Screening of Lactic Acid Bacteria from Sauerkraut and Their Antioxidant Properties Research

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Abstract: In this study, we identified Lactic acid bacteria (LAB) with antioxidant properties by isolating three strains from naturally fermented pickled Chinese cabbage using a traditional plate streaking methodology. The preliminary selection was based on tolerance to 1.0-2.0 mmol/L H_2O_2. Further screening assessed the total antioxidant capacity, free radical scavenging ability, reducing power, and anti-lipid peroxidation capacity of the strains, revealing that strain DN-2 exhibited the strongest antioxidant activity. The cell-free extract of strain DN-2 demonstrated significant antioxidant activity, with a 77.87% scavenging efficiency against DPPH radicals and a 33.08% inhibition of hydroxyl radicals. Additionally, it displayed an anti-lipid peroxidation effect of 38.59% and a total antioxidant capacity equivalent to 0.137 mol Trolox per 10^4 cells. Intact cells of DN-2 showed a hydroxyl radical scavenging rate of 38.72% and a reducing power of 35.16%, further confirming its antioxidative potential. Furthermore, DN-2 possesses BSH enzyme activity and exhibits no hemolytic activity, while showing high sensitivity to antibiotics such as tetracycline, clindamycin, ampicillin, chloramphenicol, gentamicin, cefazolin, and amoxicillin. Through 16S rDNA gene sequence alignment, it was identified as Leuconostoc mesenteroides. These results indicate that the screened strain DN-2 not only has substantial antioxidant capacity but also probiotic effects, providing a foundation for preventing various diseases caused by oxidative stress and for the development of natural and healthy antioxidant health foods.

Keywords: Sauerkraut; Lactic Acid Bacteria; Antioxidant; Screening and Identification

1. Introduction

1.1 Overview of Lactic Acid Bacteria

Lactic acid bacteria can ferment to produce lactic acid, which has been mainly used for fermentation together with yeast, and can be divided into two types according to their fermentation products [1]: one belongs to homolactic acid fermentation, which only produces lactic acid. If the fermentation product not only contains lactic acid, but also produces other substances, it is a heterotype lactic acid fermentation. In the gastrointestinal tract, Lactic acid bacteria are very common probiotics, which not only inhibit some pathogenic bacteria by producing bacteriocin, but also inhibit the growth and reproduction of pathogenic bacteria by robbing them of their attachment positions and nutrients in the gastrointestinal and gastrointestinal regions, and Lactic acid bacteria play a high role in regulating the balance of gastrointestinal flora [2,3]. In the field of food preservation, Lactic acid bacteria can effectively extend the shelf life of food by inhibiting pathogenic bacteria such as *Staphylococcus aureus* and reducing the pH of food [1]. Lactic acid bacteria can also effectively reduce cholesterol in people at risk of high cholesterol, which has been demonstrated by experiments such as the establishment of hyperlipidemic mice [4].

1.2 Antioxidant Properties of Lactic Acid Bacteria

Oxygen is the basic substance necessary for our body, but in the body's metabolic process, a lot of reactive oxygen species are produced

while providing energy. The oxygen itself is harmless, and the reactive oxygen species produced are strongly oxidizing. Too much of these reactive oxygen species (ROS) can cause damage to the human body, attacking the structure of cells and causing them to age [5]. Lactic acid bacteria, on the other hand, are natural antioxidants that can scavenge reactive oxygen species and are safer and healthier than synthetic antioxidants. ROS is very unstable and has a high oxidative activity, and ROS begins to attack cells, further leading to oxidative stress. ROS is a major cause of oxidative stress, and in this state, cells age at an accelerated pace and even lead to various diseases [6,7]. At this time, the accumulation of reactive oxygen species in the body is excessive, and although it has its own defense mechanism, it is not enough to eliminate the excess ROS [8], and then the help of exogenous antioxidants is needed, which can be obtained from external food. As a natural antioxidant, Lactic acid bacteria can effectively delay the oxidation of food, prevent various diseases caused by oxidative stress, and provide ideas and directions for the development of natural and healthy health foods.

1.3 Antioxidant Mechanism of Lactic Acid Bacteria

1.3.1 Oxygen stress and defense mechanisms of Lactic acid bacteria

Lactic acid bacteria are generally anaerobic bacteria or facultative anaerobes, so they are suitable for living in low-oxygen environments. However, due to the stress of various aerobic environments and poor resistance to harsh environments, Lactic acid bacteria began to use oxygen, and in aerobic respiration, electrons are transferred along the respiratory chain to O_2, and O_2 is reduced to form reactive oxygen species [9]. However, due to the electron mismatching, this reactive oxygen species exhibits strong oxidation and instability, which will attack Lactic acid bacteria cells, attack biological film systems such as cell membranes, and macromolecular substances such as nucleic acids [10], and eventually lead to cell senescence, affect the probiotic function of Lactic acid bacteria, and even lead to their death. At present, the antioxidant mechanism of Lactic acid bacteria is not well understood, and it may be that in order to resist oxygen stress, Lactic acid bacteria have gradually evolved to use the redox system and antioxidant enzyme system to defend against reactive oxygen species [11].

1.3.1.1 Mercaptans

Mercaptans play a role in the antioxidant of Lactic acid bacteria, among which glutathione (GSH) and thioredoxin (Trx) play an important role. GSH can reduce peroxides such as H_2O_2 to H_2O, and oxidize itself to GSSG under the catalysis of GPx, thereby playing a role in scavenging ROS. GSSG and GSH can be converted into each other, which also means that GSSG is catalyzed by glutathione reductase (GR) to form GSH, which continues to play an antioxidant role. Together, GSH, GR, and NADPH make up the glutathione system that continuously removes ROS [12]. Thiodoxin (TrxR), assisted by NADPH, oxidizes Trx while H_2O_2 produces harmless H_2O. Trx is of great significance for Lactic acid bacteria to survive under aerobic conditions, and for Lactic acid bacteria with low GSH content, it has a high Trx content, and can also improve the activity of certain antioxidant enzymes to help scavenge ROS. The disulfide reductase system constituted by them is the first line of defense against oxidic stress in Lactic acid bacteria [13,14], and its antioxidant response is shown in Figure 1.

$$NADPH+H^{+}+H_2O \underset{GR/GSH/GPx}{\overset{TrxR/Trx/Prx}{\rightleftharpoons}} NADP^{+}+2H_2O$$

Figure 1. Trx-GSH Antioxidant Map

1.3.1.2 Antioxidant enzymes

Lactic acid bacteria can also play a role by regulating various antioxidant enzymes, such as NADH oxidase, SOD and CAT. Among them, NADH oxidase and NADH peroxidase synergistically complete the clearance of ROS through a series of reactions [14]. First, NADH oxidase catalyzes the reduction of O_2 to form H_2O_2, and NADH is oxidized by O_2 to NAD. Subsequently, NADH peroxidase catalyzes the further reduction of H_2O_2 to H_2O [15]. SOD and CAT also work together to accomplish ROS clearance, and SOD acts on superoxide anions to reduce them to H_2O_2, which is then catalyzed by CAT to form H_2O [16]. A variety of antioxidant enzymes work synergistically in Lactic acid bacteria to jointly resist H_2O_2 and ensure that the concentration of H_2O_2 is always at a harmless level.

1.3.2 Regulate signaling pathways

When ROS in the body increases, the activity of some antioxidant enzymes decreases. Many

scholars have found that Lactic acid bacteria can increase their antioxidant activity by regulating some signaling pathways and increasing self-defense [17]. There are many signaling pathways involved in resistance to oxidative stress, such as the Nrf2 pathway, the PKC pathway, the MAPK pathway, and the AKT pathway, which are activated by Lactic acid bacteria in response to increased levels of ROS [18,19]. Among them, Nrf2-Keap1-ARE is the most important regulatory pathway, and in the low level of ROS, Nrf2 binds to Keap1 and inhibits the activity of Nrf2, which occurs in the cytoplasm. When ROS levels rise, cells are attacked by free radicals, and MAPK and Pl3K regulate signaling pathways, altering the Keap1 conformation. Nrf2 detached into the nucleus and binds to ARE, regulates the production of antioxidant proteins, and prevents oxidative stress through the Nrf2-Keap1-ARE pathway [20], and improves antioxidant capacity to resist oxidative stress.

1.3.3 Scavenging free radical system

Free radicals are highly oxidizing, and when human cells accumulate too many free radicals due to respiration and other reasons, a series of hazards such as lipid peroxidation, destruction of cell internal structure, and cell aging and death will occur. Some scholars have also proposed the theory of free radicals, so the human body urgently needs safe and healthy natural antioxidants such as Lactic acid bacteria to help the body remove free radicals. Zhang Lin et al. [21] isolated traditional yogurt from live bacteria on the market, and identified a total of five species. When the bacterial concentrations were 10^9 CFU/mL and 10^{10} CFU/mL, respectively, the free radical scavenging rates of CCSN-1, CCSN-2, CCSN-3, CCSN-10 and SSJF-8 were statistically analyzed, and it was found that the strains with CCSN-1 and 10^{10} CFU/mL had higher scavenging rates.

1.3.4 Anti-lipid peroxidation and chelation of Fe^{2+} and Cu^{2+}

Lipid peroxidation refers to the excessive accumulation of ROS, which begins to attack biofilm systems with unsaturated fatty acids such as cell membranes and macromolecules such as nucleic acids, causing cell damage and death. Qiao Yujing[22] screened four strains of Lactic acid bacteria from the feces of healthy rabbits and found that four strains of Lactic acid bacteria, including B2-3, had good anti-lipid peroxidation ability, among which the anti-lipid peroxidation rate of *Lactobacillus* joseri B2-3 reached 39.46%, and Lactic acid bacteria exerted their antioxidant effect by directly inhibiting lipid peroxidation. Fe^{2+} and Cu^{2+} catalyze the production of free radicals, excess Fe^{2+} and Cu^{2+} catalyze the production of ROS and superoxide radicals, and Lactic acid bacteria can protect against oxidative stress through their chelating ability to Fe^{2+} and Cu^{2+} [14]. Zhang Di [23] screened 8 strains of Lactic acid bacteria from fermented vegetables, and found that they had a strong chelating ability to Fe^{2+}, among which GZ394 had a high chelating capacity of 54.64% for Fe^{2+}.

1.4 Research Status and Progress on Antioxidant Properties of Lactic Acid Bacteria

Some scholars have found that not only the intact cells of Lactic acid bacteria have antioxidant properties, but also the cell-free extracts obtained by centrifugal fragmentation of Lactic acid bacteria also have the ability to reduce and resist lipid oxidation, and even the inactivated bacteria also have antioxidant effects [24], and it has been found that the antioxidant mechanism of Lactic acid bacteria may be related to some substances produced by Lactic acid bacteria. The research is not only limited to in vitro experiments, but also uses animals as carriers to complete a series of in vivo antioxidant experiments to ensure that the screened Lactic acid bacteria can survive under the acidic conditions of the stomach and intestines, and exert their various probiotic functions. Yang et al. [25] found that the antioxidant activity of Lactic acid bacteria was higher after fermentation, and the DPPH scavenging rate and iron reducing capacity of unfermented laver sauce were 20.4% and 0.23, and the free radical scavenging rate and iron reducing capacity were significantly increased to 78% and 0.73 after 21 days of fermentation by *Lactobacillus* casei.

1.5 Application of Lactic Acid Bacteria for Antioxidant

With the increasing demand for antioxidation and the understanding of health care functions, the antioxidant effect of Lactic acid bacteria is widely used in many fields, attracting the attention of many disciplines such as microecology. Lactic acid bacteria can be

added to the feed of livestock as a probiotic fermentation preparation to increase the antioxidant activity of the feed and effectively alleviate the stress of livestock [26,27]. It is also used in food, and the antioxidant activity of food fermented by Lactic acid bacteria can be effectively improved, which is a safe and healthy antioxidant, which greatly delays the shelf life and taste of food. Lactic acid bacteria are also used in the field of diseases, such as liver disease and high blood pressure, which can alleviate the disease, reduce the damage to the body, and are even expected to be used in cancer to prolong the survival time. At the same time, it can be used as a health food to remove excess ROS for the human body and prevent and alleviate a series of diseases caused by oxidative stress, such as cardiovascular diseases [11].

1.6 Research Content and Implications

It is of great significance to screen out strains with strong antioxidant properties. With a series of metabolic activities such as cellular respiration, the reactive oxygen species produced are unstable and strongly oxidized, attacking the cells, and when there are too many reactive oxygen species, the body's defense mechanism is insufficient, which will lead to oxidative stress and a series of cardiovascular diseases [28]. Exogenous antioxidants are urgently needed to help the body resist oxidation, and Lactic acid bacteria, as a microecological agent, also has beneficial effects such as cholesterol lowering and antibacterial, which is safer and healthier than synthetic antioxidants. The need for antioxidants is increasing, and antioxidants are gradually penetrating into various fields. It can be used in food to extend the shelf life and improve antioxidant activity, and used in feed to relieve the stress of poultry and livestock. In this experiment, through the initial screening of H_2O_2 tolerance and the ability to scavenge free radicals and anti-lipid oxidation, the safe strains with good antioxidant capacity and certain prebiotic effects were screened out, which laid the foundation for the development of natural and healthy antioxidant health foods, which has certain practical significance.

2. Materials and Methods

2.1 Test Material

2.1.1 Strain source

The strains in this experiment were isolated and screened from naturally fermented sauerkraut.

2.1.2 Culture medium

The formulation of the main medium used in this experiment is shown in Table 1.

Table 1. Culture Medium and Preparation Method

Solution	Formula
MRS medium	MRS broth 54.0 g, add distilled water to 1000 mL, adjust the pH to about 7, add 15 g agar if prepared with solid medium, and sterilize at 121°C for 20 min
MRS-$CaCO_3$ medium	MRS medium was sterilized at 121°C for 20 min
LB medium	LB broth 25.0 g, add distilled water to 1000 mL, if with solid medium, add 10 g of agar, sterilize at 121°C for 20 min
Bile salt MRS medium	Add 0.5% sodium taurodeoxycholate and 0.037% calcium chloride to the sterilized MRS medium and sterilize at 121°C for 20 min
Colombian blood agar plates	Purchased from Hunan Bickman Holding Co., LTD

2.1.3 Main reagents

Table 2. Main Reagents and Manufacturers

Name of the reagent	Manufacturer
MRS broth	Aalshan (Guangzhou) Biotechnology Co., LTD
LB broth	Hunan Bickman Holding Co., LTD
Agar powder	Beijing Solaibao Technology Co., LTD
calcium carbonate	Tianjin Damao Chemical Reagent Factory
Agarose	Anhui Bio-Lab Biotechnology Co., LTD
Ferrous sulfate monohydrate	Shanghai Maclean's Biochemical Technology Co., LTD
salicylic acid	Shanghai Maclean's Biochemical Technology Co., LTD
Absolute ethanol	Xilong Science Co., LTD
30% hydrogen peroxide	Xilong Science Co., LTD
1% potassium ferricyanide	Shanghai Maclean's Biochemical Technology Co., LTD
Trichloroacetic acid	Shanghai Maclean's Biochemical Technology Co., LTD

The kit used in this experiment and its manufacturer: bacterial genomic DNA extraction kit was purchased from Beijing Quanshijin Biotechnology Co., LTD.; DPPH Free Radical Scavenging Ability Kit, Total Antioxidant Capacity Kit and Anti-Lipid Peroxidation Ability Kit were all purchased from Suzhou Grace Biotechnology Co., LTD. The main reagents used in this experiment are shown in Table 2.

2.1.4 Main instruments and equipment and

consumables

Conventional equipment such as Erlenmeyer flasks, beakers, graduated cylinders, glass rods, petri dishes, coating rods, inoculation loops and vernier calipers; Consumables such as tubes, tips, and rubber gloves. The main instruments and equipment used in this experiment are shown in Table 3.

Table 3. Main Instruments, Equipment and Manufacturers

Experimental instrument	Machine type	Manufacturer
Ultra-clean workbench	SW-CJ-IFD	Suzhou Antai Air Technology Co., LTD
Automatic autoclave	YXQ-75G	Shanghai Boxun Industrial Co., LTD
Electronic balances	STX1202ZH	OHAUS Instruments (Changzhou) Co., LTD
Constant temperature culture shaker	THZ-300	Shanghai Yiheng Scientific Instrument Co., LTD
High-speed refrigerated centrifuge	5424R	Hunan Xiangyi Laboratory Instrument Development Co., LTD
Ultra-low temperature storage box	DW-86L626	Qingdao Haier Co., LTD
Biological microscopy	Ci-L plus	Nikon Imaging Instrument Sales (China) Co., LTD
UV-Vis spectrophotometer	X-6	Shanghai Yuanxi Instrument Co., LTD
Multimode microplate reader	Spectra Max M2e	Meigu Molecular Instruments (Shanghai) Co., LTD
Ultrasonic cleaner	KQ5200DE	Kunshan Ultrasonic Instrument Co., LTD
Gradient thermal cycler	TC-96/G/H(b)C	Hangzhou Bioer Technology Co., LTD
Electrophoresis gel imaging split system	JS-2000	Bole Life & Medical Products Co., LTD

2.2 Test Method

2.2.1 Isolation and purification of Lactic acid bacteria

Take an appropriate amount of fermented sauerkraut and grind it thoroughly, and collect the filtrate as the stock solution. 50 μL of stock solution was thoroughly mixed with 4.95 mL of sterile normal saline, and diluted 100-fold to obtain a 10^{-2} concentration sample. The same method was then used to prepare samples for 10^{-4} and 10^{-6} gradients by stepwise dilution. 100 μL was evenly coated in MRS medium containing $CaCO_3$ and incubated at 37°C for 48 h. Single colonies with obvious calcium-solubilizing circles were selected and transferred to MRS liquid medium for enrichment and purification. Pipette 100 μL into MRS solid medium, plate scribing, and then pick a single colony to inoculate in MRS liquid medium, and enrich and culture at 160 r/min shaker for 24 h. It was passaged several times [29], and the growth was checked daily until the strain was vigorous. Finally, the same volume of bacterial solution was mixed with 50% glycerol (total volume is 1 mL) and stored in an ultra-low temperature freezer at -80°C for long-term storage.

2.2.2 Identification of Lactic acid bacteria

2.2.2.1 Observation of morphological characteristics and Gram staining

150 μL of the preserved bacterial solution was inoculated in 100 mL of MRS liquid medium, and incubated overnight at 37°C and 160 r/min for activation. The color and transparency of the colony were observed and recorded by coating method (100 μL bacterial solution/MRS plate) after 48 hours of constant temperature incubation. After Gram staining, single colonies were picked and placed in MRS liquid medium and incubated overnight at 37°C with shaking. Clean the slides with ethanol, dry them with mirror paper, take 50 μL of bacterial solution on a clean glass slide, and pass them on the outer flame of an alcohol lamp about 3 times to fix. Add 1 ~ 2 drops of crystal violet staining solution, iodine solution, 95% ethanol and saffron staining solution dropwise in turn, pay attention to cover the specimen, strictly record the time and wash it with deionized water before adding the next reagent dropwise. After the slides are dry, they are examined under a microscope.

2.2.2.2 Molecular biology identification (16S rDNA identification)

(1) Extraction of genomic DNA of Lactic acid bacteria: DNA extraction of the screened Lactic acid bacteria with a kit is carried out in sequence according to the steps of the instructions.

(2) PCR amplification system: 50 μL reaction system, as shown in Table 4.

Table 4. PCR 50μL System

Components	Volume
Template DNA	2 μL
Forward Primer (10 ~ 20 pmol)	2 μL
Reverse Primer (10 ~ 20 pmol)	2 μL
10×PCR Buffer	5 μL

Mg^{2+} (25 mM)	3 μL
dNTP (each 2.5 mM)	4 μL
Taq DNA Polymerase (5U/μl)	1 μL
ddH_2O	31 μL
Total	50 μL

(3) The PCR amplification conditions are shown in Table 5.

Table 5. PCR Amplification Conditions

	Temperature	Time	Cycle
Pre-denaturation	95℃	5 min	1
denaturation	95℃	30 sec	30
Refolding	55℃	30 sec	30
extend	72℃	1.5 min	30
Terminal extension	72℃	10 min	1
Save	4℃		

After PCR, the genomic DNA of the strain was detected by 1% agarose gel electrophoresis. The whole agarose gel was placed in the electrophoresis gel imaging split system, and the DNA bands were checked under UV irradiation after adjusting the brightness and other parameters to determine whether the bands were as expected. The PCR products were sent to Sangon Bioengineering (Shanghai) Co., LTD. for sequencing identification. The NCBI website was used for nucleic acid comparison, and several strain sequences with high homology were selected to construct a phylogenetic tree with MEGA.

2.2.3 Determination of Lactic acid bacteria growth curves

150 μL of the preserved bacterial solution was inoculated in 100 mL of MRS liquid medium and incubated overnight at constant temperature and 160 r/min for activation. The strain was inoculated in MRS liquid medium at 1% and cultured in a constant temperature shaker for 16 h. And every 2 hours, 2 mL of medium (including 0 h after inoculation) was taken in the ultra-clean bench, the OD_{600} value was measured and recorded, the medium of the uninoculated bacterial solution was used as the control, and the growth curve was drawn with Origin software.

2.2.4 Lactic acid bacteria safety test

2.2.4.1 Hemolytic activity test

If the concentration of the bacterial solution is high, that is, the OD_{600} value is greater than 1.0, it needs to be diluted and marked to avoid too dense colonies. A sterile inoculation loop was used to mark the four-zone plate on the blood agar plate, and the hemolytic ring was observed after 24 h of constant temperature incubation.

2.2.4.2 Antibiotic susceptibility assays

Using the filter paper diffusion method, the aspirated 100 μL of bacterial solution was evenly coated on the plate using a sterile coating rod, and the sterile filter paper was placed on the medium with sterilized tweezers. 10 μL of tetracycline (30 μg), ampicillin (10 μg), chloramphenicol (30 μg), clindamycin (10 μg), gentamicin (10 μg), vancomycin (30 μg), cefazolin (30 μg), azithromycin (30 μg) and amoxicillin (20 μg) were applied to the filter paper. After incubation at 37°C for 48 h, the diameter of the inhibition zone (mm) was measured with a digital vernier caliper, and the antibiotic sensitivity was marked according to the standards of the European Food Safety Authority, and normal saline was used as a negative control.

2.2.5 Lactic acid bacteria BSH enzyme activity assay

Sterile filter paper discs are soaked in the activated bacterial solution and placed on the surface of MRS solid medium containing 0.5% sodium taurodeoxycholate and 0.037% calcium chloride. Sterile saline was used as a negative control, cultured at a constant temperature, and whether there was an opaque precipitate circle around the filter paper piece, and its diameter (mm) was measured with vernier caliper. Three parallel groups were set up for the selected bacterial fluids.

2.2.6 Study on antioxidant properties of Lactic acid bacteria

2.2.6.1 Lactic acid bacteria cell suspensions and cell-free extracts are prepared

After the preservation strains were activated, the strains were divided equally into two groups, which were used to prepare the suspension (IC) and cell-free extracts (CFE) of intact cells. Both groups of samples were centrifuged at 4°C, 8000 rpm for 10 min, the bacterial pellet was washed three times with PBS buffer, and the IC was obtained by resuspending. On this basis, the other group continued to be ultrasonically broken in ice bath, with 5 seconds per sonication, 5 seconds of pause, continuous cycle repeating, and after 30 min, centrifuge with a centrifuge at 12000 rpm for 10 min, leaving the supernatant to obtain CFE.

2.2.6.2 H_2O_2 tolerance assay

A large number of H_2O_2-containing media was

used as a screening index. 30% H_2O_2 was added to the sterilized MRS liquid medium to obtain medium containing H_2O_2 concentrations of 1.0 and 2.0 mmol/L. The bacterial solution was inoculated in H_2O_2-containing medium and incubated in a shaker for 12 h, and the OD_{600} value of the bacterial solution was determined.

2.2.6.3 DPPH free radical scavenging capacity assay

Take the bacterial solution in a centrifuge tube, discard the supernatant after centrifugation, add 1mL of 80% methanol and mix well. The bacterial suspension was sonicated for 3 seconds, with a pause of 10 seconds and 30 repeats, and the whole process was carried out on ice. After centrifugation at 12000 rpm for 10 min, the supernatant was taken as CFE. Follow the steps in the instructions to determine the clearance of the screened strains with the kit. The scavenging capacity of DPPH free radicals is calculated as follows:

$$\begin{aligned} &DPPH\ free\ radical\ scavenging\ rate\ (\%) \\ &=\left[1-\left(A_X-A_Y\right)/A_0\right]\times 100\% \end{aligned} \quad (1)$$

Note: A_X is the assay group; A_Y was the control group, and the absorbance of the working solution was replaced by an equal volume of 80% methanol; A_0 is the blank group, and the absorbance of the sample is replaced by an equal volume of 80% methanol.

2.2.6.4 Determination of hydroxyl radical scavenging capacity

The bacterial solution was incubated and activated in MRS liquid medium in advance, and 2 mL of bacterial solution was taken as IC in a centrifuge tube for later use. In addition, the same volume of bacterial solution was taken and the cell precipitate was left after centrifugation, washed with PBS buffer and resuspended several times, and it was used as CFE in a centrifuge tube for later use. After adding 1mL of 5 mmol/L ferrous sulfate solution, 5 mmol/L salicylic acid-ethanol solution and 3 mmol/L hydrogen peroxide solution to the centrifuge tube, the OD_{510} value was finally measured after a water bath at 37°C for 20 min. The scavenging capacity of hydroxyl radicals is calculated as follows:

$$\begin{aligned} &Hydroxyl\ radical\ clearance\ (\%) \\ &=\left[1-\left(A_X-A_Y\right)/A_0\right]\times 100\% \end{aligned} \quad (2)$$

Note: A_X is the assay group; A_Y is the control group, which refers to the absorbance of replacing the H_2O_2 solution with the same volume of pure water; A_0 is the blank group, which refers to the absorbance of the sample with an equal volume of pure water.

2.2.6.5 Determination of reducing capacity

The bacterial solution was incubated and activated in MRS liquid medium in advance, and 0.5 mL of bacterial solution was taken as IC and set aside in a centrifuge tube. 0.5 mL of 0.2 mol/L PBS solution and 1% potassium ferricyanide solution were added sequentially, and a water bath at 50°C was mixed for 20 min. After rapid cooling on ice, add 0.5 mL of 10% trichloroacetic acid and centrifuge at 4000 rpm for 5 min. Take 1 mL of supernatant, pure water and 0.1% ferric chloride and mix thoroughly, let it stand for 10 min, and finally measure the OD_{700} value. The reductive capability is calculated as follows:

$$\begin{aligned} &Reductive\ activity\ (\%) \\ &=\left[\left(A_X-A_Y\right)/A_Y\right]\times 100\% \end{aligned} \quad (3)$$

Note: A_X is the assay group; A_Y is the control group, and the absorbance of the sample is replaced with an equal volume of PBS buffer.

2.2.6.6 Total antioxidant capacity assay

After preparing the cell-free extract of the bacterial solution, 1mL of 80% ethanol was added and mixed, and the ice bath was sonicated and broken. Centrifuge at 12000 rpm for 10 min, remove the pellet, and take the supernatant as CFE on ice for testing. The total antioxidant capacity of the strain was determined with the kit, calculated as follows:

$$\begin{aligned} &\triangle A=A_0-\left(A_X-A_Y\right) \\ &Total\ antioxidant\ capacity\ \left(nmol\ Trolox/10^4 cell\right) \\ &=\left[\left(\triangle A+0.0218\right)\div 1.7571\times V1\times 10^3\right] \\ &=\left(V1\div V\times 500\right)\times D \\ &=569.1\times\left(\triangle A+0.0218\right)\div 500\times D \end{aligned} \quad (4)$$

Note: A_X is the assay group; A_Y was the control group, and the absorbance of the working solution was replaced by an equal volume of 80% ethanol; A_0 was the blank group, and the absorbance of the bacterial solution was replaced by an equal volume of 80% ethanol.

V: Add the volume of extracted liquid, 1 mL; V1: sample volume in reaction, 10 μL = 0.01 mL; Trolox molecular weight: 250.29; 500: total number of bacteria or cells,10000; D: dilution factor, 1 for undiluted.

2.2.6.7 Lipid peroxidation inhibition rate assay

In the cell-free extract of Lactic acid bacteria,

add 1 mL of 80% ethanol and mix well. The bacterial suspension was sonicated for 3 seconds, with a pause of 10 seconds and 30 repeats, and the whole process was carried out on ice. Centrifuge at 12000 rpm for 10 min, remove the precipitate, and take the supernatant as CFE on ice for testing. Then, the lipid peroxidation resistance (LPO) resistance of the screened Lactic acid bacteria was determined by using the lipid peroxidation resistance kit, and the steps in the kit instructions were strictly followed. The LPO inhibition rate is calculated as follows:

$$LPO\ inhibition\ rate\ (\%) = \left[(A_0 - A_X) / A_0 \right] \times 100\% \quad (5)$$

Note: A_0 is the blank group, that is, the absorbance of the sample is replaced by the same volume of pure water; A_X is the assay group.

2.3 Data Processing and Statistical Analysis

All experiments were averaged in triplicate replicates and expressed as mean ± standard deviation. After processing the basic data with Excel 2021 software, the significant difference of the data was statistically analyzed by SPSS 27, and the significant difference was $P<0.05$. Origin 2024 software was used to draw the fitting curve and histogram, and Mega 4.0 software was used to construct the phylogenetic tree.

3 Results

3.1 Isolation, Purification and Identification of Lactic Acid Bacteria

3.1.1 Morphological characteristics of Lactic acid bacteria

In the case of naturally fermented sauerkraut, a single colony with obvious calcium-solubilizing circle was isolated by using MRS-$CaCO_3$ medium after dilution gradient, and the growth was good after multiple streaking and continuous subculture purification (Figure 2). The morphological characteristics of the observed colonies are recorded in Table 6, and the colonies are round or oval in shape, with a convex and smooth surface. The strains were sequentially stained with Gram stain and examined under the microscope, and the results are shown in Figure 3, and the purple color of the cells indicates that they are Gram-positive bacteria, and they are all streptosphere-shaped.

Table 6. Morphological Characteristics of the Strain

Strain number	Color	Transparency	Gram stain
DN-1	milky	opaque	G^+
DN-2	milky	opaque	G^+
DN-3	milky	opaque	G^+

Note: G^+ stands for Gram positive.

Figure 2. The Colony Morphology of the Strain

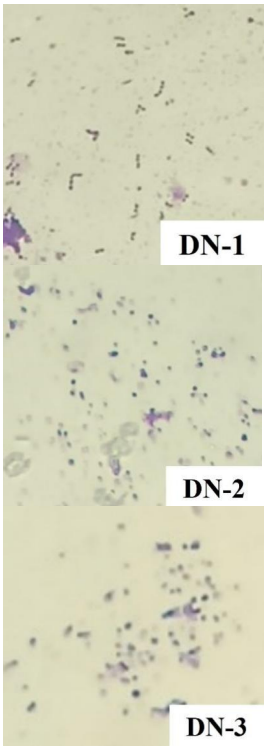

Figure 3. Gram Staining Results of the Strain

3.1.2 Molecular biology identification

After extracting the DNA using the kit, the PCR product was subjected to agarose gel electrophoresis (Figure 4) with clear bands. The first four lanes were DNA marker, strain DN-1, DN-2 and DN-3, and the bands were all between 1000~1500 bp, which was consistent with the expected results. After being sent to the company for identification, it was Leuconostoc enteromyctomy. After the sequencing results were compared with Nucleotide Blast in NCBI, the homology with

Leuconostoc mesenteroides was confirmed to be 99~100%, and the phylogenetic tree of the target strains and homologous strains was constructed by the adjacency method of MEGA software, as shown in Figure 5.

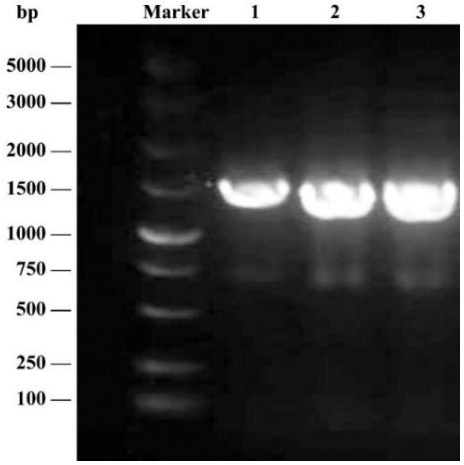

Figure 4. Agarose Gel Electrophoresis Results

Note: 1 in the figure represents DN-1; 2 stands for DN-2; 3 stands for DN-3.

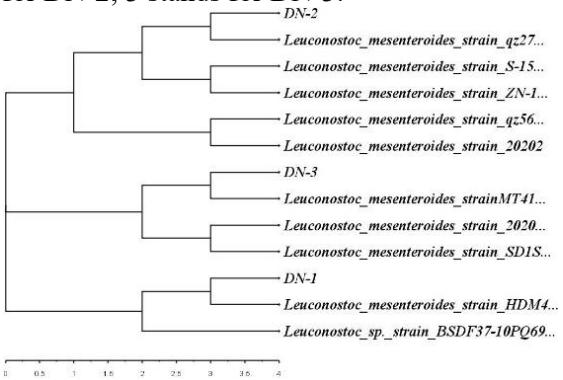

Figure 5. Phylogenetic Tree of Strains DN-1, DN-2, And DN-3

3.2 Determination of Lactic Acid Bacteria Growth Curve

After fitting the curves by the DoseResp function, the growth of strains DN-1, DN-2, and DN-3 when incubated at 37°C was obtained (Figure 6). It can be seen that the growth curves of these three strains all showed a typical S curve, and the four stages could be clearly observed. Among them, the lag period of strains DN-1 and DN-2 was 0~4 h, the growth rate of 4~11 h increased significantly, which was logarithmic phase, 11~14 h was stable period, and the decay period was 14 h later. The growth rate of strain DN-3 increased significantly after 5 h, entered the logarithmic phase, stabilized after 13 h, and decayed after 15 h.

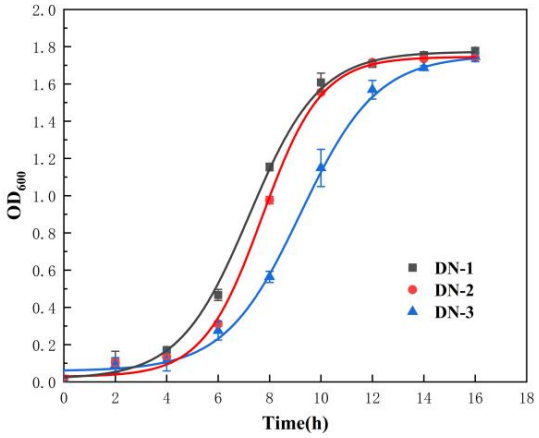

Figure 6. Growth Curves of Strains DN-1, DN-2, and DN-3

3.3 Lactic Acid Bacteria Safety Test

3.3.1 Hemolytic activity test

The activated bacterial solution was coated on the Columbia blood agar plate, incubated at 37°C for 48 h, and the hemolytic loops were observed, and the results of each hemolytic activity test are shown in Figure 7. The three strains were all γ-hemolytic and had no hemolytic activity, and the results showed that the strains DN-1, DN-2 and DN-3 were safe probiotics.

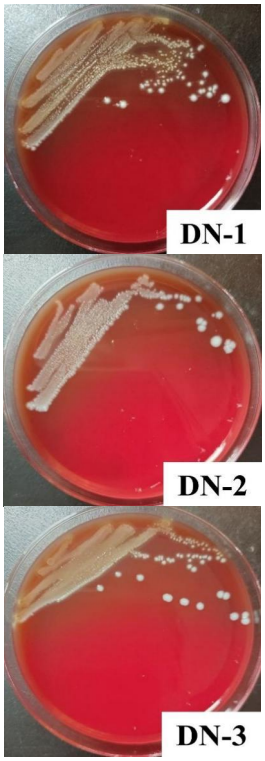

Figure 7. Results of Hemolysis Experiments for Strains

3.3.2 Antibiotic susceptibility assays

Escherichia coli was used as the positive control to measure the diameter of the antibiotic inhibition zone (Figure 8). Strains

DN-1, DN-2 and DN-3 were highly sensitive to tetracycline, clindamycin and ampicillin, and the diameter of the bacteriostatic zone was significantly higher than that of other antibiotics. The strain had a high sensitivity to gentamicin and amoxicillin; Moderate sensitivity to azithromycin; Resistant to vancomycin. Among them, DN-1 has a general sensitivity to cefazolin and chloramphenicol, and DN-2 and DN-3 have a high sensitivity to it. In comprehensive comparison, the strains DN-2 and DN-3 were sensitive to a variety of antibiotics, and the diameter of the inhibition zone of DN-2 against clindamycin and ampicillin was significantly higher than that of other antibiotics, which were 38.82 mm and 30.63 mm, respectively. The results showed that the strains DN-1, DN-2 and DN-3 all met the safety standards of probiotics and had safety guarantees.

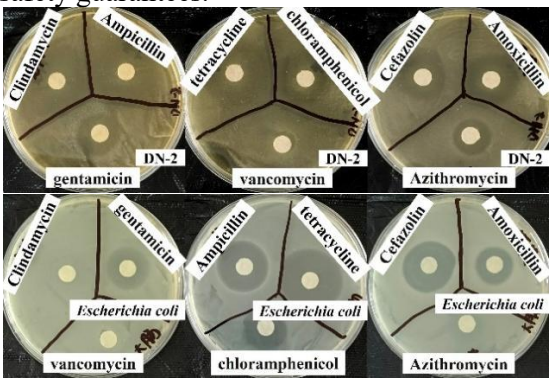

Figure 8. Antibiotic Sensitivity Inhibition Zones of Strains DN-1, DN-2, DN-3, And Escherichia Coli

3.4 BSH Enzyme Activity Assay

The BSH enzyme activity of the strain was measured with normal saline as a negative control, and the diameter of the sedimentation circle (mm) was measured, and the results are shown in Table 7 and Figure 9. The sedimentation circle diameters of strains DN-1, DN-2 and DN-3 were 10.80 mm, 9.51 mm and 11.06 mm, respectively. The precipitation circles of DN-1 and DN-3 were significantly larger than those of DN-2 (P<0.05), showing better BSH enzyme activity.

Table 7. BSH Enzyme Activity Assay Value (mm)

Strains	Sedimentation circle diameter
DN-1	10.80±0.45^{a}
DN-2	9.51±0.62^{b}
DN-3	11.06±0.14^{a}

Note: Different letters on the strains mean significant differences (P<0.05), while the same letters mean no significant differences.

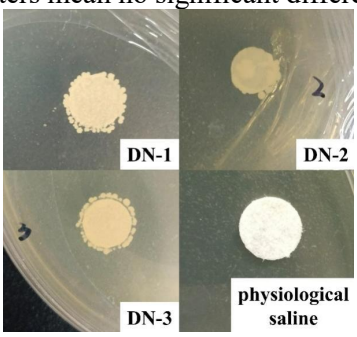

Figure 9. BSH Enzyme Activity Assay Results

3.5 Screening of Lactic Acid Bacteria with Antioxidant Properties

3.5.1 Lactic acid bacteria H_2O_2 tolerance determination

As an oxidizing agent, H_2O_2 not only destroys cells, but also participates in oxidation as a precursor to hydroxyl radicals. Numerous studies have shown that strains that are tolerant to H_2O_2 have antioxidant potential, so they are used as a primary screen for antioxidant capacity. The hydrogen peroxide tolerance analysis of the strains is shown in Table 8. Affected by H_2O_2, the survival rate of strains decreased. At a concentration of 1.0 mmol/L, the average OD_{600} values of strains DN-1, DN-2 and DN-3 were 1.501, 1.949 and 1.707, respectively, and the OD value of DN-2 was significantly higher than that of other strains (P<0.05), and the OD value of strains decreased significantly at 2.0 mmol/L. The results showed that the strain had a good growth state and showed good H_2O_2 tolerance, and the strain DN-2 had the best ability to resist H_2O_2.

Table 8. Growth Of Bacterial Strains under Different Concentrations of H_2O_2

Strains	1.0 mmol/L H_2O_2	2.0 mmol/L H_2O_2
DN-1	1.501±0.002^{c}	0.082±0.003^{b}
DN-2	1.949±0.016^{a}	0.115±0.001^{a}
DN-3	1.707±0.153^{b}	0.084±0.002^{b}

Note: Different letters on the OD value mean there is a significant difference (P<0.05), and the same letter means no significant difference.

3.5.2 DPPH free radical scavenging capacity assay

The single electron of DPPH has strong absorption at 517 nm, and its alcohol solution will appear purple in color. When the Lactic acid bacteria exert the role of free radical scavenging, after the electron pairing, the

absorption is weakened, and the purple color becomes lighter, and the lighter the purple color, the higher the free radical scavenging rate. After ultrasonic cell disruption of the three strains, cell-free extracts were obtained for analysis of DPPH free radical scavenging, and the results are shown in Table 9 and Figure 10. The DPPH radical scavenging rates of the three strains were 43.35%, 77.87% and 31.20%, respectively, and the DPPH radical scavenging rate of strain DN-2 was the highest, which was significantly higher than that of the other two strains (P<0.001), and strain DN-2 showed the strongest DPPH scavenging ability.

Table 9. DPPH Free Radical Scavenging of Strains (%)

Strains	DPPH free radical scavenging rate
DN-1	43.354±0.148[b]
DN-2	77.866±0.253[a]
DN-3	31.204±0.146[c]

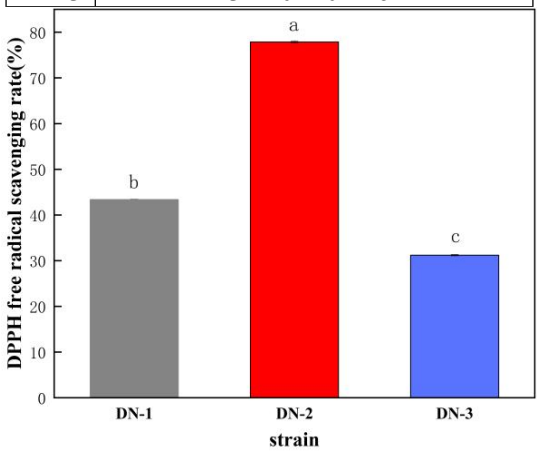

Figure 10. DPPH Radical Scavenging of Strains DN-1, DN-2, and DN-3

Note: Different letters on the strains mean a very significant difference (P<0.001), while the same letters mean no significant difference.

3.5.3 Determination of hydroxyl radical scavenging capacity

The bacterial liquid was divided into two groups: one group was used to create a suspension of intact cells (IC), while the other group was subjected to ultrasonic disruption in an ice bath to obtain cell-free extracts (CFE). The hydroxyl radical scavenging abilities were measured, with results shown in Table 10 and Figure 11. The selected strains, whether IC or CFE, exhibited a certain capacity to scavenge •OH; however, there were differences in their scavenging abilities. Furthermore, the scavenging ability of IC for •OH was significantly greater than that of CFE for the same strain (P<0.05). The •OH scavenging capacity of strain IC ranged from 38.72% to 55.17%, with strain DN-3 showing the highest ability; meanwhile, the •OH radical scavenging capacity of strain CFE ranged from 33.08% to 48.43%, with strain DN-1 having the highest ability. This indicates that DN-1 and DN-3 possess outstanding hydroxyl radical scavenging abilities.

Table 10. Hydroxyl Radical Scavenging Rate 0f Bacterial Strains (%)

Strains	Intact cell suspensions	Cell-Free Extract
DN-1	53.529±0.588a	48.427±2.441b
DN-2	38.717±0.418c	33.077±1.868d
DN-3	55.166±1.217a	38.324±1.367c

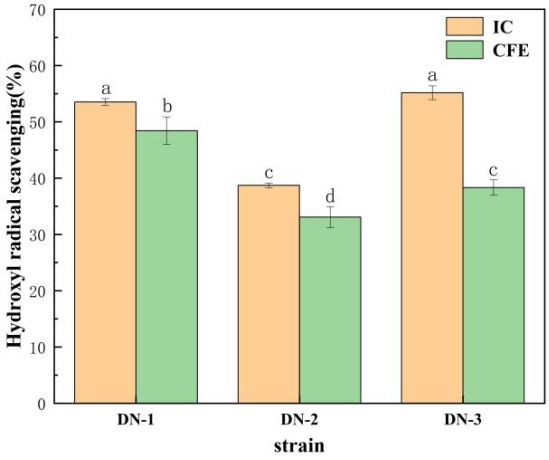

Figure 11. Hydroxyl Radical Scavenging Rates of Strains DN-1, DN-2, and DN-3

Note: Different letters on the strains mean significant differences (P<0.05), while the same letters mean no significant differences.

3.5.4 Determination of reducing capacity

The reducing ability of strain IC was determined, and the results are shown in Table 11 and Figure 12. The reducing capacity of strains was 30.80%, 35.16% and 26.19%, respectively, and the reducing capacity of strain DN-2 was significantly higher than that of other strains (P<0.05), and the reducing capacity of strain DN-1 was significantly higher than that of DN-3 (P<0.05). The reduction ability of the three strains was strong, and the reduction ability of strain DN-2 was the strongest.

Table 11. The Reducing Ability of Bacterial Strains (%)

Strains	Reductive activity
DN-1	30.801±0.338[b]
DN-2	35.162±0.339[a]
DN-3	26.186±0.254[c]

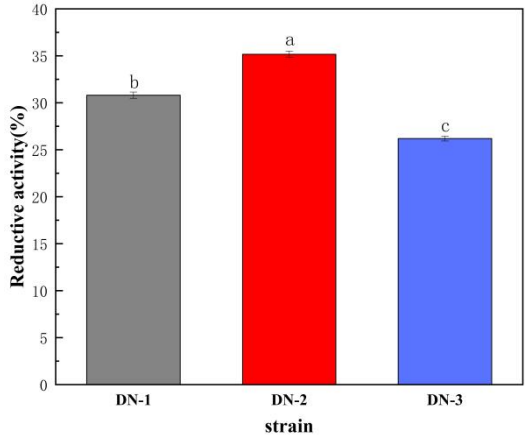

Figure 12. The reducing ability of strains DN-1, DN-2, and DN-3

Note: Different letters on the strains mean significant differences (P<0.05), while the same letters mean no significant differences.

3.5.5 Total antioxidant capacity assay

When the oxidant is present, the component ABTS in the working solution is oxidized to $ABTS^+$ (green), and the antioxidant added at this time can inhibit the oxidation of ABTS. Therefore, the lighter the green color, the stronger the antioxidant capacity of the sample, and the total antioxidant capacity was compared by measuring OD_{414} (Table 12 and Figure 13). The total antioxidant capacity of the strains was 0.107, 0.137 and 0.138 mol Trolox/10^4 cell, respectively, and the total antioxidant capacity of strains DN-2 and DN-3 was significantly higher than that of DN-1 (P<0.05).

Table 12. The Total Antioxidant Capacity of the Strain (nmol Trolox/10^4 cell)

Strains	Total antioxidant capacity
DN-1	0.107±0.004^b
DN-2	0.137±0.005^a
DN-3	0.138±0.006^a

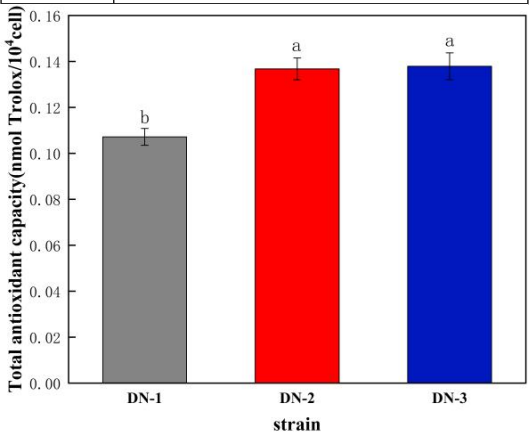

Figure 13. Total Oxidative Capacity of Strains DN-1, DN-2, and DN-3

Note: Different letters on the strains mean significant differences (P<0.05), while the same letters mean no significant differences.

3.5.6 Lipid peroxidation inhibition rate assay

The results of measuring the lipid peroxidation (LPO) inhibition rate of intact cell suspensions are shown in Table 13 and Figure 14. The LPO inhibition rates of strains DN-1, DN-2 and DN-3 were 33.681%, 38.590% and 32.828%, respectively. Among them, DN-2 had the highest LPO inhibition rate, which was significantly higher than that of other strains (P<0.05), and DN-1 had a significantly higher LPO inhibition rate than DN-3 (P<0.05). Strain DN-2 exhibited a strong inhibition rate of lipid peroxidation.

Table 13. Inhibition of Lipid Peroxidation of Strains (%)

Strains	Inhibition of lipid peroxidation
DN-1	33.681±0.171b
DN-2	38.590±0.112a
DN-3	32.828±0.091c

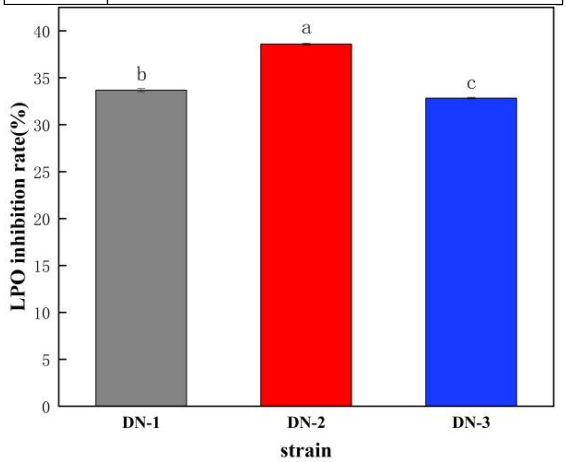

Figure 14. Resistance to Lipid Peroxidation of Strains DN-1, DN-2 and DN-3

Note: Different letters on the strains mean significant differences (P<0.05), while the same letters mean no significant differences.

4. Discussion

Many scholars at home and abroad have screened Lactic acid bacteria with strong antioxidant properties through a series of experiments such as free radical scavenging rate measurement, LPO inhibition rate measurement, superoxide dismutase (SOD) and other antioxidant enzyme activity determination, and metal ion chelation measurement. At present, the mechanism of how Lactic acid bacteria scavenge reactive oxygen species (ROS) is not yet clear, and many researchers believe that ROS is

scavenged through the antioxidant enzymes and antioxidant systems produced by Lactic acid bacteria [2,30], such as Lactic acid bacteria producing SOD to scavenge superoxide anions, and glutathione peroxidase (GSH-Px) to scavenge H_2O_2 and hydroxyl radicals. Therefore, the antioxidant ability of Lactic acid bacteria can be reflected by measuring various antioxidant enzymes in Lactic acid bacteria. Hydroxyl radicals are the most harmful free radicals to cells and are produced by H_2O_2. The precursor of hydroxyl radicals is H_2O_2, which is converted into hydroxyl radicals catalyzed by ferrous sulfate [30]. Therefore, many scholars at home and abroad have conducted preliminary screening by measuring the tolerance of strains to hydrogen peroxide to screen out Lactic acid bacteria with antioxidant potential. Wang et al. [27] analyzed the tolerance of three H_2O_2 concentrations of Lactic acid bacteria, and screened three highly tolerant strains at 0.5 mmol/L, with a survival rate of 96.04 ~ 99.05%, a survival rate of 80.89% ~ 94.36% at 1 mmol/L, and the highest survival rate of L19 at 2 mmol/L (27.30%). In this study, in order to screen Lactic acid bacteria with antioxidant potential, the tolerance of strains isolated and purified from sauerkraut to H_2O_2 was determined. When the concentration of H_2O_2 in the medium was 1.0mmol/L, the OD_{600} value of the three strains was between 1.50 ~ 1.95, indicating that the strains had good tolerance to H_2O_2 and had great antioxidant potential.

Cheng et al. [31] showed that the fermentation broth, intact cell (IC) and cell-free extract (CFE) of Lactic acid bacteria all had certain antioxidant capacity and there were significant differences. Among them, the DPPH scavenging ability of the fermentation broth of the strain was the strongest, with the highest being 85%. This is followed by IC, with a maximum clearance of 40%; CFE has the weakest DPPH clearance capacity at a maximum of 16%. The strains screened by Li et al. [32] were divided into two groups when measuring the free radical scavenging rate, the DPPH scavenging ability was measured by intact cells, and the hydroxyl scavenging rate was measured by cell-free extracts. Among them, the clearance rate of intact cells was the highest, which was 90.47%, and its antioxidant capacity was comparable to that of 27.98 μg/mL Trolox standard solution. The clearance rate of cell-free extracts was 60.99%, and its reducing power was comparable to that of L-cysteine hydrochloride at 513.37 μmol/L. In this experiment, the DPPH scavenging rate of DN-2 cell-free extract was 77.87%, and the hydroxyl scavenging rate of DN-1 and DN-3 intact cells was 53.53% and 55.17%, respectively.

Malondialdehyde is a secondary oxidation product in linoleic acid unsaturated systems with a strong absorption peak at 532 nm [33]. By the exogenous addition of malondialdehyde, a red condensate is formed with TBA under acidic conditions, which can inhibit the production of malondialdehyde in the presence of lipid peroxide resistance (LPO) [33], and the LPO inhibition rate was calculated by measuring the OD value at 532 nm. Zhang Di [23] screened 8 strains of Lactic acid bacteria with strong antioxidant activity from fermented vegetables, and used ascorbic acid and ferrous ions to induce lipid peroxidation to determine the LPO inhibition rate, among which JX306 had the highest LPO inhibition rate of 28.14%. When the lipid peroxidation resistance rate of the strain reaches more than 20%, it can be considered a probiotic with antioxidant properties [23]. In this experiment, the LPO inhibition rates of strains DN-1, DN-2 and DN-3 were 33.68%, 38.59% and 32.83%, respectively, showing strong lipid peroxidation inhibition ability. Zhao Xin et al. [34] screened two strains of lactic acid bacteria with strong antioxidant activity from traditional kimchi, and the reducing capacity of the cell suspension of the strains was 13.52% and 13.18% in the reduction capacity experiment. In this experiment, the reducing capacity of strains DN-1, DN-2 and DN-3 was 33.80%, 35.16% and 26.19%, respectively, indicating that these three strains showed strong reducing ability.

The hemolytic activity test is an important indicator of the safety test of strains, and there are three types of hemolysis [22]: because the red blood cells are not completely lysed, the green area around the colony is α-hemolysis, and the common representative is *Streptococcus pneumoniae*; Due to the hemolysin produced by bacteria, red blood cells are completely dissolved, and an obvious transparent ring appears at this time, which belongs to β-hemolysis, and the common representatives are *Staphylococcus aureus*;

γ-hemolysis means that it has no hemolytic activity and is a probiotic. Lactic acid bacteria produce bile salt hydrolase (BSH) to resist the inhibition of growth by bile salts, and the level of BSH enzyme activity is associated with bile tolerance [35], and strains DN-1, DN-2 and DN-3 have precipitation circles of about 10 mm, indicating that they have BSH enzyme activity and the potential to tolerate bile salts, and have certain prebiotic effects.

Acknowledgments

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