigerator at 4 °C.

2.3 Preliminary identification of LAB

The 66 isolates were initially characterized using cell morphology, Gram staining and catalase test. The single colony on the surface of MRS solid agar medium was observed by the color, size and shape. Then the isolates were observed to be Gram-positive or Gram-negative by Gram stainin. Catalase test (H_2O_2, 3% v/v in water) was performed according to Kong et al. (2020). The Grampositive and catalase-negative strains were preliminarily identified as lactic acid bacteria and stored in 15% sterile glycerol (v/v) at −80 °C.

2.4 Screering of cholesterol-lowering LAB

MRS broth was supplemented with 0.3% bile salt (mainly containing bovine cholic acid) (Solarbio, China) and sterilized water soluble cholesterol (0.25 g/L). The lactic acid bacteria were inoculated in liquid medium containing cholesterol incubated at 37 °C for 48 h. The culture 0.2 mL medium was added to 4.8 mL anhydrous ethanol, swirled and mixed, and after placing statically for 5 min the mixture was shaken and mixed again. Centrifuged at 3700 × g for 15 min, and the supernatant was taken. The relative content of cholesterol was determined using tammonium ferric sulfate method in GB5009.128-2016 "determination of cholesterol in Foods". The uninoculated MRS−CHOL medium was used as a control group, and the absorbance was measured at A_{560} nm. The cholesterol removal rate was calculated with the formula $[A_0\text{-}A_t)/A_0] \times 100\%$, where A_0 and A_t were the absorbance of supernatant of experimental group and absorbance of sterile MRS−CHOL medium of control group, separately. The experiment was carried out in triplicate with duplicate analysis (Iranmanesh et al., 2014).

2.5 Screering of triglyceride-lowering LAB

MRS broth was supplemented with 3% bile salt (mainly containing bovine cholic acid) (Solarbio, China) and sterilized water soluble triglyceride (0.25 g/mL). After the strain was activated twice in MRS liquid medium, the lactic acid bacteria were inoculated into the triglyceride medium (Badis et al., 2004) and cultured at 37 °C for 48 h. The 0.2 mL culture medium was added to 4.8 mL anhydrous ethanol, swirled and mixed, and after placing statically for 5 min, the mixture was shaken and mixed again. Centrifuged at 3700 × g for 15 min, and the supernatant was taken. And the absorbance was measured at A_{510} nm. The degradation rate of triglycerides was calculated according to the formula in the kit. The uninoculated triglyceride medium was used as control group. The experiment was carried out in triplicate with duplicate analysis.

2.6 Bile tolerance assay

The bile salt tolerant ability of LAB isolates was determined by the viable counts method with slight modifications as described by Shehata et al. (2016) and Matijašić & Rogelj (2000). Briefly, MRS broth containing bile salt was prepared by the addition of 0.0, 0.1, 0.2, 0.3, 0.4 and 0.5% (v/v) of bile salt mainly containing bovine cholic acid (Solarbio, China), and then the LAB isolates were inoculated into MRS broth containing different bile salt concentrations for culture. The LAB strains were incubated at 37 °C for 3 h, appropriate serial dilutions (10-5, 10-6 and 10–7) were made, and 200µL of the diluted samples were transferred to MRS agar plates and incubated at 37 °C for 48 h, the viable counts were detected on MRS agar plates. The experiment was carried out in triplicate with duplicate analysis (Charteris et al., 1998).

2.7 pH tolerance assay

The pH tolerance ability of LAB isolates was determined by the viable counts method with minor modifications as described by Aarti et al. (2017) and Delgado et al. (2007). The LAB strains were incubated at 37 °C for 24 h, centrifuged at 3700 × g for 10 min at 4 °C, and the supernatant was poured out. Then the strain was resuspended with sterile PBS with pH of 1, 2, 2.5, 3 and 3.5, and the resuspended bacterial solution was placed at room temperature for 2 h. Then 200 µL bacterial suspension was coated on MRS solid medium and then incubated at 37 °C for 48 h. The viable counts were detected on MRS agar plates. The experiment was carried out in triplicate with duplicate analysis.

2.8 Antibacterial activity

The bacteriostatic test was carried out agar diffusion method. In brief, inoculated plates (the diameter 90 mm) were covered with 10 mL of soft agar (water added of 0.75% bacteriological agar), then put on the Oxford cup. when the LB solid medium (1.5% Agar) cooled to 50 °C, the indictor bacterias were added (10^6 CFU/mL) were to the LB medium, shaking gently to prevent bubbles, pour it on the bottom soft agar (water added of 0.75% bacteriological agar). After waiting for 20 min, the Oxford cup was taken out and a well with a diameter of 6 mm was formed on the plate. Then the fermentation broth (200 µL) was injected into the wells. The plates were placed at room temperature for 30 min then cultured at 37 °C for 12 h, and the inhibition zones were measured with a vernier caliper (Li, 2018).

2.9 Genotypic identification of the LAB

The isolate (BH6) with good comprehensive performance was inoculated in MRS liquid medium and incubated at 37 °C for 48 h. After that, 1.5 mL fermentation broth was absorbed with pipette gun in the ultra-clean table and put into 2 mL preservation tube, then it was sent with ice bag preservation

to Shanghai Geri Bio-Technology Co. LTD for sequencing. The sequencing results were submitted to the national center for biotechnology information of GenBank database, the basic local alignment search tool (basiclocalalignmentsearchtool, BLAST) was used to search, Clustalx1.83 was used for multiple sequence comparison, and neighborjoining (NJ) method in Mega6.0 was used to construct phylogenetic tree (Vidal et al., 2014).

2.10 Statistical analysis

Statistical analysis was carried out by SPSS v.23.0 software. All the experiments were performed in triplicate and results were presented as mean ± standard deviation (mean ± SD).

3 Results and discussion

3.1 Preliminary identification

Through Gram staining, it was found that all the 66 strains were Gram positive bacteria. The microscopic examination picture of Gram staining was shown in Figure 1. Through catalase test, it was found that all the 66 strains were negative. They all grew normally on MRS medium and the colony morphology was milky white, opaque, round, and the surface was neat and smooth.

3.2 Cholesterol removal analysis

The results of cholesterol removal analysis was showed in Table 1, The cholesterol degradation rate of 7 strains was between 57.42% and 70.84%. The cholesterol degradation ability of strains BH6, BH46, BH48 and BH59 was significantly higher than that of strains BH9, BH18 and BH45, and their degradation rates were 65.36%, 59.52%, 70.84% and 58.64%, respectively. Yidikong et al. isolated a LAB from the intestines of snakehead fish and proved it has cholesterol lowering ability, with the highest cholesterol degradation rate of 48.4%. Liu et al. (2020) isolated a strain of lactobacillus with cholesterol-lowering ability from Yunnan milk cake, and the cholesterol degradation rate was 62.22%. In comparison, The strains of BH6, BH46, BH48, BH59 had better cholesterol degradation ability.

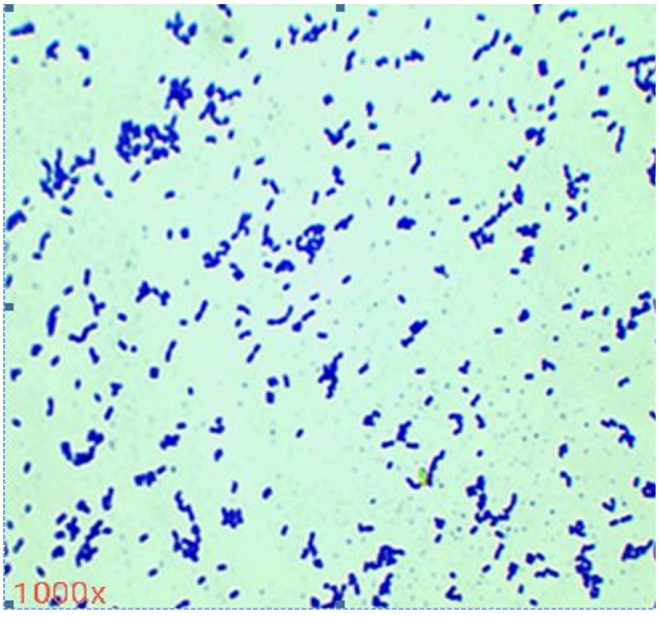

Figure 1. Microscopic view of showed gram positive rod shaped.

Values were means of three replicates, dissimilar letters show significant difference ($p < 0.05$).

3.3 Determination of triglyceride degradation ability

The results of triglyceride degradation by LAB were shown in Table 2. It concluted that the strains(BH6, BH9, BH18, BH45, BH48) had the ability of triglyceride degradation. And the isolates (BH6, BH9, BH18) had better ability of triglyceride degradation than the strians (BH45, BH48). And the bacterium BH18 had the highest triglyceride degradation rate (20.17%) among these isolates. Wu et al. (2019) screened a lactic acid bacteria ZL010 from Chinese pickle, and its triglyceride degradation rate was 5.10%. In contrast, the isolates (BH6, BH9, BH18) had better ability to reduce triglycerides.

Values were means of three replicates, dissimilar letters show significant difference ($p < 0.05$).

3.4 Bile and pH tolerance analysis

Lactic acid bacteria, as intestinal probiotics, must be well tolerated to acid and bile salts in order to survive in the stomach and the upper part of the small intestine. Six strains (BH6, BH9, BH18, BH45, BH46, BH48, BH59) of lactic acid bacteria were screened based on their ability to lower cholesterol and triglyceride in vitro, then their ability to tolerate acid and bile salts was evaluated. The experimental results were shown in Table 3 and Table 4.

As shown in Table 3, when the concertration of bile salt was 5g/L, the survival rates of the strains (BH6, BH9, BH18, BH48, BH59) were 57.17%, 58.52%, 60.60%, 63.88%, 60.99% respectively. Indicating that these six tested isolates showed high survival rate in high concentration of bile salt (5g/L).

Table 1. Determination of cholesterol degrading ability of LAB.

LAB	Cholesterol removal (%)
BH6	65.36 ± 0.83^{b}
BH9	57.42 ± 0.17^{d}
BH18	59.52 ± 0.97^{c}
BH45	58.64 ± 1.01^{c}
BH46	67.0 ± 0.85^{b}
BH48	70.84 ± 0.91^{a}
BH59	66.17 ± 1.19^{b}

Table 2. The triglyceride removal rate in LAB.

LAB	Triglyceride removal (%)
BH6	18.97 ± 0.83^{ab}
BH9	17.64 ± 0.86^{b}
BH18	20.17 ± 1.01^{a}
BH45	14.46 ± 0.99^{c}
BH48	14.67 ± 0.68^{c}

Table 3. Determination of bile salt tolerance of LAB.

LAB	Different concentration of bile salt				
	1 g/L	2 g/L	3 g/L	4 g/L	5 g/L
BH6	78.63 ± 0.83[a]	75.56 ± 0.98[b]	72.28 ± 1.48[c]	65.09 ± 1.51[d]	57.17 ± 1.50[e]
BH9	89.36 ± 1.25[a]	86.66 ± 0.88[a]	86.61 ± 1.02[a]	65.72 ± 1.05[b]	58.52 ± 1.37[c]
BH18	83.93 ± 1.12[a]	80.67 ± 1.09[b]	75.4 ± 1.01[c]	63.47 ± 1.53[d]	60.6 ± 0.86[e]
BH46	73.88 ± 1.19[a]	68.27 ± 1.27[b]	60.96 ± 1.07[c]	49.52 ± 1.40[d]	44.88 ± 1.33[e]
BH48	91.26 ± 0.83[a]	83.65 ± 1.45[b]	74.95 ± 1.40[c]	64.12 ± 1.49[d]	63.88 ± 0.83[d]
BH59	76.10 ± 1.27[a]	74.33 ± 1.64[b]	65.93 ± 1.20[c]	61.21 ± 1.50[d]	60.99 ± 1.04[e]

Table 4. Determination of bile salt tolerance of LAB.

LAB	Different pH			
	2	2.5	3	3.5
BH6	93.02 ± 0.88[b]	95.96 ± 1.05[a]	93.45 ± 1.01[a]	96.25 ± 0.83[a]
BH9	75.1±1.6[b]	80.4±0.88[a]	63.8±1.48[c]	58.64 ± 1.01[d]
BH18	88.64 ± 0.96[a]	85.92 ± 0.86[b]	80.33 ± 1.34[c]	76.48 ± 0.86[d]
BH46	89.26 ± 1.01[b]	77.54 ± 1.69[c]	79.05 ± 1.37[c]	92.69 ± 0.94[a]
BH48	73.55 ± 1.03[b]	80.93 ± 1.82[c]	99.15±0.86[a]	95.5 ± 0.88[a]
BH59	88.69 ± 1.15[b]	80.02 ± 0.59[c]	92.23 ± 0.83[a]	86.79 ± 1.47[b]

Table 5. Antimicrobial activity of LAB.

LAB	Inhibition zones diameters(mm)	
	Escherichia coli	*Staphylococcus aureus*
BH6	19.73 ± 0.42[ab]	22.08 ± 0.19[c]
BH9	19.21 ± 0.38[b]	28.09 ± 0.13[a]
BH18	13.98 ± 0.40[d]	23.38 ± 0.25[b]
BH46	16.01 ± 0.12[c]	20.18 ± 0.37[d]
BH48	20.14 ± 0.19[a]	23.12 ± 0.50[b]
BH59	19.48 ± 0.60[b]	20.80 ± 0.33[d]

When the pH was 2, the survival rate of the strains (BH6, BH9, BH18, BH45, BH46, BH48, BH59) was above 70% (Table 4), it indicated that the survival rates of strains BH6, BH9, BH18, BH45, BH46, BH48 and BH59 were high under low pH conditions. The results (Table 3 and Table 4) showed that all of the six strains (BH6, BH9, BH18, BH45, BH46, BH48, BH59) had good acid tolerance and bile salt tolerance.

Among all the isolates, the isolates (BH6, BH9, BH18, BH48, BH59) showed high survival rate when bile salt concentration was 5g/ L, and the survival rate of those isolates was higher than 50%. When the pH was 2, the strains (BH6, BH18, BH46, BH59) all showed high survival rate, and the survival rate of them was higher than 80%. Values were means of three replicates, dissimilar letters show significant difference ($p < 0.05$).

3.5 Antimicrobial activity

Escherichia coli (Gram-negative, G^-) and *Staphylococcus aureus* (Gram-positive, G^+) were used as indicator bacterias, double AGAR diffusion method was used to conduct bacteriostatic experiments to determine the antimicrobial activity of 6 strains (BH6, BH9, BH18, BH45, BH46, BH48, BH59), and the results were shown in Table 5.

The inhibition zones diameters of these 6 strains (BH6, BH9, BH18, BH45, BH46, BH48, BH59)to indicator bacteria (*Staph. aureus*) were all more than 20 mm (Table 5). In addition, the diameter of the inhibition zones for the indicator bacteria (*E. coli*) were more than 15 mm, it indicated that the 6 strains (BH6, BH9, BH18, BH45, BH46, BH48, BH59) could inhibit the growth and reproduction of *E. coli* (G^-) and *Staph. aureus* (G^+). And the inhibition zones diameters of the 6 strains to *Staph. aureus* was bigger than that to *E. coli*.

In current study, most LAB strains showed inhibitory activity against the growth of food-degrading microbiota and pathogenic microorganisms either by competing with pathogenic bacteria for food (Reis et al., 2012) or by producing antimicrobial compounds such as hydrogen peroxide, diacetyl, bacteriocins, naturally protective organic acids, and specific substances, such as antiviral peptides or low-molecular-weight peptides (Souza, 2021). In addition, Gheziel et al. (2019) had demonstrated that 6 *Lactiplantibacillus plantarum* strains isolated from fecal samples exposed high antibacterial activity against potential foodborne pathogens viz. *E. coli* and *Staph. aureus.*

3.6 Molecular identification of the most potential isolate analysis

To sum up, the LAB isolate (BH6) had good cholesterol lowering ability (65.36%), triglyceride degradation ability (18.97%), bile and pH tolerance and bacteriostatic ability. Therefore, strain BH6 was selected for further study. Then the sequence of the isolate (BH6) was aligned with the 16SrRNA sequences from the GenBank database (website) to identify the studied microorganism. 16SrRNA sequencing data of the selected isolates (Figure 2) clearly showed (BH6) 99% homology to *Lactobacillus plantarum*.

4 Discussion

In this study, 66 strains were isolated from TSP and only the isolate (BH6) was selected on the basis of its excellent probiotic properties. And the isolate (BH6) was identified as *Lactobacillus plantarum* by 16SrRNA. The in vitro experiment showed that the cholesterol degradation rate of BH6 was as high as 65.36%, which was 16.96% higher than that of the LAB (48.4%) screened from snakehead by Yidikong et al. At the same time, the cholesterol degradation rate of BH6 was 3.14% higher than that of lactic acid bacteria (62.22%) selected by Liu et al. (2020) from Yunnan milk cake. Therefore, compared with the existing research, the isolate (BH6) had better cholesterol-

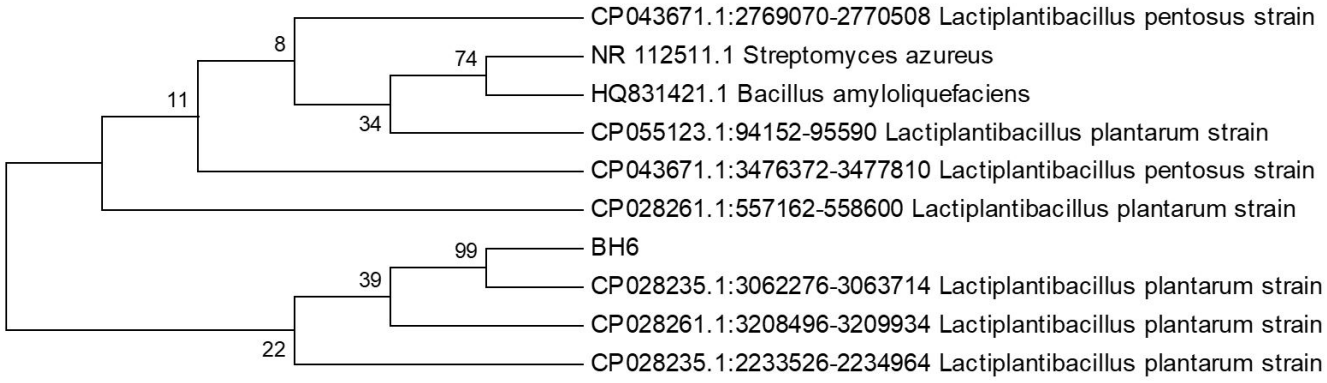

Figure 2. Phylogenetic tree constructed by the neighbour joining method respectively showing species relatedness of the potential LAB isolate (BH6).

degrading ability. And the triglyceride degradation rate of strain BH6 was 18.97%, but the triglyceride degradation rate of LAB strain ZL010 selected by Wu et al. (2019) from Chinese pickle was only 5.10%. In comparision, the isolate (BH6) had better triglyceride degradation rate. In addition, in vitro bacteriostatic test showed that the bacterium BH6 had good inhibitory effect on *Escherichia coli* and *Staphylococcus aureus*, and the results were similar to those of Souza et al. (2021) and Gheziel et al. (2019). Accordingly, owing to its good probiotic properties, the bacterium BH6 could be potentially used in functional food and health products. However, the ability of the single strain to lower cholesterol or triglyceride is limited to some extent, in order to better exert the probiotic function of LAB, we can use two or more strains as mixed bacterial agent. Therefore, the later work of the research group will use the cholesterol and triglyceride degradation rate as an indicator to develop mixed bacterial agent with better cholesterol and triglyceride degradation properties..

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