MINISTRY OF EDUCATION AND SCIENCE OF UKRAINE
KYIV NATIONAL UNIVERSITY OF TECHNOLOGIES AND DESIGN
Faculty of Chemical and Biopharmaceutical Technologies
Department of Biotechnology, Leather and Fur

QUALIFICATION THESIS

on the topic **Screening and molecular identification of lactic acid bacteria in environmental samples**
First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group BEBT-20
Du LIPING

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KYIV NATIONAL UNIVERSITY OF TECHNOLOGIES AND DESIGN

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APPROVE
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«___»_____2024

ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Du Liping

1. Thesis topic **Screening and molecular identification of lactic acid bacteria in environmental samples**
Scientific supervisor Ihor Hretskyi, Ph.D., As. prof

approved by the order of KNUTD "__"_____2024, №____

2. Initial data for work: assignments for qualification thesis, scientific literature on the topic of qualification thesis, materials of Pre-graduation practice

3. Content of the thesis (list of questions to be developed): literature review; object, purpose, and methods of the study; experimental part; conclusions

4. Date of issuance of the assignments_____

EXECUTION SCHEDULE

№	The name of the stages of the qualification thesis	Terms of performance of stage	Note on performance
1	Introduction	From 01 April 2024 to 11 April 2024	
2	Section 1 Literature review	From 06 April 2024 to 20 April 2024	
3	Section 2 Object, purpose, and methods of the research	From 21 April 2024 to 30 April 2024	
4	Section 3 Experimental part	From 01 May 2024 to 10 May 2024	
5	Conclusions	From 07 May 2024 to 12 May 2024	
6	Draw up a bachelor's thesis (final version)	From 12 May 2024 to 24 May 2024	
7	Submission of qualification work to the supervisor for feedback (14 days before the defense)	From 24 May 2024 to 10 June 2024	
8	Submission of bachelor's thesis to the department for review (12 days before the defense)	13 June 2024	
9	Checking the bachelor's thesis for signs of plagiarism (10 days before the defense)	15 June 2024	
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I am familiar with the task:

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SUMMARY

Liping Du. Screening and molecular identification of lactic acid bacteria in environmental samples – Manuscript.

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As a kind of probiotics widely existing in nature and human gut, lactic acid bacteria have been widely concerned in food, medicine and agriculture in recent years due to their unique physiological functions and wide application prospects. Environmental samples, such as soil, water, and plant surfaces, are important sources of lactic acid bacteria. The purpose of this experiment was to isolate, screen and identify lactic acid bacteria in the environment. First, the lactic acid bacteria in the environment were separated by the plate marking method and the coated plate method, and then the solid medium containing calcium carbonate or bromomethyl phenol green MRS Was used to screen the strains with calcium soluble ring or green color from the environmental soil and environmental water source. Then the species relationship of lactic acid bacteria was determined by morphological observation, physiological and biochemical tests and 16S rRNA gene sequence comparison. By comparing 16S rRNA sequences with those in the database, the species of lactic acid bacteria could be determined. The results showed that multiple strains of lactic acid bacteria were successfully screened from the environmental samples, and strains ZW-JX-1-1, ZW-JX-7-2 and ZW-JX-8-1 belonged to Lactobacillus reuter. Strains ZW-JX-2-1 and ZW-JX-4-1 belong to Lactobacillus paracei, strains ZW-JX-3-1 belong to Streptococcus thermophilus, strains ZW-JX-5-1 belong to Enterococcus faecalis, and strains ZW-JX-6-2 belong to Lactobacillus acidophilus. Phylogenetic tree was constructed by MEGA to show the kinship and genetic differences among different strains. The results showed that strain ZW-JX-2-1 and strain ZW-JX-4-1 were the most closely related, and the sequence comparison results showed that both strains belonged to Lactobacillus paracasei. Through this study, the screening process and

molecular identification method of lactic acid bacteria were systematically understood, hoping to provide ideas for molecular identification research of lactic acid bacteria.

Keywords: environmental sample; lactic acid bacteria; screen; molecular identification

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INTRODUCTION

The relevance of the topic is the screening and molecular identification of lactic acid bacteria.

The purpose of the study is the to screen lactic acid bacteria from environmental samples by systematic experimental design, molecular biology and physiological and biochemical methods, and to carry out accurate molecular identification.

The objectives of the study is through systematic experimental design, combined with molecular biology and physiological and biochemical methods, to screen lactic acid bacteria from environmental samples, and carry out accurate molecular identification.

The object of the study is lactic acid bacteria in environmental samples.

The subject of the study is lactic acid bacteria in environmental samples.

Research methods was to screen the strains by calcium-dissolving ring method, and then separate them by plate scribing method or coated plate method. Morphological observation, physiological and biochemical identification and molecular biological identification were carried out. The most commonly used molecular identification method is 16S rRNA sequencing. First, the DNA of isolated strains was extracted, then the genes were amplified by PCR, and the amplified products were purified by gel electrophoresis. Then the amplified products were sequenced, and the sequencing results were analyzed by sequence alignment.

The scientific novelty lies in 16S rRNA sequence alignment analysis.

The practical significance of the results obtained is to enrich the germplasm resource bank of lactic acid bacteria. Through systematic screening work, lactic acid bacteria strains with different characteristics and functions can be isolated from various environmental samples, which provides rich experimental materials for subsequent molecular identification and applied research.

CHAPTER 1
LITERATURE REVIEW

1.1 A review of lactic acid bacteria

1.1.1 Overview of lactic acid bacteria

Lactic acid bacteria(LAB), as a unique class of prokaryotes, exhibit remarkable characteristics - they are facultative anaerobic bacteria and have the ability to efficiently convert fermentable carbohydrates into lactic acid. In nature, this type of bacteria is widely distributed, and its species diversity is extremely rich, and together constitute a large bacterial family. The Lactobacillus family is currently known to include at least 18 different genera, with more than 200 distinct species showing their potential for a wide range of ecological and biotechnological applications. With a few exceptions, most of them play an indispensable role in the human gut and have vital physiological functions. These flora play a significant role in maintaining intestinal health and promoting nutrient absorption[1(Zhenzhu, Ling et al. 2023)].

Lactic acid bacteria not only become an ideal experimental material because of its outstanding characteristics in the study of taxonomy, biochemistry, genetics, molecular biology and genetic engineering, and has a high academic value, but also in industry, agriculture, animal husbandry, food and medicine and many other fields closely related to human life, its application value can not be ignored. However, it is worth noting that some of these bacteria are zoonotic pathogens, so they have received high attention and attention from researchers and the public.

In-depth research by biologists at home and abroad has clearly revealed that there is a significant and close correlation between lactic acid bacteria in the gut and the health and longevity of individuals. This discovery not only provides a new perspective for human health research, but also opens up a new way for the application of lactic acid bacteria in the field of medical health.

Based on the biochemical classification system of Berry Bacteriology Manual, lactic acid bacteria can be subdivided into five genera, namely *Lactobacillus, Streptococcus, Leuconostoc, Bifidobacterium and Pediococcus.*

Lactobacillus, the most commonly used probiotic strain[2(Tibor, Balázs et al. 2023)], has variable cell morphology, usually showing long or slender rods, sometimes curved, short rods, or rod-shaped bulb rods, and forming short chains. These bacteria are Gram-positive, do not sporogenesis, and the cells rarely exhibit periflagellar movement. On the nutrient AGAR medium, Lactobacillus colonies are characterized as raised, whole-rimmed, and colorless, with a diameter of approximately 2 to 5mm.

Streptococcal bacteria exhibit unique morphological characteristics, their bodies are usually spherical or oval, and tend to be arranged in a chain pattern. These bacteria do not possess spores and most lack flagella; however, capsule formation is sometimes observed during their young development. In the liquid medium environment, streptococcus tends to show the trend of precipitation growth, but it may also appear uniform turbidity growth phenomenon. On solid media, streptococcal colonies have distinctive characteristics: they are usually small, smooth, rounded, grayish-white in color, and may appear translucent or opaque. In addition, when streptococcus grows on the blood plate, its colonies often form hemolytic circles with different properties, which is one of the important characteristics of streptococcus identification.

Leuconostoc bacteria, which exhibit distinctive cellular morphology, usually spherical or bean-shaped, and are usually paired or arranged in chains in nature. In normal natural environments, the cells of bacteria are usually represented as paired structures or short chains. However, given the more intense growth challenges, especially in the face of intense competition, these bacteria can adopt adaptive growth strategies to increase their vitality by forming longer chain structures. This extensive method of cell distribution not only has the biology of a log, but also provides a strong guarantee of survival and reproduction in a constantly changing environment. Bifidobacterium bacteria, obvious differences in the shape, usually arranged in the

letter V ring, rod, branching and growth forms, in some cases form a chain. In cell distribution, Bifidobacterium exhibits a unique organizational pattern in which cells are arranged in parallel to form complex structures, such as palisades and rosettes, and sometimes the shape of raised bulbous cells can be seen. These unique morphological characteristics provide an important basis for the classification and identification of bifidobacterium. Piece of coccus, its cell shape is spherical, and will never be extended. Under suitable conditions, Pediococcus forms quadruplets by direct binary division, and although pairs are occasionally seen, they do not form a chain structure. These bacteria do not move and do not produce spores.

1.1.2 Physiological function of lactic acid bacteria

1.1.2.1 Promote body growth

Lactic acid bacteria show excellent metabolic ability in the body, they can not only effectively decompose proteins and sugars in food, but also have the function of synthesizing vitamins and transforming fats. Specifically, protease secreted by lactic acid bacteria can efficiently act on macromolecular proteins in food, partially hydrolyze them into small molecular peptides and essential amino acids that can be directly absorbed and utilized by the host, thus significantly improving the digestive efficiency and biological value of food, and promoting the absorption function of the gastrointestinal tract. In addition, the fermentation of lactic acid bacteria has a significant impact, which can effectively convert the lactose in food into glucose and galactose, and continue to metabolize into easily digestible low molecular weight compounds such as lactic acid. At the same time, lactic acid bacteria also have a certain degradation effect on fat, which can improve the content of free fatty acids and volatile fatty acids in food, thus enhancing the digestive performance of food, so that nutrients are more easily absorbed and utilized by the human body.

In vivo metabolism of lactic acid bacteria has shown excellent ability, such as break down proteins in food and sugar, synthetic vitamins and trans fats. In particular, protease secreted by lactic acid bacteria has a high effect on food macromolecular

proteins, some of which are directly absorbed and utilized by hydrolysis inn as small molecular peptides and essential amino acids, which significantly improves digestive efficiency and biological value, and stimulates stomach absorption function. In addition, the fermentation of lactic acid bacteria has an obvious effect, can effectively convert the lactose in food into glucose and galactose, and can continue to metabolize to form low molecular weight compounds, easy to digest into lactic acid. At the same time, lactic acid bacteria have a special effect on fat reduction, by increasing the content of free fatty acids and volatile fatty acids in food, improve food digestion, making it easier to absorb and utilize nutrients.

In the process of metabolism of lactic acid bacteria, although will consume some vitamins, but its unique in synthetic folic acid and B vitamins, at the same time enhance the biological availability of mineral elements, for the host to supply necessary nutrition elements and the overall nutrition metabolic efficiency, thus promote the host's growth. In particular, the acidic metabolites generated by lactic acid bacteria create an acidic environment in the intestine, which coincides with the optimal working conditions of various digestive enzymes (such as amylase at pH 6.5 and saccharifying enzyme at pH 4.4), thus significantly promoting the decomposition and absorption of nutrients and further improving the nutrient utilization rate.

1.1.2.2 Maintain the balance of intestinal flora

In animals, the entire digestive system is usually home to a large community of microorganisms, which can be divided into three main categories according to their functional properties. First, symbiotic microorganisms, mainly composed of facultative anaerobic bacteria, have a positive impact on various physiological functions of the host in the state of ecological equilibrium. Secondly, pathogenic microorganisms, although small in number under normal conditions, parasitizing the normal part does not cause host disease, but once out of control, it may lead to adverse reactions in the host. Finally, intermediate microbes possess both physiological and pathogenic properties.

Maintain the stability of gut microbes are very important to ensure the health of the organism. Lactic acid bacteria are a community of biological bacteria that live in the gut and ensure the stability and coherence of the host's physiological state by carefully regulating the microbial balance. They can actively change the internal environment of the stomach and effectively prevent the excessive reproduction of harmful bacteria, thereby maintaining and maximizing the balance of bacterial flora in the stomach. In addition, lactic acid bacteria bind tightly to mucosal cells through specific adhesive agents and establish protective positions on the mucosal surface, a structure that has become an important pillar of the so-called intestinal empirical barrier. This blocking not only strengthens the host's immune system, but also plays a very important role in preventing intestinal infections by destroying the gut flora. However, if this barrier is disturbed by antibiotics or other factors, the host's resistance to foreign pathogens is greatly reduced, leading to the development of resistance in the gut flora, which eventually replaces the original dominant fungi and disrupts the balance of gut microbes.

1.1.2.3 Improve immunity

Lactic acid bacteria preparations have different dual methods of boosting immunity. First, it increases the overall protective capacity of the organism by altering the non-specific immune response, a mechanism that is mainly activated by the increased activity of monocytes and polymorphonuclear leukocytes, as well as the increased secretion of reactive oxygen species, degrading enzymes and single nucleotides. Secondly, lactic acid bacteria preparations can also effectively stimulate specific immune responses with significantly higher specificity and immunospheroidization (IgA, IgM and IgG) on mucosal and serum surfaces. The material not only improves humoral immunity, but also expands T lymphocytes and B lymphocytes by promoting the proliferation of T lymphocytes, and improves the immune capacity of the whole organism by improving the immunity of the cells.

Lactobacillus and bifidobacterium not only significantly enhanced the activity of macrophage phagocytosis, and as a resident of intestinal flora, build a kind of natural immune defense system. By stimulating peritoneal macrophages, inducing interferon production, promoting cell proliferation and antibody formation, and strengthening cellular immunity, these probiotics comprehensively improve the body's non-specific and specific immune response, and significantly enhance the body's ability to resist foreign pathogens. When foreign bodies invade, lactic acid bacteria can quickly activate immune cells and enhance the body's ability to produce specific antibodies against foreign bodies. As described in Chndra (1984), lactic acid bacteria stimulate lymphocytes through lymph nodes and mucous membranes, and these activated lymphocytes further enter the circulatory system through the mesenteric lymph nodes (MIN) and spread throughout the body to regulate the immune response of the body, thus achieving overall protection of the body.

1.1.2.4 Inhibit the growth of harmful bacteria

Lactic acid bacteria have the effect of preventing spoilage bacteria and hypothermia bacteria, so it has potential application value in preventing and treating intestinal diseases such as diarrhea, enteritis, constipation and dermatitis. The antibacterial mechanism includes several aspects: First, lactic acid bacteria effectively reduce the environmental pH and REDOX (EH) potential by producing organic acids such as lactic acid, and effectively inhibit the growth of pathogenic bacteria by creating an acidic intestinal environment. Secondly, hydrogen peroxide released by lactic acid bacteria can activate "catalase-thiocyanic acid" in milk, which has obvious inhibition and bactericidal effect on Gram-negative bacteria and hydrogen peroxide-positive bacteria. In addition, lactic acid bacteria can produce microproteins or peptides, such as bacillin and bifidobacteridin, which have strong antagonistic effects against staphylococcus and Salmonella. Finally, certain species of bacteria, such as bifidobacterium, which have the ability to break down the cholic

acid bound in free cytokines, have a more pronounced antibacterial effect than the first.

1.2 Principle of isolation and screening of lactic acid bacteria

Lactic acid bacteria often form tiny colonies on conventional AGAR medium, which makes it difficult to observe them directly. Therefore, in the separation process, we usually first through the enrichment culture method, and select a specific medium to enhance the growth of lactic acid bacteria. These isolation media often contain tomato extract, yeast paste, Tween-80 and other ingredients, at the same time, in order to inhibit the growth of some non-target bacteria, acetate will be added, which has no adverse effect on the growth of lactic acid bacteria.

In addition, the calcium carbonate in under the action of lactic acid bacteria in culture medium, would be lactic acid dissolution, so as to form a clear transparent circle around the colonies, the phenomenon provides us with the intuitive basis of separation and identification of lactic acid bacteria. By measuring lactic acid production, we can further evaluate the performance of lactic acid bacteria. The growth and reproduction of lactic acid bacteria require a variety of amino acids, vitamins and trace amounts of oxygen, which is one of the reasons why its colonies are relatively small.

Besides the above ingredients in compound separation medium also often join oleic acid, it also has the role of promoting the growth of lactic acid bacteria. The addition of acetate can inhibit the growth of some non-target bacteria and ensure the purification culture of lactic acid bacteria.

Lactic acid bacteria on the culture environment, such as temperature, pH value, oxygen and carbon dioxide are required. In general, we filter in the appropriate temperature range of 30 °C to 40°C, while maintaining a pH of 4.5 to 7.0 and adequate oxygen and carbon dioxide content.

Lactic acid bacteria of the genus screening and evaluation is a process of closely, can accurately identify species and general characteristics. At present, the

identification methods often used include morphological observation, physiological and biochemical characterization, and advanced molecular biological methods (PCR technology, 16S rRNA sequencing, etc.), which strongly support the correct identification of lactic acid bacteria.

1.3 Methods for isolation, screening and identification of lactic acid bacteria

1.3.1 Isolation and screening method of lactic acid bacteria

The separation of lactic acid bacteria is usually done by the plate marking method and the coated plate method.

Flat line separation process, as a kind of advanced microorganism separation technology, its core operation involves using inoculation loops on the plating medium do fine partition line, to dilute mixed microorganisms group or the same in different cells. With this step, we are able to achieve the independent distribution of individual cells and make them grow and multiply into single colonies after culture. These single colonies are often considered as pedigree candidates for microorganisms to be isolated. However, it is important to note that not all single colonies are derived from the reproduction of a single cell, so we often need to perform repeated separation operations to ensure a pure breed. The principle of this method is to dilute microbial samples on the surface of solid medium many times through the dilution mode of "point to line", so as to achieve efficient separation effect.

Coated tablet method principle is under the condition of bacteria liquid diluted to appropriate height, originally reunion microorganism is effectively dispersed, form independent individual cells. These individual cells on the surface of the medium can further proliferate and form a clear single colony, which is convenient for subsequent observation and study.

Screening of lactic acid bacteria circle of soluble calcium is the most commonly used method with bromocresol green indicator method.

Soluble calcium ring method is a specific screening methods, this method is based on certain bacteria produce acid in optimization containing $CaCO_3$ composition of culture medium on the principle of dissolving circle. This method is especially suitable for the screening of lactic acid bacteria. The purpose of $CaCO_3$ added in the medium is to identify and distinguish bacteria that produce acid and to stabilize the pH of the medium by reacting with the resulting acid, thus ensuring the constancy of the experimental conditions.

Bromocresol green indicator method is joined in the MRS medium bromocresol green alcohol solution, coating after training from discoloring the bromocresol green colonies.

1.3.2 Methods for identification of lactic acid bacteria

In microbiology research, the accurate identification of lactic acid bacteria is of great significance for understanding their biological characteristics, ecological functions and industrial applications.

(1) Morphological observation: is obtained by microscope observation separation filter of colony morphology, cell size and so on, the preliminary judgment of lactic acid bacteria species.

(2) physiological and biochemical identification: from a variety of microbial metabolic patterns of different species showed significant differences. Bacteria are unique to single-celled prokaryotes, and metabolic differences are even more pronounced. This difference is largely due to the unique capabilities of each bacterium. There are several types of fermentation and products because organic materials such as sugars, fats and proteins are broken down and used differently. Although molecular biology technology is changing rapidly, the physiological and biochemical characteristics of bacteria still play a very important role in the classification and identification of varieties. Physiological and biochemical characteristics of lactic acid bacteria produce acid, gas, producing lactic acid and other features, the separation of purified strains were inoculated in corresponding

medium, determination of catalase test, sugar fermentation, gelatin liquefied glucose acid gas production test, the determination of the anaerobic growth test, methyl red test, nitrate reduction test, indole test, such as physiological and biochemical experiment. The species and subspecies of lactic acid bacteria were further determined.

(3) molecular biology identification: based on the determination of lactic acid bacteria genetic sequences, be able to get their genetic traits, and identified the species and genotypes.

(4) protein mass spectrum identification: by mass spectrometry of lactic acid bacteria isolated from the quality of protein analysis, compare the protein in the database information, can further determine the species and subspecies.

(5) to observe the growth curve.

1.4 PCR principle and the principle of PCR reaction system

1.4.1 PCR principle

The basic principle of simulation, specific amplification DNA fragments in vitro. The process can be divided into three steps: (1) Denaturation: at a high temperature of about 95 ° C, double-stranded DNA is dissociated into two single-stranded DNA. Annealing: At the appropriate temperature (generally 5 ° C lower than the primer Tm value), the two primers are respectively combined with the complementary sequence of two single strands of template DNA. (3) Extension: Under the action of heat-resistant DNA polymerase, using four mono-deoxynucleotides as raw materials and DNA as a template, starting from the 3' end of the primer, extending in the direction of 5'→3', a new DNA strand is synthesized.

Three steps above cycle, each loop make double DNA content, by controlling the cycle number, can get a lot of DNA fragments. PCR technology is widely used in biology, medicine and forensic science, such as gene cloning, gene mutation analysis, DNA sequencing, pathogen detection and so on.

1.4.2 PCR reaction system

PCR reaction system mainly consists of the following parts:

(1) template DNA: this is the starting material, PCR amplification can be genomic DNA and cDNA or plasmid DNA, etc.

(2) primers, primer is a synthetic oligonucleotide sequence, two pieces of complementary on both sides and to amplify DNA sequences. The length of the primer is generally 15 to 30 base pairs, and its 3' end must be fully complementary to the template DNA to prevent mismatches between the primer and the template DNA during PCR.

(3) heat of DNA polymerase: this is the key enzyme in the PCR reaction, can under the condition of high temperature catalytic dNTP (deoxyribose nucleoside triphosphate) to the primer 3 'end - OH, and the catalytic dihydrogen phosphate ester bond formation, which extends the DNA chain. The most commonly used heat-resistant DNA polymerase is Taq DNA polymerase.

(4) dNTP: including dATP, dTTP, dCTP and dGTP, is a raw material for PCR reaction used for synthesis of new strands of DNA.

(5) buffer: provide suitable for PCR reaction pH and ionic strength, to ensure the smooth progress of the PCR reaction.

(6) Mg^{2+}: is heat-resistant DNA polymerase activation agent, is also the cofactor of dNTP polymerization.

Table 1.1 – **PCR reaction system (50 μL system)**

component	dosage
ddH2O	up to 50 μL
2*Phanta Max Buffer	25 μL
dNTP Mix（10 mM each）	1 μL
forward primer（10 μM）	2 μL
downstream primer（10 μM）	2 μL

Phanta Max Super-Fidelity DNA Polymerase	1 μL
Template DNA	x μL

1.5 16S rRNA identification

16S rRNA identification method is a technique based on bacterial 16S rRNA sequence sequencing for species identification of bacteria. It is particularly suitable for species identification of unknown bacterial samples and for identification of bacteria that are difficult to culture by traditional methods.

1.5.1 Principle of 16S rRNA identification

Bacterial 16S rRNA is a subunit of bacterial ribosomal RNA with highly conserved and specific sequence regions. By PCR (polymerase chain reaction) technique, the 16S rRNA gene sequence in bacteria can be amplified. The amplified product was purified and sequenced to obtain the nucleotide sequence of 16S rRNA. The 16S rRNA sequence was compared with the known bacterial sequences in the database, and the species relationship of the bacteria was determined according to the sequence similarity.

1.5.2 Operations for 16S rRNA

(1) Bacterial genomic DNA extraction: total DNA is extracted from bacterial samples to be identified.

(2) PCR amplification: Using specific primers for 16S rRNA gene, PCR amplification of bacterial DNA is performed.

(3) Purification of amplification products: The PCR amplification products are purified by gel electrophoresis or other methods.

(4) DNA sequencing: The purified PCR products were sequenced to obtain the nucleotide sequence of 16S rRNA.

(5) Sequence alignment: The 16S rRNA sequence obtained by sequencing is compared with the known bacterial sequences in the database to determine the species relationship of the bacteria.

(6) Data analysis: According to the comparison results, the species relationship of bacteria is analyzed, and further analysis such as phylogenetic tree may be constructed.

1.6 Phylogenetic tree

Phylogenetic trees, also known as evolutionary trees or trees of life, are used to show the evolutionary relationships and kinship between different species. It consists of complex algorithms based on a variety of data such as molecular biology, genetics and morphology, providing a convenient way to understand the history of biodiversity and evolution. Phylogenetic tree construction to a large extent dependent on the genetic information of the organism and morphological characteristics. The comparison of DNA sequences, especially ribosomal RNA sequences, provides important data for the construction of evolutionary trees. By comparing the genetic sequences of different species, it is possible to determine the genetic distance between them and infer their evolutionary relationships.

1.7 Research background and status quo at home and abroad

With the deepening of people's understanding of the physiological function and ecological value of lactic acid bacteria, the research and application of lactic acid bacteria have been paid more and more attention. In the food industry, lactic acid bacteria are widely used in the production of fermented food, such as yogurt, pickles, etc. In the field of medicine, lactic acid bacteria have been used to treat intestinal diseases and improve immunity. In agriculture, lactic acid bacteria are used in areas such as biological control and biofertilizers. Therefore, screening and identification of lactic acid bacteria from environmental samples is not only of great significance for understanding the ecological distribution and diversity of lactic acid bacteria, but

also provides new resources and ways for the utilization and development of lactic acid bacteria, and is of great significance for promoting the research and application of lactic acid bacteria.

In recent years, research in the field of lactic acid bacteria has made remarkable progress, they not only have a deep understanding of their biochemical properties, but also have a deep understanding of industry, and the use of various fields such as agriculture and medicine has greatly expanded. Especially in recent years, we have made remarkable achievements in the screening, separation and purification of lactic acid bacteria, such as screening different kinds of viscous lactic acid bacteria and excellent strains with special functions

For the selection of epibiotic lactic acid bacteria, the existing research has achieved rich results, we have successfully isolated a large number of efficient and high and low temperature resistant lactic acid bacteria, these strains show great potential. In addition to its application in this field, the antioxidant function of lactic acid bacteria has also been gradually valued, and the positive contribution of lactic acid bacteria to human and livestock health has been widely recognized. Lactic acid bacteria effectively stabilize food digestion in the animal intestine, stimulate metabolism, and balance the imbalance between free radicals in the body, thus effectively preventing and controlling diseases related to oxidative stress. These findings not only expand the scope of use of lactic acid bacteria, but also provide more guidance for future research.

CHAPTER 2
OBJECT, PURPOSE, AND METHODS OF THE STUDY

2.1 Object of study

Lactic acid bacteria in environmental samples.

Environmental samples were taken from Yantai, Zibo and Changqing regions as shown in Figure 2.1. Peach, blackberry soil, fermented persimmon juice and small fruit were taken as samples from Yantai region; peach, soil and fallen leaves were taken as samples from Zibo region; peach and soil were taken as samples from Changqing region.

Figure 2.1 - Environmental samples from Yantai, Zibo and Changqing

2.2 Purpose of research

The aim of this study is to screen out potential lactic acid bacteria from environmental samples through systematic experimental design and advanced molecular biology technology, and carry out accurate molecular identification of them. With the continuous development of biotechnology, lactic acid bacteria, as an important probiotic, has shown broad application prospects in food industry, medical care, agricultural ecology and other fields. However, due to the complex and changeable environment, the screening and identification of lactic acid bacteria still face many challenges.

2.3 Experimental instruments

Table 2.1 - **Experimental instruments**

name of instrument	manufacturer
bechtop	Tianjin Shengteri Technology Co., LTD
magnetic stirrer	Shanghai Sile Instrument Co., LTD
high-pressure steam sterilizer	Jiangsu Xundi Instrument Technology Co., LTD
temperature incubator	Shanghai Yiheng Technology Co., LTD
centrifugal machine	Hunan Kaida Scientific Instrument Co., LTD
PCR-cycler	Shandong Hengmei electronic Technology Co., LTD
Gel electrophoresis apparatus	Shanghai Chusuo Scientific Instrument Co., LTD
gel imaging system	Shandong Holder electronic Technology Co., LTD

Pipetting gun, coated rods, a petri dish, microscope, triangle, 10 ml bottle centrifugal pipe, 2 ml centrifuge tube, EP equipment such as conventional experiment.

2.4 Research method

2.4.1 Screening of lactic acid bacteria

Collection of environmental samples: Select representative environmental samples, such as soil, water, plant rhizosphere, etc., for collection and preservation.

Enrichment culture: After proper treatment of environmental samples, inoculation into enrichment medium containing specific nutrients, enrichment culture of lactic acid bacteria is carried out.

Isolation and purification: The single colony of lactic acid bacteria was separated from the enriched culture by plate scribing method or dilution coating method, and purified.

Preliminary screening: Through morphological observation, physiological and biochemical characteristics testing and other methods, the isolated lactic acid bacteria were preliminatively screened, and the strains with excellent characteristics were selected.

2.4.2 Molecular identification of lactic acid bacteria

Genomic DNA extraction: Using appropriate DNA extraction methods, genomic DNA was extracted from lactic acid bacteria cells.

PCR amplification: The 16S rRNA gene fragment of lactic acid bacteria was amplified by PCR using specific primers.

DNA sequencing: The PCR products were sequenced to obtain the 16S rRNA gene sequence of lactic acid bacteria.

Sequence alignment and species identification: The 16S rRNA gene sequence of lactic acid bacteria obtained by sequencing was compared with the known lactic acid bacteria database, and the species and subspecies of lactic acid bacteria were determined through sequence similarity and phylogenetic analysis.

CHAPTER 3
EXPERIMENTAL PART

3.1 Medium preparation

In view of the high nutrient requirements of lactic acid bacteria, selecting the right medium becomes the most important initial step. Widely used media such as MRS Medium, DC medium, etc., they are rich in a variety of nutrients required for the growth of lactic acid bacteria, providing sufficient energy and material basis for the growth of lactic acid bacteria.

Table 3.1 shows the formula of MRS Liquid culture medium [12]. Each component in the table is weighed and added to the large beaker in turn, and mixed evenly on the magnetic stirrer, and ensure that the PH value is 5.7±0.2. After evenly mixed, the components are divided into multiple triangle bottles in equal parts, sealed and placed in the high-pressure steam sterilization pot for sterilization. MRS Solid medium can be prepared by adding 15-20g/L AGAR powder on the basis of Table 3.1 medium.

Table 3.1 – **MRS Liquid medium formula (1000mL system)**

ingredient	1000mL system
peptone	10.0g
beef noodle soup	8.0g
yeast powder	4.0g
glucose	20.0g
dipotassium phosphate	2.0g
diammonium hydrogen citrate	2.0g
sodium acetate	5.0g
magnesium sulfate	0.2g
manganese sulfate	0.04g
Tween 80	1.0g

3.2 Isolation and screening of Lactic acid bacteria from environmental samples

After sterilization, the medium was removed from the autoclave, the solid medium was heated in the microwave oven to melt, and then poured into the petri dish after melting completely (Figure 3.1), waiting for cooling.

Figure 3.1 - Preparation of solid culture plate of lactic acid bacteria

Take 10 g sample, thoroughly mix it with 90 mL sterile saline solution, dilute the mixture with sterile saline solution, and take diluent solution with three gradients of 10^{-4}, 10^{-5} and 10^{-6}, and transfer the diluent into the cooled petri dish with a pipette in a super-clean workbench. The diluted solution was evenly coated in MRS Solid petri dishes containing CaCO3 or bromo-methyl phenol green using a coater after sterilization or a disposable coater (Figure 3.2), and coated successively from low concentration to high concentration. After coating, the petri dishes were incubated at 37℃ for 48 h (Figure 3.3).

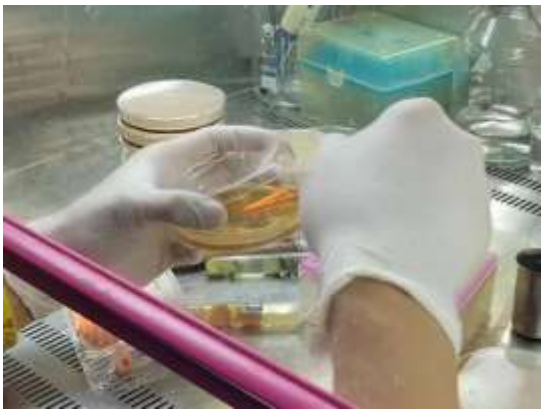

Figure 3.2 - Strain dilution is coated on MRS Solid petri dish

Figure 3.3 - After diluted coating, the culture was incubated at constant temperature for 48 h in a solid culture dish

The colonies with obvious calcium-soluble ring or green color were selected and plate marking was performed on the MRS Petri dish, and the marking was repeated for many times until a single colony of pure breed was obtained.

3.3 Identification of lactic acid bacteria

3.3.1 morphological observation

During the lactic acid bacteria screening process, the resulting strains were inoculated into MRS Solid medium and cultured at 37°C for 48 h. After the culture was completed, a single colony with a smooth surface and no mixed impurities was selected and purified twice to ensure the purity of the strain, and the morphological characteristics of the colony were observed in detail. Subsequently, the well-grown isolates were precisely selected using the inoculation ring, smeared and fixed on the slide. After it was cooled and stabilized, it was treated with Gram staining method, and the morphological structure of lactobacillus cells was carefully observed under a microscope. This procedure is not only beneficial to determine the purity of the strain, but also provides an important reference for the identification and subsequent use of the strain.

3.3.2 physiological and biochemical identification

The isolated strains were inoculated into the corresponding physiological and biochemical medium, and physiological and biochemical experiments such as catalase determination, methyl red test, glucose gas production teststarch hydrolysis test and indole production test were carried out.

Determination of hydrogen peroxide enzyme: first of all should be test bacteria inoculated to PGY medium on the inclined plane of and under the condition of constant temperature of 37 ℃ for 24 hours. Then, a ring of inoculum is taken from the cultured bevel and evenly coated on the clean slide surface. Next, a 5% concentration of hydrogen peroxide solution was added to the colony-coated slide. If bubbles are observed on the slide, it is determined that the catalase positive reaction; If no bubbles are seen, the reaction is considered negative. After completing the above operations, the experimental results were recorded in detail, and the strains showing negative reactions were screened.

3.3.3 Molecular biological identification

In recent years, with the rapid development of molecular biology technology, the identification method of lactic acid bacteria has changed from the traditional morphological, physiological and biochemical characteristics analysis to more accurate and efficient molecular biology technology. In this study, we used a molecular biological method based on 16S rRNA gene sequence analysis [23,24] to identify lactic acid bacteria.

(1) DNA extraction [25]: The bacterial solution of the isolated strain was taken, cultured overnight and placed in a centrifuge tube. The centrifuge tube was centrifuged at 12000 rpm for 30 seconds, and the supernatant was poured in to collect the bacteria.

Precipitation in the STE buffer solution of lysozyme to suspension, to destroy bacteria cell wall and cell membrane, the release of DNA.

Again to join in centrifuge tube such as the volume of phenol: chloroform, isoamyl alcohol mixture, mixing, centrifugal, repeat this step.

Up in the clear liquid adding suitable amount of isopropyl alcohol, make DNA precipitation. Wash the precipitate with anhydrous ethanol, remove the adsorption column in a clean centrifuge tube, add the elution buffer, leave at room temperature for 2 minutes, centrifuge at 12000 rpm for 1 minute.

(2) of the PCR amplification: The DNA of the strain isolated above was amplified by PCR technology (Figure 3.4), and the 16S rRNA gene fragment was obtained. The PCR amplified primer sequences were 27F (5 '-CAGAGTTTGATCCTGGCTCAG-3') and 1492R (5 '-GGTTACCTTGTTACGACTT-3'), and the reaction system (25 μL) was as follows: Primers 27F and 1492R (10 μmol ·L-1) are 1 μL each, 2×Master Mix is 12.5 μL, ddH2O is supplemented to 25 μL, and fresh colonies cultured for 24-48 h are selected and mixed in the reaction system. The PCR procedure is predenaturation at 95 ° C for 3 minutes, followed by 35 cycles, each consisting of denaturation at 95 ° C for 15 seconds, annealing at 65 ° C for 15 seconds, and extension at 72 ° C for 30 seconds.

After the reaction is complete, it is kept at 72 ° C for another 5 minutes to ensure the complete extension of the product.

Figure 3.4 - PCR amplification

(3) Electrophoresis: The PCR products were purified and verified by gel electrophoresis to ensure that the amplified sequence was the 16S rRNA gene of lactic acid bacteria. The PCR amplification product was evenly mixed with the buffer of the upper sample, and the DNA Marker was electrophoreted with 1% agarose gel at 60 V for 20 min. The results were observed by gel imager.

(4) Sequencing and sequence alignment: After the PCR amplification product is purified, it is sent to the sequencing company for sequencing. The sequencing results were submitted to the NCBI website for sequence comparison with sequences in the database, so as to determine the classification of each strain. By comparing the sequencing results with the 16S rRNA gene sequence database of the known lactic acid bacteria, the species information of the isolated lactic acid bacteria can be identified.

3.4. Strain culture result

After the environmental samples were treated and coated on the MRS Petri dish and incubated at constant temperature for 48 h, the colony growth was shown in Figure 3.5.

Figure 3.5 - Culture results of lactic acid bacteria strains in environmental samples

3.5 Gel electrophoresis result

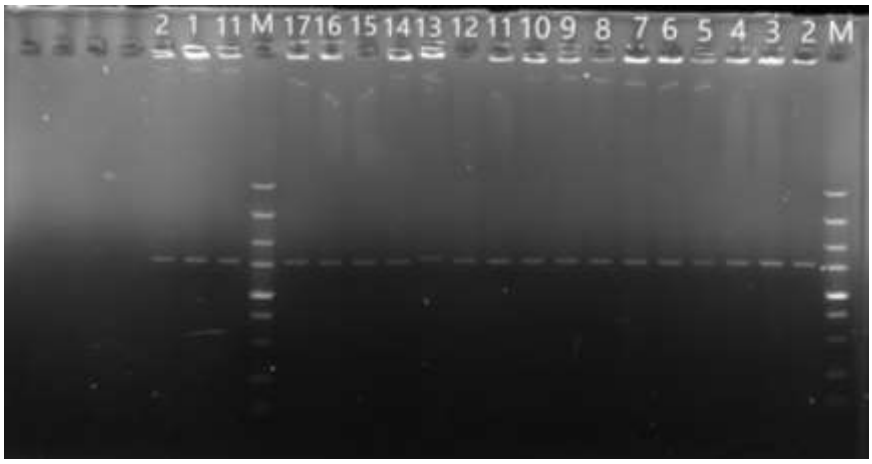

Figure 3.6 - Gel electrophoresis results of PCR amplification products

From right to left, the first is Maker, 2 through 17 are bacteria, 18 is Maker, and the last three are 11, 1, and 2.

3.6 PCR amplified product sequencing results

The PCR amplification products were sequenced. After sequencing by ZW - JX - 1-1 of sequence fragments as CGATACGCACTGGCCCACTGATTGATGGTGCTTGCCCTGATTGACGATGG ATCACCAGTGAGTGGCGGACGGGTGAGTAACACGTACGTAACCTGCCCC GGAGCGGGGGATAACATTTGGAAACAGATGCTAATACCGCATAACAACA AAAGCCACATGGCTTTTGTTTGAAAGATGGCTTTGGCTATCACTCTGGGA TGGACCTGCGGTGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCG ATGATGCATAACCGAGTTGAGAGACTGATCGGCCACAATGGAACTGAGA CACGGTCCATACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGG GCGCAAGCCTGATGGAGCAACACCGCGTGAGTGAAGAAGGGTTTCGGCT CGTAAAGCTCTGTTGTTGGAGAAGAACGTGCGTGAGAGTAACTGTTCACG CAGTGACGGTATCCAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGC CGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAG CGAGCGCAGGCGGTTACTTAAGTCTGATGTGAAAGCCTTCGGCTTAACCG AAGAAGTGCATCGGAAACCGGGCGACTTGAGTGCAGAAGAGGACAGTGG AACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGT GGCGAAGGCGGCTGTCTGGTCTGCAACTGACGCTGAGGCTCGAAAGCAT GGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATG AGTGCTAGGTGTTGGAGGGTTTCCGCCCTTCAGTGCCGGAGCTAACGCAT TAAGCACTCCGCCTGGGGAGTACGACCGCTAGGTTGAAACTCAAGGAATT GACGGGGGCCCGCACAATCGGTGGAGCATGTGGTTTAATTCGAAGCTACG CGAGAACCTTACCACGTCTTGACATCTTGCGCTACCTTAGAGATAACGCG TTCCCTTCGGGGACGCATGA, its sequencing map fragments as shown in figure 3.7.

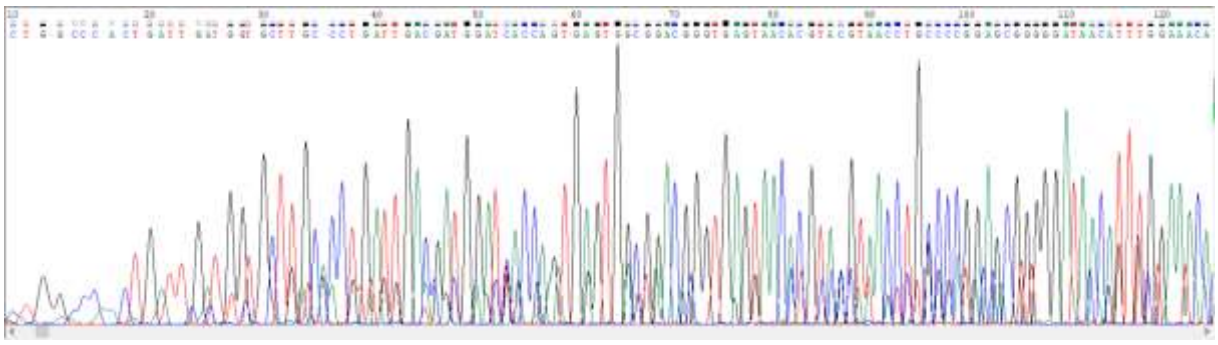

Figure 3.7 - ZW-JX-1-1 sequence fragment

The sequence fragment of ZW-JX-2-1 is:

CGTGCGGCGTGCCTATACTGCAAGTCGAACGAGTTCTCGTTGATGAT
CGGTGCTTGCACCGAGATTCAACATGGAACGAGTGGCGGACGGGTGAGT
AACACGTGGGTAACCTGCCCTTAAGTGGGGGATAACATTTGGAAACAGAT
GCTAATACCGCATAGATCCAAGAACCGCATGGTTCTTGGCTGAAAGATGG
CGTAAGCTATCGCTTTTGGATGGACCCGCGGCGTATTAGCTAGTTGGTGA
GGTAATGGCTCACCAAGGCGATGATACGTAGCCGAACTGAGAGGTTGAT
CGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCA
GTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGT
GAGTGAAGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTGGAGAAGAATGG
TCGGCAGAGTAACTGTTGTCGGCGTGACGGTATCCAACCAGAAAGCCACG
GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATC
CGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGT
GAAAGCCCTCGGCTTAACCGAGGAAGCGCATCGGAAACTGGGAAACTTG
AGTGCATAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAG
ATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGA
CGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTA
GTCCATGCCGTAAACGATGAATGCTAGGTGTTGGAGGGTTTCCGCCCTTC
AGTGCCGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGTACGACCGCAA
GGTTGAAACTCAGAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCAT
GTGGTTTAATTCTAAGCAACGCGAAGAACCTTACCACGTCTTGACATCTTT
TGATCACCTGAGAGATCAGTTTCCCCTTCGGGGCAAATGACAGTGGTGCA
TG，the sequencing map is shown in Figure 3.8.

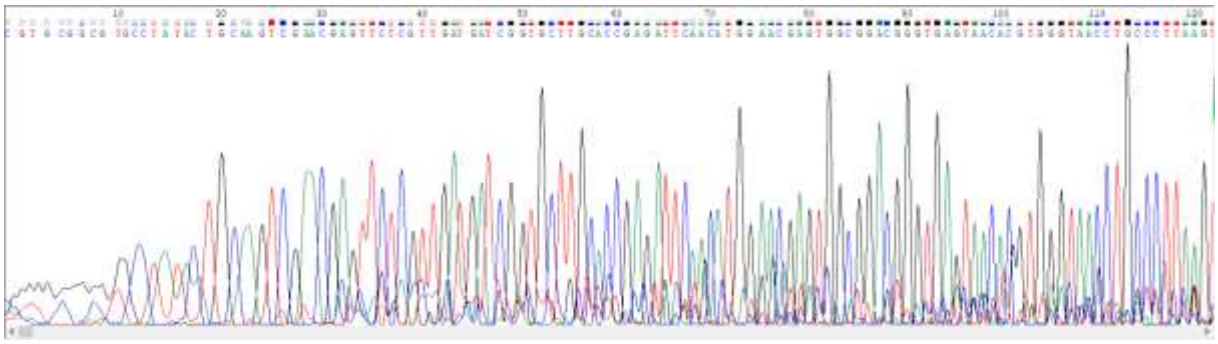

Figure 3.8 - ZW-JX-2-1 sequence fragment

The sequence fragment of ZW-JX-6-2 is:

GGGGCGGTGCGGCGTGCTATACATGCAAGTCGAGCGAGCTGAACCA
ACAGATTCACTTCGGTGATGACGTTGGGAACGCGAGCGGCGGATGGGTG
AGTAACACGTGGGGAACCTGCCCCATAGTCTGGGATACCACTTGGAAACA
GGTGCTAATACCGGATAAGAAAGCAGATCGCATGATCAGCTTATAAAAG
GCGGCGTAAGCTGTCGCTATGGGATGGCCCCGCGGTGCATTAGCTAGTTG
GTAGGGTAACGGCCTACCAAGGCAATGATGCATAGCCGAGTTGAGAGAC
TGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGC
AGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCC
GCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGA
AGGATAGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCAGAAAGT
CACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCG
TTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAAGAATAAGTCT
GATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCATCGGAAACTGTTTTT
CTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTAGCGGTGGAATGC
GTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCA
ACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCC
TGGTAGTCCATGCCGTAAACGATGAGTGCTAAGTGTTGGGAGGTTTCCGC
CTCTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGAGTACGACC
GCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGA

GCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTTG ，the sequencing map is shown in Figure 3.9.

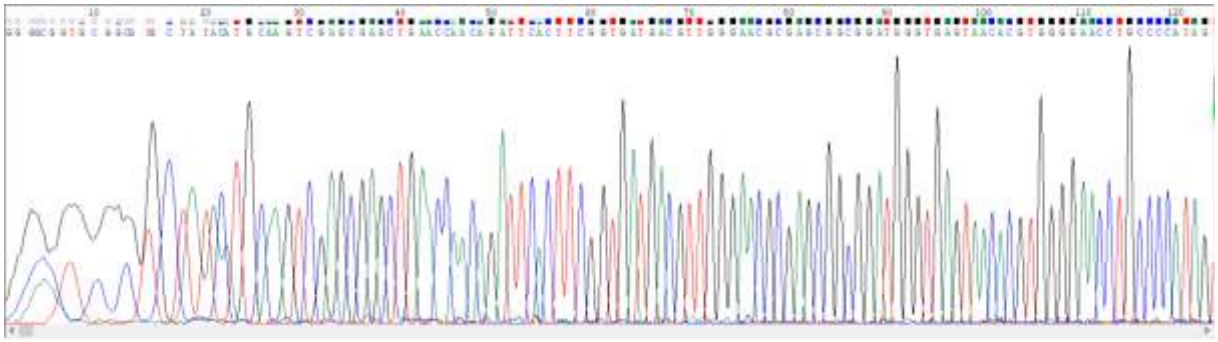

Figure 3.9 - ZW-JX-6-2 sequence fragment

The sequences of strains ZW-JX-3-1, ZW-JX-4-1, ZW-JX-5-1, ZW-JX-7-2, and ZW-JX-8-1 are shown in Figure 3.10.

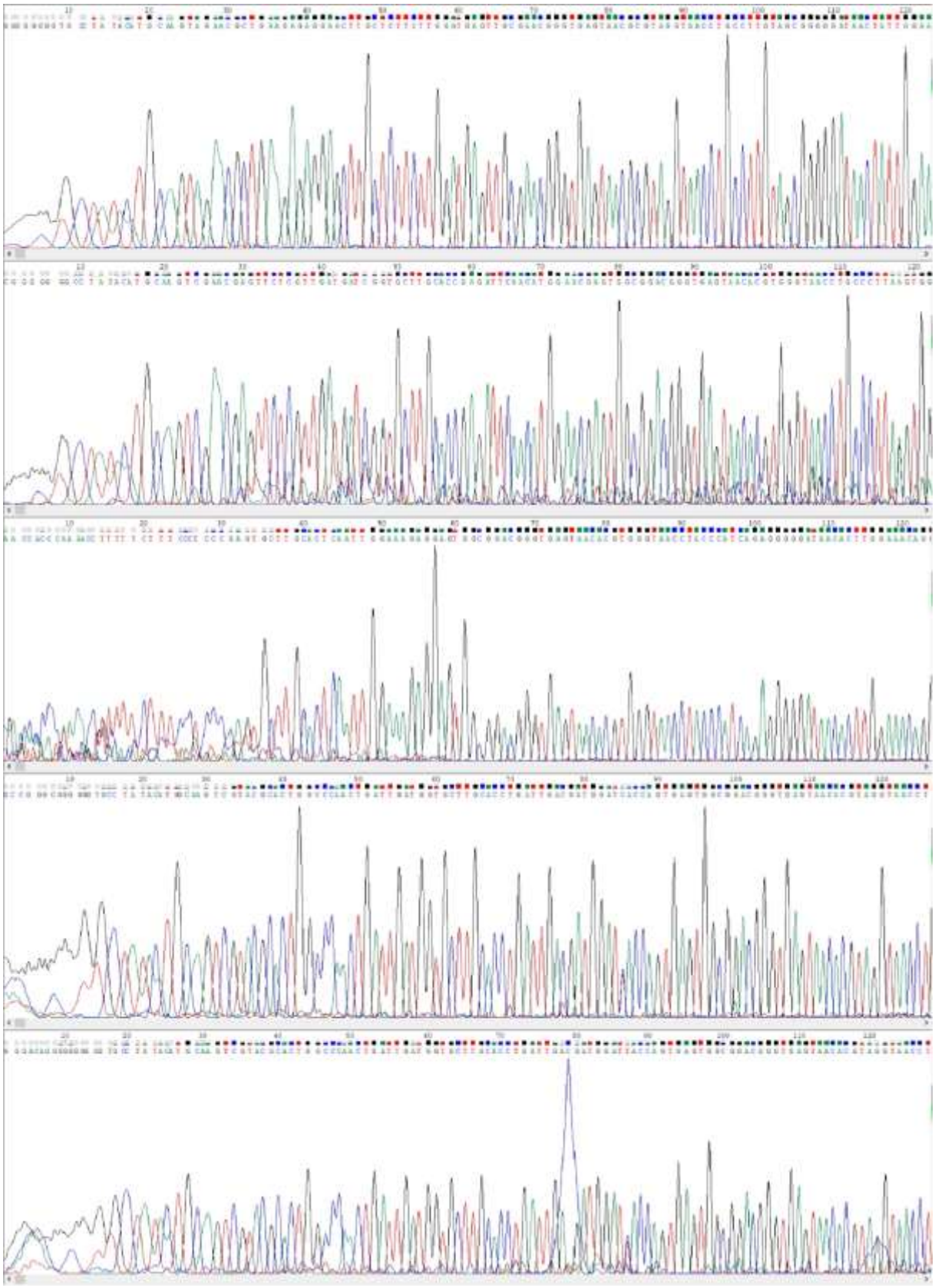

Figure 3.10 - sequence fragments of ZW-JX-3-1, ZW-JX-4-1, ZW-JX-5-1, ZW-JX-7-2, and ZW-JX-8-1 from top to bottom

4.4 Sequence alignment result

Enter the official website of NCBI, input the sequence to be compared with ZW-JX-1-1, BLAST the sequence to be compared with the sequence in the database, and the comparison result shows that the strain with the highest similarity to ZW-JX-1-1 is Lactobacillus reuteri (Figure 3.11). The Query Cover value was 100%, and ZW-JX-1-1 was identified as Lactobacillus reuteri.

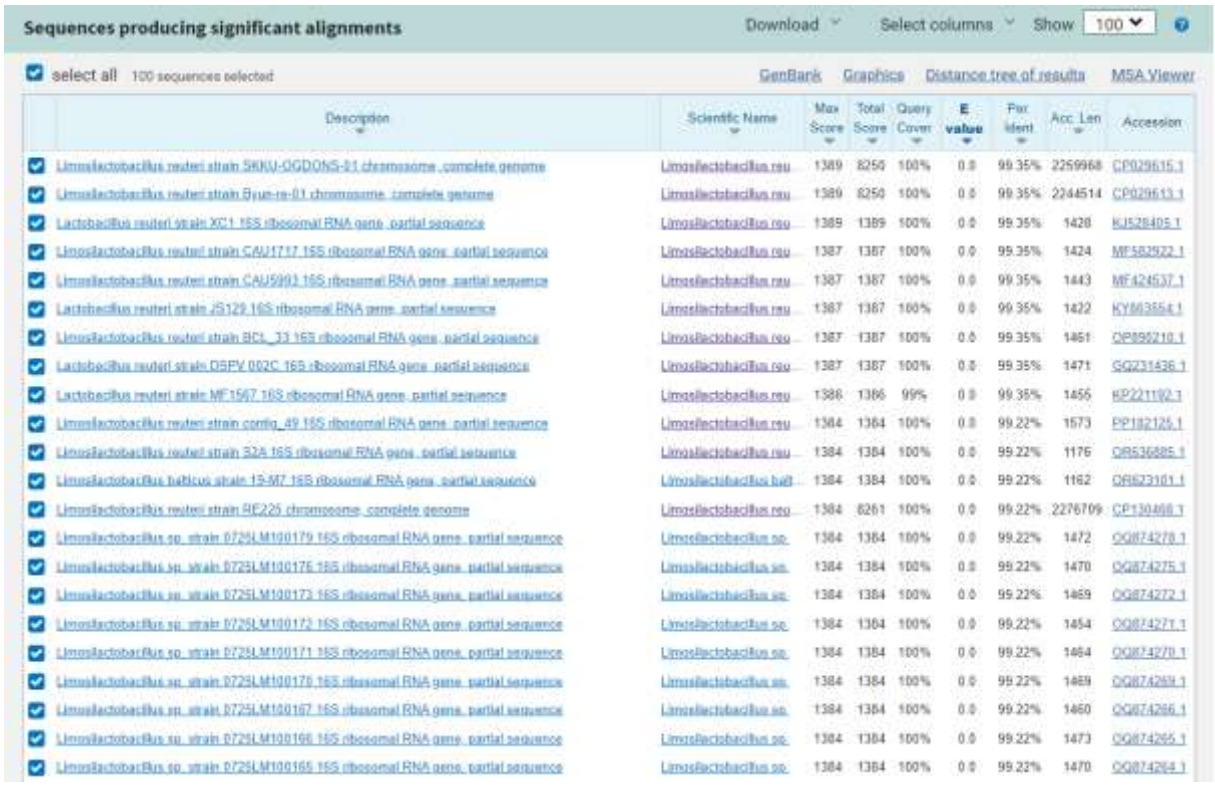

Figure 3.11 - ZW-JX-1-1 sequence comparison result

Repeat the above operations, input the sequence to be compared of ZW-JX-2-1 and BLAST it. The comparison result shows that the strain with the highest similarity to ZW-JX-2-1 is Lacticaseibacillus paracasei (Figure 3.12), and the Query Cover value is 99%-100%. ZW-JX-2-1 was identified as Lactobacillus paracei.

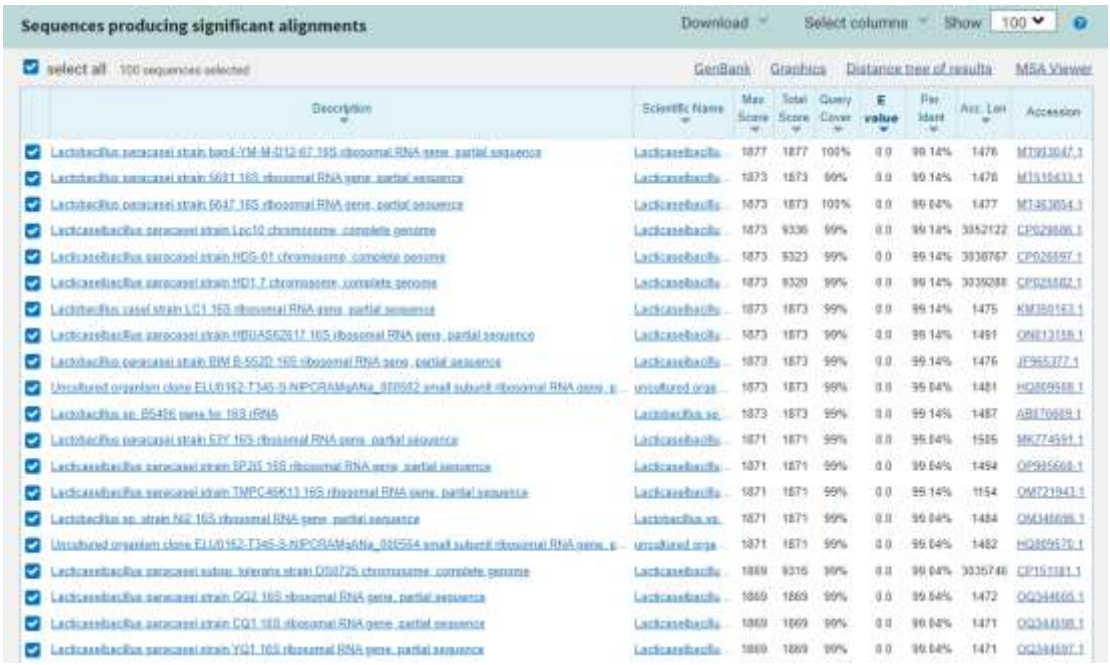

Figure 3.12 - ZW-JX-2-1 sequence comparison result

Input the sequence to be compared with ZW-JX-6-2, and the comparison results showed that the strain with the highest similarity to ZW-JX-6-2 was Lactobacillus acidophilus (Figure 3.13), and the Query Cover value was 98%-99%, indicating that ZW-JX-6-2 was Lactobacillus acidophilus.

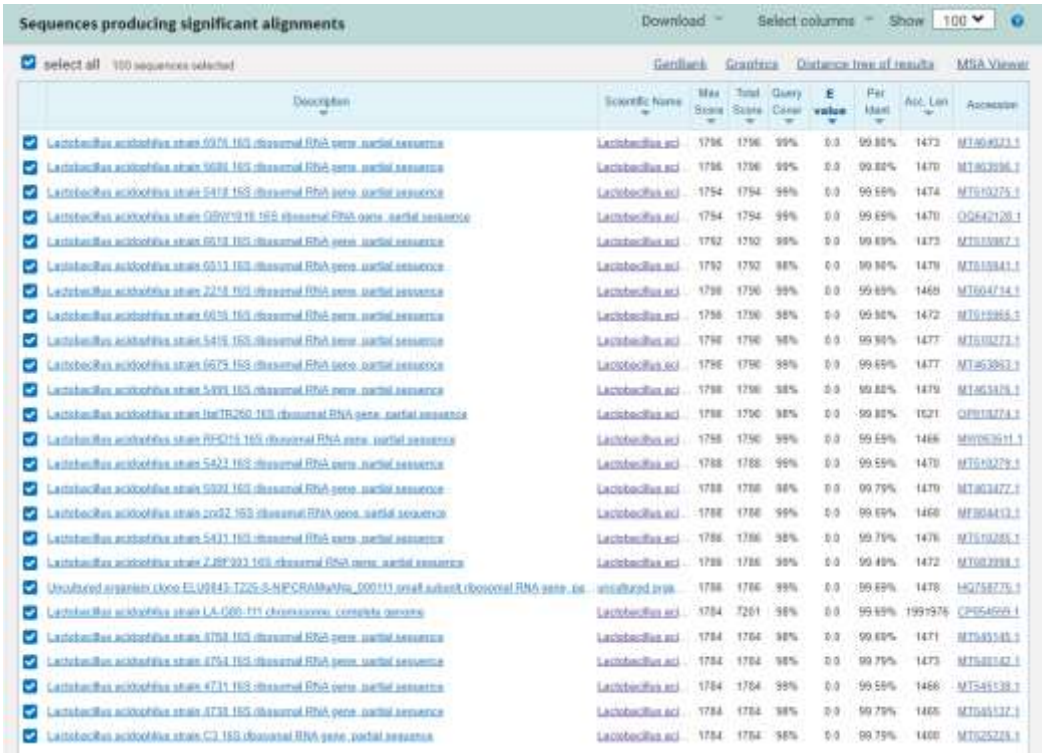

Figure 3.13 - ZW-JX-6-2 sequence comparison result

The BLAST operation was repeated for several times, and the selected strains were identified successively. The identification results are shown in Table 3.2.

Table 3.2 - **Results of strain sequencing**

Strain number	species	Strain number	species
ZW-JX-1-1	Lactobacillus reuteri	ZW-JX-2-1	Lactobacillus paracasei
ZW-JX-3-1	Streptococcus thermophilus	ZW-JX-4-1	Lactobacillus paracasei
ZW-JX-5-1	enterococcus faecalis	ZW-JX-6-2	Lactobacillus acidophilus
ZW-JX-7-2	Lactobacillus reuteri	ZW-JX-8-1	Lactobacillus reuteri

The sequencing sequences of strains ZW-JX-1-1, ZW-JX-7-2 and ZW-JX-8-1, all belonging to Lactobacillus reuteri, were sorted into a file and saved in fasta format for sequence comparison (Figure 3.14). It can be seen from the figure that the conserved sequences of Lactobacillus reuteri are between 37-984 bp. The difference appeared after 985 bp.

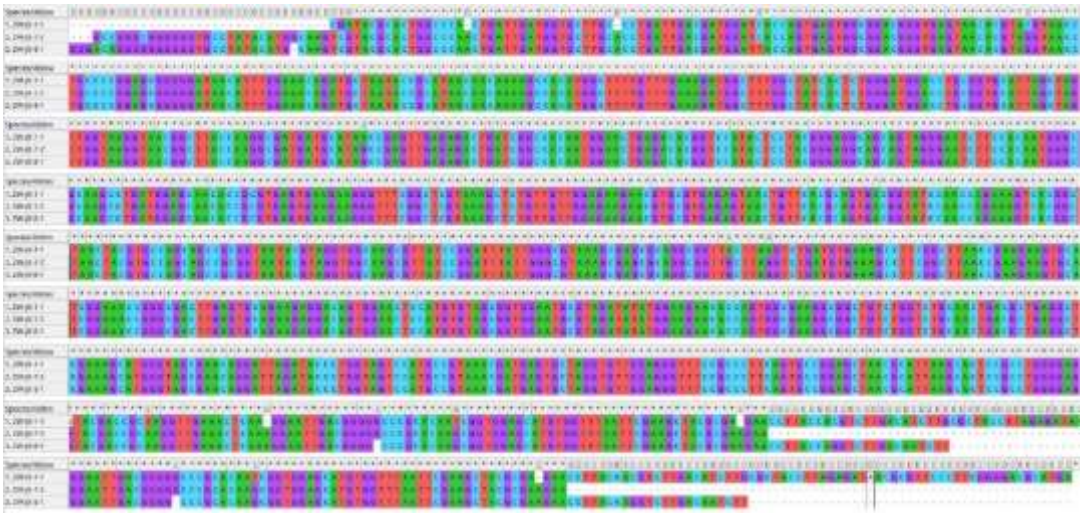

Figure 3.14 - ZW-JX-1-1, ZW-JX-7-2, and ZW-JX-8-1 sequence comparison

The sequences obtained by ZW-JX-1-1, ZW-JX-2-1, ZW-JX-3-1, ZW-JX-4-1, ZW-JX-5-1, ZW-JX-6-2, ZW-JX-7-2, ZW-JX-8-1 were sorted into the same file and saved in fasta format. Using the MEGA 11 software component phylogenetic tree (Figure 3.15). Phylogenetic tree showed the relationship between different strains. For example, strain ZW-JX-2-1 and strain ZW-JX-4-1 were most closely related, both belonging to Lactobacillus paracasei, and the self-spread value was 100.

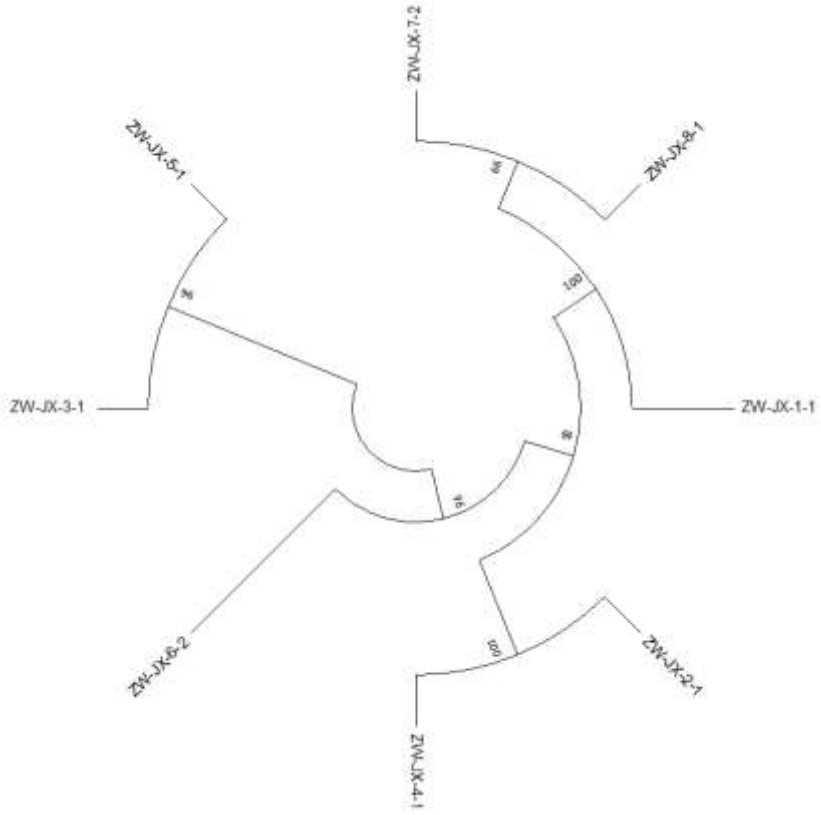

Figure 3.15 - Phylogenetic tree

CONCLUSIONS

In this study, the screening and molecular identification of lactic acid bacteria in environmental samples were systematically investigated.

Through a flat line, coated tablet and soluble calcium ring method, the experiment operation, we successfully from yantai, zibo, pingyin, more lactic acid bacteria strains were isolated from the samples collected, shows the universality of the distribution of lactobacillus in the natural environment.

Molecular identification, successively the morphology observation, physiological and biochemical test and 16 s rRNA sequence alignment analysis to determine screening strains were isolated from the genus in the environmental samples. 16S rRNA sequence comparison provides us with the classification information of lactic acid bacteria. Through comparison and analysis between the sequencing sequence and the sequences in the NCBI database, it can be known that the lactic acid bacteria species screened and isolated from the collected environmental samples include Streptococcus thermophilus, Lactobacillus paracei, Lactobacillus reuteri, Enterococcus faecalis, Lactobacillus acidophilus, etc. According to the construction of MEGA phylogenetic tree, it can also be seen that strain ZW-JX-2-1 and strain ZW-JX-4-1 are most closely related, and the previous sequence comparison results also show that both strains are Lactobacillus paracasei.

These molecular identification technology not only provides the classification information of lactic acid bacteria strains, also revealed the genetic differences and evolutionary relationships between them. Through 16S rRNA sequence analysis, we found that some strains had high homology with known lactobacillus species, while others showed unique genetic characteristics. By constructing phylogenetic trees with MEGA, we were able to clearly understand the relationship between different strains.

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