

# $^{12}\text{C}^{6+}$ Irradiation Mutation for the Selection and Breeding of High-Cholesterol-Lowering *Lactobacillus*

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**Abstract:** A high-throughput screening method for the selection of lactic acid bacteria with high cholesterol-degrading ability was established using the  $^{12}\text{C}^{6+}$  beam irradiation mutagenesis technique. This study aimed to breed excellent strains with high cholesterol-degrading capability and explore the mechanisms of cholesterol degradation by these strains. The mutated strains JMR-01 (*Lactobacillus reuteri*) and SN-2 (*Lactobacillus rhamnosus*) after irradiation were primarily screened for high cholesterol-degrading strains by optimizing the KENJI culture medium with colony diameter as an indicator. The primary screened strains were further screened using ammonium ferric sulfate method. The mechanisms of cholesterol degradation by lactic acid bacteria were further investigated by determining the total cholesterol degradation rate, the cholesterol degradation rate through co-precipitation and absorption. The results showed that after primary and secondary screening, four mutant strains with high cholesterol-degrading ability (JMR-01-1, JMR-01-2, SN-2-1, SN-2-9) were selected. The cholesterol-degrading ability of strain JMR-01-1 was increased by 9.7% compared to the original strain JMR-01 ( $P < 0.05$ ), and the cholesterol-degrading ability of strain SN-2-1 was increased by 11.5% compared to the original strain SN-2. The total cholesterol degradation rate, co-precipitation, and absorption of cholesterol degradation rate of the four mutant strains showed no significant difference in the co-precipitation effect on cholesterol compared to the original strains, but the relative absorption rate was significantly increased. This study optimized the screening method for cholesterol-degrading strains and demonstrated through the exploration of different cholesterol degradation pathways by lactic acid bacteria that this screening method can select strains with high cholesterol absorption capacity.

**Keywords:** Lactic acid bacteria;  $^{12}\text{C}^{6+}$ ; Mutagenesis breeding; Cholesterol-lowering.

## 1. Citation

In recent years, the number of obese individuals in our country has continued to rise. According to the «China National Nutrition and Chronic Disease Status Report (2020)» the overweight and obesity rates for children and adolescents under 6 years old and between 6 to 17 years old are 10.4% and 19.0% respectively, while the overweight and obesity rates for residents aged 18 and above are 34.3% and 16.4%[1]. More than half of the adult residents are either overweight or obese. Numerous studies have shown that obesity is mainly caused by an imbalance between energy intake and expenditure, leading to the disruption of fat balance and the excessive accumulation of substances such as cholesterol and triglycerides in the body[2]. Prolonged excessive cholesterol accumulation can lead to the loss of control over normal lipid regulation, which in turn causes a series of chronic non-communicable diseases. Therefore, obese individuals are at a higher risk of developing various chronic non-communicable diseases such as diabetes, cardiovascular and cerebrovascular diseases, and fatty liver, which severely affect the health of our citizens[3, 4]. Reports have shown that there are differences in the gut microbiota between obese and normal-weight populations[5, 6]. Therefore, researchers believe that supplementing with probiotics and altering the gut microbiota can potentially reduce cholesterol levels and aid in weight loss[4]. A large number of studies both domestically and abroad have shown that lactic acid bacteria have a strong ability to degrade cholesterol. Lactic acid bacteria are essential to the human body, mainly distributed in the intestinal tract, and play a significant role in probiotic

effects[7]. Although numerous studies have confirmed that lactic acid bacteria can reduce cholesterol content both in vitro and in vivo in humans and animals, the exact mechanism by which they lower cholesterol remains controversial. Currently, there are only three perspectives: (1) co-precipitation; (2) bacterial absorption of cholesterol; (3) a combined effect of co-precipitation and absorption[6, 8]. Researchers have conducted a series of experiments on the cholesterol-lowering effects of lactic acid bacteria, aiming to screen for strains with excellent cholesterol-lowering capabilities and develop functional lactic acid bacteria products.

In the mid-20th century, Jansen had already confirmed that radiation could induce mutations. Radiation breeding is a simple process, where mutation induction can be achieved merely by exposing organisms to radiation. The resulting variant characteristics are diverse and cover a very wide range[9]. Among these methods, heavy ion mutagenesis has physical effects such as energy deposition, charge exchange, and momentum transfer, which can affect the physiological and biochemical functions of cells and cause changes in the genetic material of the cells. Compared to traditional radiation methods, heavy ion mutagenesis has the advantages of lighter damage, higher mutation rates, a broader mutation spectrum, relatively stable inheritance, and ease in obtaining desired new strains. It is an ideal method for the selection and breeding of microbial strains[10, 11].

This study used JMR-01 and SN-2 as the original strains and subjected them to gradient irradiation mutagenesis treatment with medium-energy  $^{12}\text{C}^{6+}$  beams provided by the Lanzhou Heavy Ion Accelerator. The irradiated strains were

initially screened using an optimized KENJI solid screening medium, with the strain diameter as the screening criteria. Subsequently, the ammonium ferric sulfate method was employed to re-screen the preliminary selected strains, using the cholesterol degradation rate as the standard. Final, highly efficient cholesterol-degrading mutant strains were selected. Further investigation into the mechanism of cholesterol reduction by the re-screened strains was conducted to provide a basis for the subsequent selection of cholesterol-degrading strains.

## 2. Materials and Methods

### 2.1. Bacterial Strains

JMR-01 (*Lactobacillus reuteri*) was isolated from the intestinal tract of mice and identified as *Lactobacillus reuteri*;

SN-2 (*Lactobacillus rhamnosus*) was isolated from yogurt and identified as *Lactobacillus rhamnosus*.

### 2.2. Apparatus

DHP-9082 Electric Thermostatic Oven, Shanghai Yiheng Scientific Instrument Co., Ltd.; SW-CJ-1C<sub>μ</sub> Double Person Single-sided Clean Workbench, Suzhou Purification Equipment Co., Ltd.; 756PC UV-Visible Spectrophotometer, Shanghai Spectrum Instruments Co., Ltd.; BS224S Electronic Balance, TE101-L Balance, Beijing Sartorius Instrument System Co., Ltd.; LDZX-30KB Vertical Pressure Steam Sterilizer, Shanghai Shen'an Medical Instrument Factory; Vortex Mixer, Shanghai Kehua Biochemical Instrument Manufacturing Co., Ltd.

### 2.3. Culture Medium Composition and Sterilization Condition

MRS Agar and Slope Medium:

Beef extract 10 g, Peptone 10 g, Yeast extract 5 g, K<sub>2</sub>HPO<sub>4</sub> 2 g, Sodium citrate 5 g, Sodium acetate 5 g, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.58 g, Glucose 20 g, MnSO<sub>4</sub>·4H<sub>2</sub>O 0.25 g, Tween-80 5 mL, dissolved in 1000 mL distilled water, with the addition of Agar 20 g.

KENJI Solid Screening Medium:

Cholesterol (water-soluble) 1.0 g, NH<sub>4</sub>NO<sub>3</sub> 1.0 g, MgSO<sub>4</sub> 0.25 g, NaH<sub>2</sub>PO<sub>4</sub> 0.25 g, Yeast extract 2.0 g, FeSO<sub>4</sub> 5 mg, Tween-80 2 mL, dissolved in 1000 mL distilled water, with the addition of Agar 20 g<sup>[12]</sup>.

MRS-CHOL Liquid Medium:

Weigh 0.1 g of cholesterol, 0.2 g of bovine bile salts, and 0.1 g of sucrose ester, add them to 1 mL of Tween-80, mix well, add 5 mL of glacial acetic acid, and heat at 60°C to dissolve. After the solution is treated with ultrasonic processing (130 W, 20 kHz, 60% ultrasonic fragmentation for 20 cycles, each cycle 8 times, ultrasonic 2 s, interval 3 s), a cholesterol micelle solution is obtained. This solution is quickly added to the MRS liquid medium with continuous stirring to form a uniform and stable colloidal solution. Adjust the volume to 1 L and regulate the pH of the medium to around 6.5 with sodium hydroxide. The sterilization conditions for the above culture medium are all at 115°C, for 30 minutes<sup>[13]</sup>.

### 2.4. Experimental method

#### 2.4.1. <sup>12</sup>C<sup>6+</sup> Irradiation Mutation

Take the fresh slants of JMR-01 and SN-2 that have been cultured at 37°C for 24 hours and add 10 mL of sterile physiological saline. Use an oscillator to prepare a uniform

bacterial suspension. For the preliminary treatment of irradiation mutagenesis, set the irradiation parameters for the <sup>12</sup>C<sup>6+</sup> beam. Take 2 mL of the prepared bacterial suspension and place it in a 35 mm diameter irradiation dish, seal it with a sealing film, and put it on a rotating rack. Utilize the heavy ion beam <sup>12</sup>C<sup>6+</sup> provided by the Heavy Ion Research Facility in Lanzhou (HIRFL), a national key laboratory, with a dose rate of 60 Gy/min and absorbed doses of 0, 60, 120, 180, 240, 300, 400 Gy. Measure the dose with an air ionization dosimeter to perform <sup>12</sup>C<sup>6+</sup> beam irradiation treatment on the *Lactobacillus reuteri* JMR-01 and *Lactobacillus rhamnosus* SN-2 suspensions<sup>[9]</sup>.

#### 2.4.2. Fatality rate

The bacterial suspensions from the control group and the irradiated groups were uniformly spread onto MRS solid medium at a dilution of 10<sup>-7</sup> (with 3 replicates for each treatment). After culturing upside down at 37°C for 24 hours, the number of colonies was counted. The fatality rate was calculated using formula (1), and a lethality curve was plotted.

$$\text{Fatality rate(\%)} = \frac{1 - \frac{\text{Irradiated Group Colony Count}}{\text{Control Group Colony Count}}}{1} \times 100 \quad (1)$$

#### 2.4.3. Establishment of screening methods

##### 2.4.3.1 Screening Plate Optimization

###### 2.4.3.1.1 Cholesterol Solubility Optimization for Research

Based on the research of Guo Dongqi (2007)<sup>[12]</sup> and Xu Jingxue (2010)<sup>[14]</sup>, among others, the KENJI liquid medium was improved and optimized. During the modification process, it was found that the solubility of cholesterol in the medium was poor. To address this issue, three different methods were employed to dissolve cholesterol: first, dissolving cholesterol in water and then emulsifying it with an ultrasonic cell disruptor (600W) for 20 minutes; second, dissolving cholesterol with DMSO; and third, dissolving cholesterol with absolute ethanol. The states of cholesterol dissolved by the three methods in the improved solid medium were compared, and the number of colonies in the medium was counted to determine the optimal dissolution method for cholesterol in the improved solid screening medium.

###### 2.4.3.1.2 Optimize Yeast Extract Concentration

Following the determination of the cholesterol dissolution method in the improved KENJI solid screening medium, this study further optimized the addition amount of yeast extract in the improved solid screening medium. The colony counts in the improved KENJI solid screening medium were compared at different yeast extract addition levels (5 g/L, 2.5 g/L, 2.0 g/L, 0.2 g/L), ultimately determining the optimal yeast extract addition in the KENJI solid screening medium<sup>[15, 16]</sup>. This provides more favorable culture conditions for the subsequent screening of cholesterol-lowering strains.

###### 2.4.3.2 Establishment of a Screening Method for High Cholesterol-Lowering Strain Detection

The bacterial suspension of the original strain was diluted to a 10<sup>-7</sup> dilution and evenly spread on the improved KENJI solid screening medium. It was cultured in a 37°C incubator for 3-4 days until single colonies formed. Single colonies with different diameters were selected and cultured in MRS and MRS-CHOL for 24 hours, respectively. The cholesterol degradation ability of strains with different diameters was determined using the ammonium ferrous sulfate method<sup>[6, 13, 17, 18]</sup>. The EXCEL software was used to statistically analyze the colony diameter of the strains and their corresponding cholesterol degradation ability, and a linear regression curve

was plotted to determine whether there is an association between the colony diameter and the strain's cholesterol degradation ability.

#### 2.4.4. Measurement of Positive and Negative Mutation Rate

The bacterial suspensions of all irradiated dose strains were uniformly spread onto the improved KENJI solid screening medium plates at a dilution of  $10^{-7}$  (with 3 replicates for each treatment). After sealing, the plates were incubated upside

$$\text{Positive Mutation Rate (\%)} = \frac{\text{Number of Colonies with Diameter Increased by 20\% or More Compared to the Control Group}}{\text{Total Colony Count}} \times 100 \quad (2)$$

$$\text{Negative Mutation Rate (\%)} = \frac{\text{Number of Colonies with Diameter Decreased by 20\% or Less Compared to the Control Group}}{\text{Total Colony Count}} \times 100 \quad (3)$$

#### 2.4.5. Primary Screening and Secondary Screening of Strain

After establishing a high-throughput screening method for lactobacilli with high cholesterol-lowering ability, a large number of irradiated strains were initially screened using the improved KENJI solid screening medium. Strains with significantly increased colony diameter compared to the original strain were selected and cultured in MRS and MRS-CHOL for 24 h each. Finally, the cholesterol-degrading ability of these strains was further determined using the ammonium ferrous sulfate method, to screen for mutant strains that showed significant differences in cholesterol degradation rates compared to the original strain.

#### 2.4.6. Stability Assay of High Cholesterol-Lowering Strain

The mutant strains with high cholesterol-degrading ability, screened using the improved KENJI solid screening medium, will be subcultured for 6 generations. Each generation will undergo an assay to measure the cholesterol-degrading capability, by determining the strain's ability to degrade cholesterol in the fermentation broth, in order to assess the genetic stability of the strain's cholesterol-degrading capacity [19].

#### 2.4.7. Preliminary Study on the Mechanism of Lactobacillus Cholesterol Lowering

The original and mutant strains were cultured in MRS for 24 hours and then inoculated at a 1% volume into MRS-

down at 37°C for 3-4 days and counted to calculate the positive and negative mutation rates for each irradiation dose. A strain with a colony diameter that increased by 20% compared to the average colony diameter of the control strain was considered a positive mutation. A strain with a colony diameter that decreased by 20% compared to the average colony diameter of the control strain was considered a negative mutation. The positive and negative mutation rates were calculated using formulas (2) and (3), respectively.

CHOL and cultured for another 24 hours. After culturing, the bacterial suspension was mixed thoroughly and centrifuged at 12,000 r/min for 10 minutes. A 0.2 mL aliquot of the supernatant was taken and the un inoculated MRS-CHOL medium was used as a control to determine the total cholesterol degradation rate of the experimental strains using the ammonium ferrous sulfate method. The remaining supernatant was discarded, and then 4 mL of sodium phosphate buffer was added to the bacterial pellet, mixed thoroughly, and centrifuged at 8,000 r/min for 10 minutes. A 1 mL aliquot of the supernatant was taken to determine the cholesterol content using the ammonium ferrous sulfate method, which represents the amount of cholesterol coprecipitated by the strain. The remaining supernatant was discarded, and 4 mL of sodium phosphate buffer and 100  $\mu$ L of 50 mg/mL lysozyme were added to the remaining bacterial pellet. The suspension was then sonicated at ice bath conditions with a power of 200W for 20 minutes (120 cycles, each cycle 5s on and 5s off). A 2 mL aliquot of the broken cell suspension was centrifuged at 12,000 r/min for 10 minutes, and a 1 mL aliquot of the supernatant was used to determine the cholesterol content, which represents the amount of cholesterol absorbed by the strain [8, 16, 20, 21]. The total cholesterol degradation rate, the coprecipitation cholesterol degradation rate, and the absorption cholesterol degradation rate were calculated using formulas (4), (5), and (6), respectively.

$$\text{Total Cholesterol Degradation Rate (\%)} = \frac{\text{Total Cholesterol Content} - \text{Cholesterol Content in the Supernatant}}{\text{Total Cholesterol Content}} \times 100 \quad (4)$$

$$\text{Coprecipitation Cholesterol Degradation Rate (\%)} = \frac{\text{Cholesterol Content in Cell Washes}}{\text{Total Cholesterol Content}} \times 100 \quad (5)$$

$$\text{Absorption Cholesterol Degradation Rate (\%)} = \frac{\text{Cholesterol Content in Cell Lysate}}{\text{Total Cholesterol Content}} \times 100 \quad (6)$$

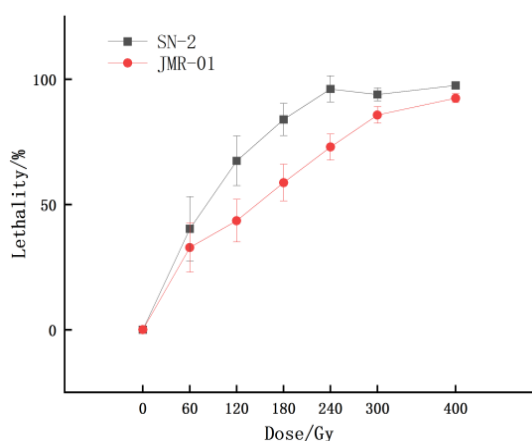
## 2.5. Data Processing

The experimental data were statistically analyzed using SPSS 23.0, and the figures were processed with Origin 8.0. The results are expressed as the mean  $\pm$  standard error. A P-value of less than 0.05 indicates a statistically significant difference.

## 3. Results and Discussions

### 3.1. The Impact of 12C6+ Irradiation Dose on Lethality

Using the method described in section 2.4.2, the lethality at each dose was statistically analyzed, and a lethal curve for the strain was plotted, as shown in Figure 1.



**Figure 1.** Lethal curve of mutagenesis induced by heavy ion irradiation

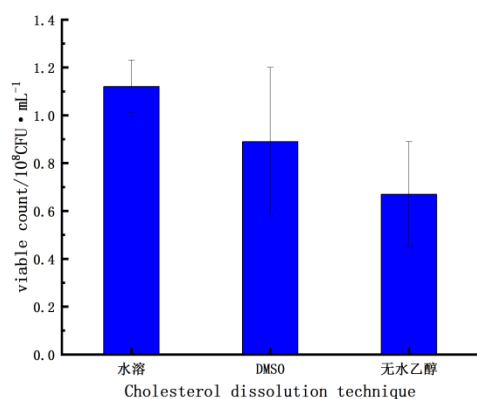
The experimental results showed that the mortality of *Lactobacillus reuteri* JMR-01 and *Lactobacillus rhamnosus* SN-2 increased significantly with the absorbed dose following irradiation with different doses of  $^{12}\text{C}^{6+}$ , but did not exhibit a linear relationship. Strain SN-2 showed a weak saddle-shaped curve, where the mortality increased with the absorbed dose before 240 Gy, slightly decreased at 300 Gy, and then increased again at 400 Gy. When the irradiation dose reached 120 Gy, the mortality of *Lactobacillus reuteri* JMR-01 reached 43.5%; at 60 Gy, the mortality of *Lactobacillus rhamnosus* SN-2 reached 40.3%. Therefore, the half-lethal dose for *Lactobacillus reuteri* JMR-01 was estimated to be between 120 Gy and 180 Gy, and for *Lactobacillus rhamnosus* SN-2, it was between 60 Gy and 120 Gy references [10, 11].

## 3.2. Establishment of screening methods

### 3.2.1. Screening Plate Optimization

#### 3.2.1.1 Cholesterol Solubility Optimization for Research

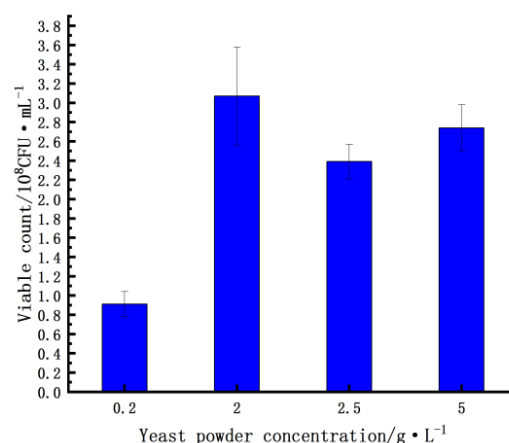
According to the method described in section 2.4.3.1.1, the experimental results indicated that the maximum number of colonies was observed in the screening plate when cholesterol was dissolved in water and emulsified by ultrasonic cell disruptor (600W) for 20 minutes, reaching  $1.12 \times 10^8$  CFU/mL. Moreover, the cholesterol dissolved in water showed a better state in the modified solid medium. The results are shown in Figure 2. Therefore, it was determined that the final method for dissolving cholesterol would be to dissolve it in water and emulsify it with an ultrasonic cell disruptor (600W) for 20 minutes.



**Figure 2.** Relationship between the method of Cholesterol dissolution and the number of Viable bacteria

### 3.2.1.2 Optimize Yeast Extract Concentration

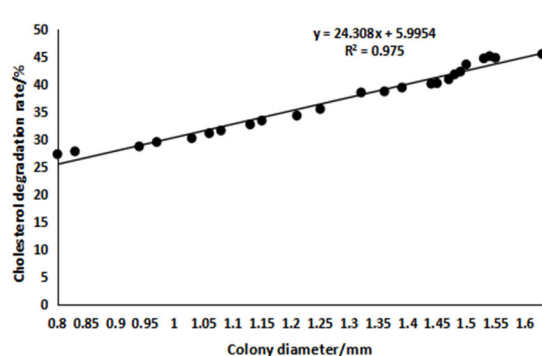
According to the method described in section 2.4.3.1.2, the experimental results showed that the maximum number of colonies was achieved in the modified solid screening medium when the yeast extract concentration was 2.0 g/L, reaching  $3.07 \times 10^8$  CFU/mL, as shown in Figure 3. It was ultimately determined that the optimal concentration of yeast extract is 2.0 g/L.



**Figure 3.** Relationship between Yeast Powder Concentration and Viable Bacteria Count

### 3.2.2. Establishment of a Screening Method for High Cholesterol-Lowering Strains

Using the method described in section 2.4.3.2, the colony diameter of 25 strains of JMR-01 with different sizes was measured, as well as the cholesterol degradation rate of each strain. The colony diameter and corresponding cholesterol degradation rate were analyzed for linear correlation using EXCEL software. The results showed that there was a significant positive correlation between the colony diameter of lactic acid bacteria grown on the modified solid screening medium and their cholesterol-degrading ability. The regression equation was  $y = 24.308x + 5.9954$ , with a correlation coefficient of 0.975 (as shown in Figure 4). Therefore, this correlation can be applied in the preliminary screening of lactic acid bacteria with high cholesterol-degrading capability.

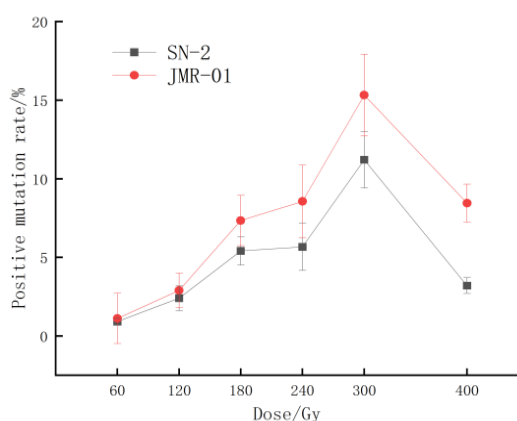


**Figure 4.** Relationship between lactic acid bacteria colony diameter and cholesterol degradation ability

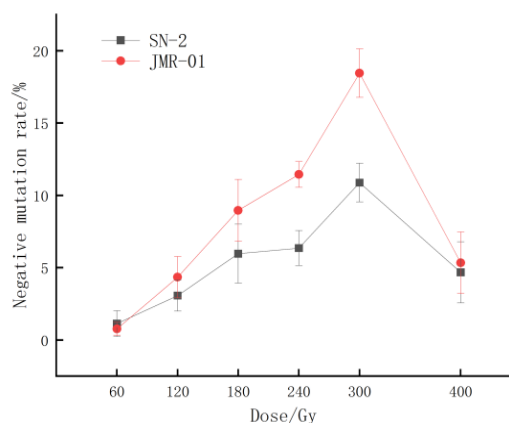
## 3.3. The Impact of $^{12}\text{C}^{6+}$ Irradiation Dose on the Rates of Positive and Negative Mutations

According to the method described in section 1.4.4, the

rates of positive and negative mutations in strains JMR-01 and SN-2 were statistically analyzed after irradiation. The absorbed dose was plotted on the x-axis, and the rates of positive and negative mutations were plotted on the y-axis. The results are shown in Figures 5 and 6. As can be seen from these figures: After the original strains were irradiated with different doses of  $^{12}\text{C}^{6+}$  beams, the rates of positive and negative mutations for both strains gradually increased with the absorbed dose. When the absorbed dose reached 300 Gy, both the positive and negative mutation rates reached their peaks, with the maximum positive and negative mutation rates for strain JMR-01 being 15.31% and 18.45%, respectively, and for strain SN-2 being 11.21% and 10.88%, respectively. According to related studies on radiation mutagenesis, when the mortality of the mutated microorganisms is higher, the mutation rate of the strains may also be higher. Under the absorbed doses used, the strains were more likely to exhibit higher rates of positive and negative mutations references [23-25]. The experimental results were consistent with the theoretical findings of this research, and the reasons for this phenomenon require further investigation. However, although the optimal dose for screening high-quality and high-yield lactic acid bacteria is 300 Gy, the number of obtained strains is relatively small.



**Figure 5.** Positive Mutation Rate of Heavy Ion Irradiation-Mutated Strains



**Figure 6.** Negative Mutation Rste of Heavy Ion Irradiation-Induced Mutant Strain

### 3.4. Screening of Mutants with High Cholesterol-Degrading Ability

#### 3.4.1. Preliminary Screening of Mutants with High Cholesterol-Degrading Ability

After the bacterial suspensions were irradiated with different doses of  $^{12}\text{C}^{6+}$  beams, gradient dilutions were performed. Following the preliminary screening method described in section 2.4.5, the high cholesterol-degrading ability of the irradiated JMR-01 and SN-2 strains was initially screened using colony diameter. Ultimately, 14 mutant strains of JMR-01 and 12 mutant strains of SN-2 were preliminarily screened, totaling 26 strains with a colony diameter 20% larger than that of the original strains. The preliminary screening results are shown in Table 1.

**Table 1.** Primary screening results of strain

Strain number	Colony diameter/mm	Strain number	Colony diameter r/mm
JMR-01-1	1.73	JMR-01-14	1.47
JMR-01-2	1.67	SN-2-1	1.71
JMR-01-3	1.57	SN-2-2	1.67
JMR-01-4	1.64	SN-2-3	1.55
JMR-01-5	1.55	SN-2-4	1.64
JMR-01-6	1.58	SN-2-5	1.63
JMR-01-7	1.48	SN-2-6	1.60
JMR-01-8	1.47	SN-2-7	1.52
JMR-01-9	1.51	SN-2-8	1.57
JMR-01-10	1.54	SN-2-9	1.69
JMR-01-11	1.50	SN-2-10	1.58
JMR-01-12	1.48	SN-2-11	1.48
JMR-01-13	1.45	SN-2-12	1.50

#### 3.4.2. Rescreening of Mutants with High Cholesterol-Degrading Ability

After the preliminary screening of the 26 strains, a rescreening was conducted using the ammonium ferrous sulfate method, with the cholesterol degradation rate as the indicator. This process resulted in the selection of 5 strains that showed a significant difference in cholesterol degradation rate compared to the original strains. The results of the rescreening are presented in Tables 2 and 3. Among them, strain JMR-01-1 showed a 9.7% increase in cholesterol degradation rate compared to the original strain JMR-01, with a significant difference ( $p < 0.01$ ). Similarly, strain SN-2-1 exhibited an 11.5% increase in cholesterol degradation rate compared to the original strain SN-2, also with a significant difference ( $p < 0.01$ ).

**Table 2.** Screening results of JMR-01 plate

Strain number	Colony diameter/mm	Cholesterol degradation rate/%
JMR-01 (CK)	1.21	51.3
JMR-01-1	1.73	56.3**
JMR-01-2	1.67	56.1**
JMR-01-4	1.64	55.5**

**Table 3.** Screening results of SN-2 plate

Strain number	Colony diameter/mm	Cholesterol degradation rate/%
SN-2 (CK)	1.21	49.5
SN-2-1	1.71	55.2**
SN-2-9	1.69	54.8**

### 3.5. Stability Assessment of Strains with High Cholesterol-Degrading Ability

The highest four strains with high cholesterol-degrading ability (JMR-01-1, JMR-01-2, SN-2-1, SN-2-9) were passaged six times consecutively, and their cholesterol-

degrading capabilities were measured in each generation to assess the genetic stability of their cholesterol-degrading traits. The results are presented in Tables 4 and 5.

As indicated by the data in Tables 4 and 5, after six passages, there were no significant differences in the cholesterol degradation rates of the four mutant strains (JMR-01-1, JMR-01-2, SN-2-1, SN-2-9), suggesting that these strains possess stable genetic characteristics for cholesterol degradation. The average cholesterol degradation rate of JMR-01-1 was 56.4%, which is a 9.94% increase compared to the original strain JMR-01 (51.3%), with a significant difference ( $p < 0.01$ ). Similarly, the average cholesterol degradation rate of SN-2-1 was 55.04%, an 11.19% increase compared to the original strain SN-2 (49.5%), also with a significant difference ( $p < 0.01$ ).

**Table 4.** Genetic stability test of JMR-01 mutant strain

Passage number	1	2	3	4	5	6
JMR-01-1	56.97%	56.95%	56.28%	56.38%	55.80%	56.01%
JMR-01-2	56.15%	55.55%	55.61%	56.58%	56.56%	55.35%

**Table 5.** Genetic stability test of SN-2 mutant strain

Passage number	1	2	3	4	5	6
SN-2-1	55.83%	55.52%	54.58%	54.83%	54.67%	54.78%
SN-2-9	53.62%	53.79%	54.58%	53.74%	53.50%	53.81%

### 3.6. Mechanisms of Improved Cholesterol-Degrading Ability in Bacterial Strains

The study involved measuring and analyzing the cholesterol content in the supernatant, wash solution, and cell lysate of the original strains (JMR-01 and SN-2) as well as the four high-mutant strains. The assimilation and co-precipitation effects of these strains on cholesterol were calculated, and the results are presented in Table 6.

The results indicate that the total cholesterol degradation rate and the absorption-mediated cholesterol degradation rate of the mutant strains JMR-01-1 and JMR-01-2 were increased

by 8.69% and 7.47%, and 16.6% and 18.61%, respectively, compared to the original strain JMR-01, with significant differences ( $P < 0.05$ ). However, no significant difference was observed in the co-precipitation cholesterol degradation rate. Similarly, the total cholesterol degradation rate and the absorption-mediated cholesterol degradation rate of the mutant strains SN-2-1 and SN-2-9 were increased by 11.79% and 9.90%, and 20.26% and 15.05%, respectively, compared to the original strain SN-2, all with significant differences ( $P < 0.05$ ), but again, no significant difference was found in the co-precipitation cholesterol degradation rate.

**Table 6.** Comparison of cholesterol-lowering ability among strain

	JMR-01	JMR-01-1	JMR-01-2	SN-2	SN-2-1	SN-2-9
Total cholesterol degradation rate/%	51.79±0.4	56.29±0.3*	55.66±0.8*	48.68±1.2	54.42±0.7#	53.50±0.7#
cholesterol degradation rate through co precipitation/%	18.28±1.0	18.91±1.3	17.20±1.2	16.74±1.1	16.89±0.5	16.89±1.3
Absorption effect cholesterol degradation rate/%	30.84±1.1	35.96±0.7*	36.58±0.5*	29.91±0.6	35.97±1.2#	34.41±0.4#
Other effects cholesterol degradation rate/%	2.67	1.42	1.88	2.03	1.56	2.20

(Note: \* indicates a significant difference from JMR-01; # indicates a significant difference from SN-2)

Currently, the mechanisms by which probiotics lower cholesterol primarily involve the adsorption and co-precipitation of cholesterol by probiotic cells, ultimately leading to the excretion of cholesterol along with the probiotics, thereby reducing the cholesterol content in the intestinal environment and decreasing cholesterol absorption. For example, studies by Li Yao and others on the lactic acid bacteria WS1 and LP6 isolated from traditionally fermented sauerkraut have shown that the absorption effect of these strains is greater than the co-precipitation effect<sup>[20]</sup>. Similarly,

research by Li Ni and others on *Lactobacillus plantarum* MPCF7-3 isolated from human intestines, homemade pickles, and marine fish intestines has demonstrated that the absorption effect of *Lactobacillus plantarum* MPCF7-3 accounts for a significant proportion of cholesterol removal<sup>[26]</sup>. Furthermore, research by Yin Boxing and others on a batch of cholesterol-lowering human-derived strains with independent intellectual property rights, such as *Lactobacillus rhamnosus* LV108, *Lactobacillus casei* grx12, and *Lactobacillus fermentum* grx08, isolated from the intestines

of long-lived individuals has shown that the stronger the absorption effect of these strains, the greater their cholesterol-lowering ability [27]. However, further research is still needed to confirm the degradation effect and mechanism of lactic acid bacteria on cholesterol in vivo.

## 4. Summary

This study successfully established a method for preliminary screening of lactic acid bacteria's cholesterol-degrading ability using colony diameter as a screening indicator by optimizing the cholesterol screening medium. Preliminary research on the mechanism of cholesterol reduction by lactic acid bacteria indicates that this screening method can identify strains with improved cholesterol absorption capabilities. Using this method to screen JMR-01 and SN-2 after mutagenesis with 300Gy heavy ion beam irradiation, four mutant strains, JMR-01-1, JMR-01-2, SN-2-1, and SN-2-9, were obtained. Compared to their original strains, these mutants showed increased cholesterol degradation rates of 9.7%, 9.4%, 11.5%, and 10.7%, respectively, with good stability. The experiment demonstrates that this method can be used to screen lactic acid bacteria with stronger cholesterol-degrading abilities, and the mutant strains JMR-01-1 and SN-2-1, with higher cholesterol degradation rates, are promising candidates for the development of subsequent lipid-lowering and weight loss probiotic products.

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