

Discovery of Lactobacillus with Cholesterol-Lowering Effect in Crab Sauce

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Abstract: This paper aims to find out lactobacillus strains with cholesterol-reducing effect from crab paste. Lactobacillus were isolated and purified from the crab paste from Ningbo, Zhejiang Province using MRS-CaCO₃ medium, and the probiotic properties of the lactobacillus isolated from crab paste were detected by gastric acid tolerance assay, bile salt tolerance assay, determination of bacterial inhibition ability, antioxidant activity assay. Following that, the cholesterol-lowering lactobacillus were screened using the o-phthalaldehyde method. The results showed that among the 13 isolated Lactobacillus strains, strain LA-05 had a high cholesterol-lowering ability, with a cholesterol degradation rate of 85.69%, as well as good antibacterial ability and probiotic properties. This study successfully screened lactobacillus with cholesterol-lowering effect in crab paste, which enriched the knowledge of lactobacillus from traditional fermented foods in China and provided an experimental basis for the generalization and application of Lactobacillus, a cholesterol-lowering bacterium with regional properties.

1. Introduction

Hyperlipidemia is a major factor in the development of cardiovascular diseases such as atherosclerosis. At the present stage, many studies have shown that elevated serum cholesterol is a major factor in triggering hyperlipidemia, and it even directly accelerates the development of atherosclerosis and coronary heart disease [1]. There are many measures to regulate the body's high serum cholesterol content. At present, a variety of statins have been used clinically to treat patients with hyperlipidemia. Although the use of drugs can effectively reduce serum cholesterol content and have a significant effect on patients, there are irreversible side effects on physical health [2]. Adjusting one's own dietary intake can also regulate lipid metabolism in the body for a period of time without side effects, but the course of treatment is short, the effect is slow, and it is easy to rebound. As food-grade probiotics, lactic acid bacteria are present in large quantities in the intestines of humans and animals, and have important functions such as maintaining the balance of intestinal microorganisms and improving the body's immunity [3,4]. Relevant studies have confirmed that lactic acid bacteria have good cholesterol-lowering ability and can be used to treat elevated serum cholesterol in the human body [5]. Since intestinal flora and probiotics are closely related to people's dietary habits and have certain regional characteristics, it is particularly

important to discover cholesterol-lowering lactobacilli from Chinese traditional foods with independent intellectual property rights.

Crab sauce, as a kind of seasoning commonly used in coastal areas of our country, is a paste-like fermented seasoning made from medium and small crabs (generally pike crabs) as raw materials and fermented with salt. It has a unique sea crab aroma and is loved by coastal people. A study on the classification of microorganisms in crab sauce found that at the species level, *Lactobacillus plantarum* and *Lactobacillus* fermentation have higher content, which are the dominant strains in crab sauce [6]. In this study, strains of lactic acid bacteria with the effect of degrading cholesterol were selected from crab sauce, and the lactic acid bacteria in shuttle crab sauce from Ningbo, Zhejiang were isolated and purified by MRS-CaCO₃ medium. The probiotic properties of lactic acid bacteria isolated from crab sauce in the early stage were detected by gastric acid tolerance experiment, bile salt tolerance experiment, determination of antibacterial ability, and determination of antioxidant activity, and then the phthalaldehyde method was used to screen for cholesterol-lowering lactic acid bacteria [7].

2. Materials and Methods

2.1. Experimental Materials

Pike crab sauce from Ningbo, Zhejiang Province of China.

2.2. Reagents

Peptone, Beef dip, Yeast dip, Glucose, Dipotassium hydrogen phosphate, Triammonium citrate, Sodium acetate, Magnesium phosphate, Manganese sulfate, Agar, Tween 80, Gram stain kit, Bovine bile salt, Benzotriol, 1,1-diphenyl-2-trinitrophenylhydrazine (DPPH).

2.3. Culture Medium

MRS-CaCO₃ medium: Agar 2%, CaCO₃ 1.5%, MRS 54g/L [8].

Cholesterol-MRS medium: Cholesterol 1g/L, Tween 80 2%, Bovine bile salt 3g/L. After heating and dissolving, remove the prepared cholesterol - Tween solution and MRS Broth medium to 1L for use.

LB medium: Tryptone 5g, Yeast extract 2.5g, NaCl 5g, Water 500mL.

2.4. Instruments and Equipment

Microscope, Autoclave, Ultra-clean bench, Constant temperature incubator, Electronic balance, Spectrophotometer.

2.5. Methods

2.5.1. Strain Isolation Screening and Morphological Observation

9 ml of sterile saline was added to 1 mL of crab paste specimen for gradient dilution, and plates of 10⁻⁴, 10⁻⁵, 10⁻⁶ gradients of dilutions were sequentially coated on MRS-CaCO₃ solid medium and incubated at 37 °C for 48 hours. Colonies with typical characteristics were picked and purified by multiple isolations on MRS-CaCO₃ solid medium to obtain the strains. The purified strains were tested by Gram staining method and peroxidase assay, and the color, morphology, edge condition and other characteristics of the colonies were observed, from which the colonies with positive Gram

staining and negative peroxidase were screened, and this was tentatively determined to be *Lactobacillus* [9].

2.5.2. Gastric Acid Tolerance Test of *Lactobacillus*

Preparation of artificial gastric acid: Take 2.0g of NaCl, 3.5g of pepsin and 1000ml of distilled water to make a solution with a pH of 3.0. Artificial gastric acid needs to be prepared and used now. Put the strain to be tested into the solution, incubate it at 37°C for three hours, and then coat it to measure its survival rate [10].

2.5.3. Bile Salt Tolerance Test of *Lactobacillus*

Weigh 3g of bile salt and put it into 1L of physiological saline to be used. The activated *Lactobacillus* were inoculated into the bile salt solution at 3% inoculum, incubated at 37°C for three hours, and then coated to measure their survival rate.

2.5.4. Determination of *Lactobacillus* Inhibition Ability

Six bacteria were used as indicator bacteria in this test, namely: *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi*, *Shigella fowleri*, *Bacillus cereus* and *Pseudomonas aeruginosa*, and their antibacterial tests were performed by Oxford cup method.

Preparation of lactic acid bacteria supernatant: The activated lactic acid bacteria were inoculated with 3% inoculation in the sterilized MRS broth, cultured at 37°C for 48 hours, 4°C, 5000 r/min, and centrifuged for 10 minutes to obtain a fermented supernatant. Production of indicator plates: Place 3 to 4 sterilized Oxford cups in a petri dish, and then pour the indicator bacteria liquid that has been activated 2 to 3 times into a solid medium of about 40°C, shake well, and pour it into the plate. After solidification, it was cultured at 37°C for 24 hours, and the diameter of the antibacterial ring was measured.

2.5.5. Determination of Antioxidant Activity of *Lactobacillus*

The first is the DPPH clearance ability test. Select 0.2 ml of specimen, add 2 ml (0.02 mmol/L) of DPPH ethanol, and centrifuge for 10 min at 6000 rpm after 30 min of light avoidance, measure the absorbance at 517 nm expressed as A_0 , replace the specimen with anhydrous ethanol and express its value as A_1 , replace DPPH with anhydrous ethanol expressed as A_i , and the standard curve expressed as VC. The scavenging capacity of DPPH was calculated according to the following equation [11].

$$\left[1 - \frac{A_0 - A_1}{A_i} \right] \times 100\% \quad (1)$$

The second is determination of hydroxyl radical scavenging capacity. In order, put 1 ml of 2.5 mmol/L of o-firiline, 1 ml of 0.02 mmol/L of PBS phosphoric acid buffer, and 1ml of distilled water into a test tube. After mixing, add 1 mL of 2.5 mmol/L of ferrous sulfate. After the full reaction, add 1mL of 20mmol/L of hydrogen peroxide, react at 37°C for 1.5 hours, measure the absorbance A_P at 536nm, replace H_2O_2 with distilled water and denote it as A_B , and replace the distilled water with the solution to be tested and denote it as A_S , take VC as a direct comparison. Calculate the clearance rate of hydroxyl radicals according to the following formula.

$$\frac{A_S - A_P}{A_B - A_P} \times 100\% \quad (2)$$

2.5.6. Determination of Cholesterol Degradation Rate of Lactobacillus

Configure the cholesterol-MRS medium: Add 1 gram of cholesterol, 3 grams of bovine bile salt, and 10 ml of tween to the test tube, heat it to dissolve, and then vibrate with ultrasound. Put the newly formulated cholesterol solution into 1 liter of MRS. liquid broth medium, inoculate with 3% of the inoculation amount in the cholesterol-MRS. Liquid broth medium and culture it at 37°C for 24 hours to determine its cholesterol content. In this experiment, phthalaldehyde was used as the research object to study the effect of lactobacillus on cholesterol. Calculate the cholesterol degradation rate according to the following formula.

$$\frac{X}{X_0} \times 100\% \quad (3)$$

Where X_0 is the content of cholesterol in the medium before degradation. Where, X is the content of cholesterol in the degraded medium.

3. Results and Analysis

3.1. Isolation of Lactobacillus from Crab Paste

The lactic acid bacteria in shuttle crab sauce were isolated using MRS.-CaCO₃ solid medium, and 13 white, round, raised, and opaque strains with uniform morphology were obtained. Typical calcium-soluble circles appeared around the colonies. After purification, they were numbered as LA-01~LA-13.

3.2. Probiotic Properties of the Strains

3.2.1. Results of Gastric Acid Tolerance Test

As shown in Figure 1, the experimental results of gastric acid tolerance of strains show that LA-01, LA-03 and LA-11 all showed high gastric acid tolerance, with a survival rate of more than 90%, while LA-07's tolerance to gastric acid decreased slightly. Compared with other strains, LA-07's tolerance performance decreased significantly. However, on the whole, most strains have a good tolerance to microacidity, which creates good conditions for adapting to the environment in which humans live.

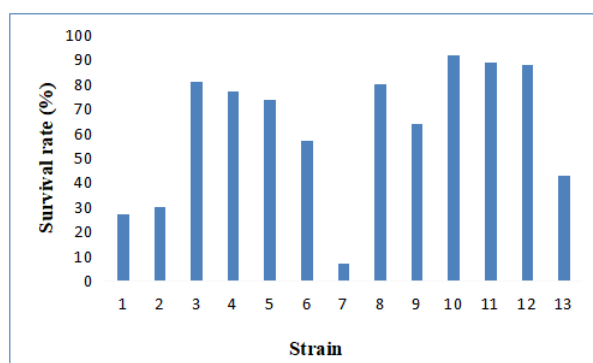


Figure 1: Survival rate of lactobacillus cultured in artificial gastric acid for 3h

3.2.2. Results of Bile Salt Tolerance Performance Experiments

As can be seen from Figure 2, from the experimental results of bile salt tolerance performance, it

can be seen that LA-03 has the highest tolerance and a 3h survival rate of 81.54%. Although the No. 4 strain has poor tolerance to gastric acid, it has better tolerance to salts. This may be related to changes in pH value. The survival rate of LA-05 is the lowest, only 10.56%. Obviously, the tolerance of bacteria to various environments is different, and LA-03 is the most likely bacteria to survive in the body.

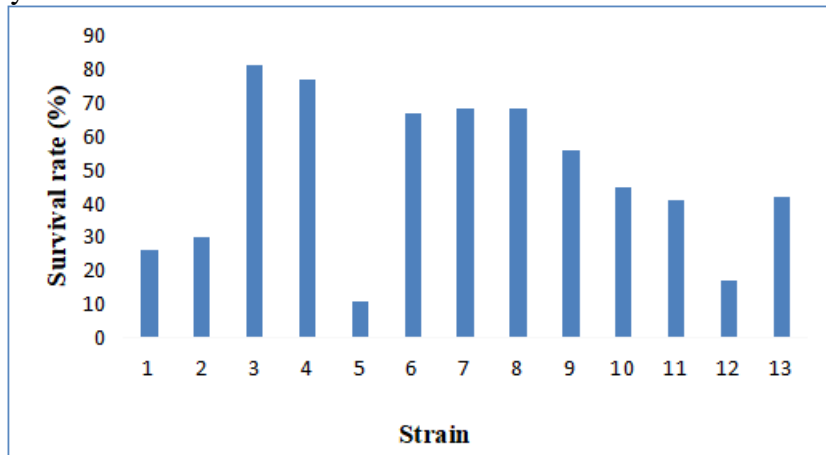


Figure 2: Survival of lactobacilli cultured in bile salt for 3h

3.2.3. Results of the Determination of Bacterial Inhibitory Capacity

As can be seen from Table 1, the 13 strains screened have weak inhibitory effects on *Shigella* *fusiformis*, followed by *Bacillus cereus* and *Pseudomonas aeruginosa*, which have the greatest inhibitory effect on *Staphylococcus aureus*, especially the LA-05 bacteriostatic ring 20 mm, which can play a good inhibitory effect. The inhibitory effect of this strain on *E. coli* and *Salmonella typhimurium* is very good. Among them, the antibacterial effect of *Escherichia coli* is better than that of *Salmonella typhimurium*, and the LA-12 strain has a certain inhibitory effect on various indicator bacteria.

Table 1: Inhibitory effect of isolates against indicator bacteria

	<i>E. coli</i>	<i>Pseudomonas aeruginosa</i>	<i>Staphylococcus aureus</i>	<i>Shigella forsythia</i>	<i>Bacillus cereus</i>	<i>Salmonella typhimurium</i>
LA-01	+	-	+	-	-	++
LA-02	++	++	+	-	-	+
LA-03	+++	-	++	-	++	+
LA-04	+++	-	++	-	-	++
LA-05	+++	-	+++	++	+++	+
LA-06	++	++	++	-	-	++
LA-07	+	+	++	-	-	++
LA-08	-	+	+++	-	++	++
LA-09	++	++	+	++	++	++
LA-10	+	++	++	+	+	++
LA-11	++	+	++	++	-	+
LA-12	+	++	++	++	++	++
LA-13	++	-	+	+++	+	-

Note: The unit means mm; Oxford cup diameter means 9 mm; - indicates means no effect (no inhibition circle); + inhibition circle diameter range means (9 mm, 12 mm]; + + + inhibition circle diameter range means (12 mm, 18 mm]; + + + inhibition circle diameter mans (> 18 mm).

3.2.4. Results of the Determination of Antioxidant Activity

As can be seen from Figure 3: LA-12 bacteria have the best clearance effect on DPPH, with a clearance rate of 60.6%, followed by LA-03 and LA-08, which are 56.35% and 54.93%, respectively. But in fact, the clearance rate of different bacteria on DPPH is not significantly different.

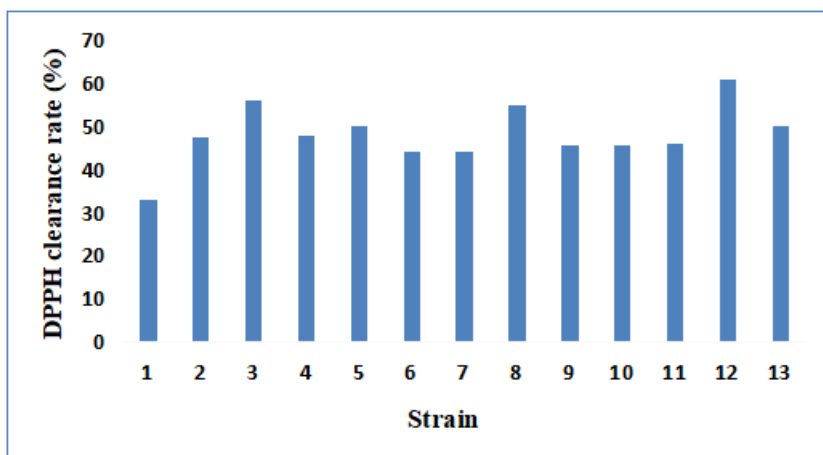


Figure 3: Free radical scavenging rate of lactobacilli

As shown in Figure 4, LA-12 has the worst scavenging ability of hydroxyl radicals, and the LA-07 strain has a clearance rate of 88.22%, which has the best removal effect of hydroxyl radicals. Although their clearance rates are very uniform, there are obvious differences between the strains.

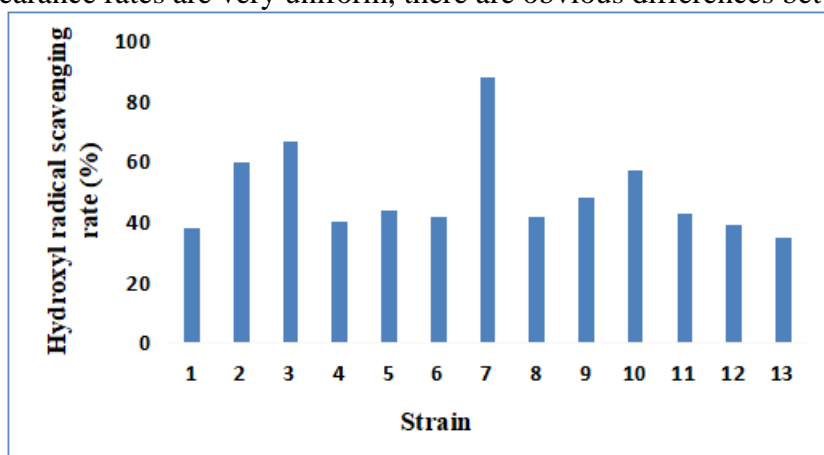


Figure 4: Scavenging rate of hydroxyl radical by lactobacilli

3.2.5. Determination of Cholesterol Degradation Capacity

As can be seen from Figure 5, in vitro tests, LA-05 has the best cholesterol-lowering effect, with a degradation rate of up to 85.69%. LA-02, LA-03, LA-05, LA-06, and LA-10 all have a good effect on degrading cholesterol. The lowest cholesterol degradation rate is LA-02, with a degradation rate of only 20.2%.

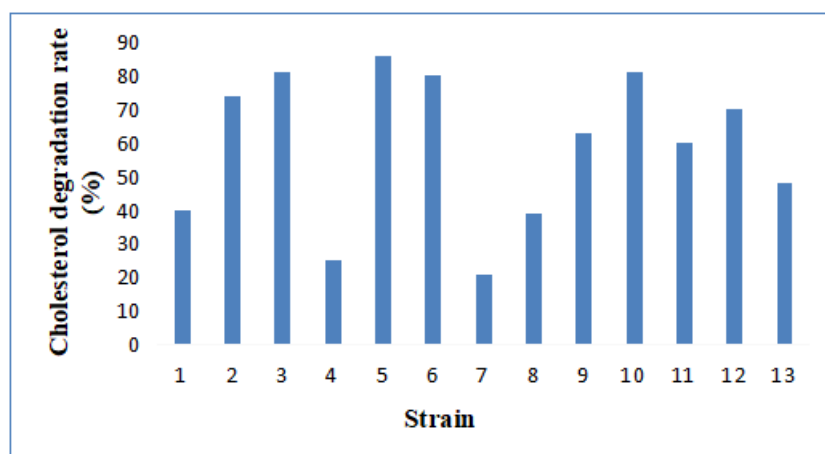


Figure 5: Degradation of cholesterol by lactobacillu

4. Conclusion

In this study, the lactic acid bacteria strain LA-05, which has good cholesterol-lowering effects, was selected from the traditional food shuttle crab sauce in Ningbo, Zhejiang. Its cholesterol degradation rate was 85.69%, and the cholesterol degradation rate of LA-03, LA-06, and LA-10 was as high as 80%. It can be seen from the detection of the probiotic properties of lactic acid bacteria that LA-05 also has good probiotic properties. The acquisition of this strain has good application value in the development of cholesterol-lowering functional crab sauce and other food products. At the same time, it will also enrich the understanding of lactic acid bacteria derived from traditional fermented foods in our country, and can promote the application of lactic acid bacteria with regional characteristics and cholesterol-lowering effects.

References

- [1] LaRosa J C, Hunninghake D, Bush D, Criqui M H, Getz G S, Gotto A M, Grundy S M, Rakita L, Robertson R M, Weisfeldt M L. The cholesterol facts. A summary of the A joint statement by the American Heart Association and the National Heart, Lung, and Blood Institute. The Task Force on Cholesterol Issues, American Heart Association. *Circulation*, 1990, 81 (5).
- [2] Connie B Newman, Gary Palmer, Halit Silbershatz, Michael Szarek. Safety of atorvastatin derived from analysis of 44 completed trials in 9,416 patients. *The American Journal of Cardiology*, 2003, 92 (6).
- [3] Zhou Li, Pingyang, Tan Jing, Zhang Yaxun, Luo Beibei. The overview of lactic acid bacteria in probiotics and the research progress of detection technology. *Chinese condiment*, 2019, 44 (10): 190-194.
- [4] Lu Mingshou, Lin Meijun, Chen Fenglian, Zhang Guang, Shi Yankuo. Research progress on metabolomics of lactic acid bacteria. *Chinese condiment*, 2019, 44 (11): 174-178+182.
- [5] Han Junhua, Sheng Xiaogan, Dong Caifeng, Ge Chunmei, Zhang Linlin. Research status of the cholesterol-lowering effects of lactic acid bacteria. *China Dairy Industry*, 2002 (03): 16-20.
- [6] Ma Chen-Chen et al. Analysis of microbial diversity in Hainan Li fermented seafood. *Chinese Journal of Food*, 2020, 20 (08): 270-277.
- [7] Choi E A, Chang H C. Cholesterol-lowering effects of a putative probiotic strain *Lactobacillus plantarum* EM isolated from kimchi. *LWT-Food Science and Technology*, 2015, 62 (1): 210-217.
- [8] Azat R, Liu Y, Li W, et al. Probiotic properties of lactic acid bacteria isolated from traditionally fermented Xinjiang cheese. *Journal of Zhejiang University-SCIENCE B*, 2016, 17 (8): 597-609.
- [9] Edith Marius F K, François Z N, Pierre Marie K, et al. Screening and Characterization of *Lactobacillus* sp. from the Water of Cassava's Fermentation for Selection as Probiotics. *Food Biotechnology*, 2018, 32 (1): 15-34.
- [10] Yadav R, Puniya A K, Shukla P. Probiotic properties of *Lactobacillus plantarum* RYPR1 from an indigenous fermented beverage Raabadi. *Frontiers in microbiology*, 2016, 7: 1683.
- [11] DAVIES C V, GERARD L M, FERREYRA M M, et al. Bioactive compounds and antioxidant activity analysis during orange vinegar production. *Food Science and Technology*, 2017, 37 (3): 449-455.