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# Cell Biology Research

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# Study on the Distribution of Respiratory Bacterial Communities in Patients with Pulmonary Tuberculosis

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**Abstract:** *Objective:* This study aims to reveal the changes in the lower respiratory tract bacterial community of tuberculosis patients compared to healthy individuals. It initially explores the correlation between the lower respiratory tract microbiota and the occurrence and development of pulmonary tuberculosis, aiming to provide new reference ideas for the treatment of pulmonary tuberculosis. *Methods:* This research methodology is based on second-generation sequencing, targeting the V3-V4 variable region of bacterial 16S rDNA for high-throughput sequencing. The study collected bronchial epithelial brush-off samples from 11 healthy volunteers and 20 confirmed pulmonary tuberculosis patients in Yunnan Province for sequencing. Subsequently, a comparative analysis of the sequenced bacterial community results was conducted to explore the association between tuberculosis and human microecology. *Results:* In this experiment, 40 samples of bronchial epithelial brush-off and throat swab were collected from 20 smear-positive pulmonary tuberculosis patients. For the healthy control group, 55 samples of bronchial epithelial brush-offs were collected from five different lung segments of 11 healthy volunteers in Yunnan Province. High-throughput sequencing was performed on these samples. The results showed that the colonized flora present in the lower respiratory tract of healthy people in our province mainly includes *Bacillus*, *Lactococcus*, *Enterococcus*, and *Streptococcus* at the genus level. The results from 20 tuberculosis patients revealed that the number of lower respiratory tract flora in tuberculosis patients is richer than that of healthy people. Besides *Mycobacterium tuberculosis*, the dominant bacteria mainly include *Prevotella*, *Veillonella*, *Pseudomonas*, and *Streptococcus*. Although *Prevotella* is the dominant flora in the samples of tuberculosis patients, its abundance varies significantly among samples. *Conclusion:* Comparing the respiratory flora of healthy people and tuberculosis patients revealed significant differences. Not only have the types and abundance of bacteria increased significantly, but there are also unique bacterial species in the patient's respiratory tract. This suggests that these unique bacteria are likely closely related to the occurrence and development of pulmonary tuberculosis. Furthermore, bacterial abundance may also be related to antibiotic use, providing a new direction for further research.

**Keywords:** Respiratory flora; Pulmonary tuberculosis; 16SrDNA; Sequencing technology

**Online publication:** June 28, 2025

## 1. Introduction

According to the World Health Organization (WHO) estimates, there were approximately 10.4 million new tuberculosis cases globally in 2016, with 1.67 million deaths attributed to the disease<sup>[1]</sup>. Pulmonary tuberculosis has become one of the

significant public issues constraining China's socio-economic development and progress<sup>[2]</sup>. The prevention and control of tuberculosis face unprecedented challenges<sup>[3,4]</sup>. Currently, researchers' investigations into the pathogenesis of tuberculosis primarily focus on two main directions: one centers around Th1 and Th2 cytokines. When the balance between the two is disrupted in the body, it can lead to the latent development of tuberculosis<sup>[5]</sup>. The second focus is on *Mycobacterium tuberculosis* (MTB), an intracellular parasite that alters the host's energy and protein metabolism through host-pathogen interactions<sup>[6,7]</sup>, thereby triggering disease or inflammation. Additionally, there are studies exploring diagnosis, treatment, and pathogenesis at the proteomic level. This article takes a new approach, delving deeply into the development process of pulmonary tuberculosis from a fresh perspective, aiming to provide new directions for the treatment of tuberculosis.

The human body harbors a vast number of microorganisms. It is estimated that the human microbiota contains up to  $10^{14}$  bacterial cells, which is ten times the number of human cells<sup>[9]</sup>. Skin commensal bacteria can protect the body from pathogens<sup>[10]</sup>, while the intestine also hosts a significant amount of colonized microorganisms, accounting for approximately two-thirds of all microorganisms in the human body. Thus, the intestine is often regarded as the primary gathering place for human microorganisms, exerting a crucial influence on human health<sup>[11,12]</sup>. Due to difficulties in obtaining sterile lung samples and limitations of traditional culturing techniques, the lower respiratory tract has long been considered a sterile environment without colonized microorganisms<sup>[13–15]</sup>. However, in recent years, with the widespread application of fiberoptic bronchoscopy and rapid advancements in molecular detection techniques for microorganisms, it has become possible to investigate bacterial composition without the need for culturing individual microorganisms, greatly improving detection efficiency and accuracy<sup>[16,17]</sup>. Numerous studies have indicated that an increased proportion of neutrophils in the airways is a key factor in the progression and exacerbation of inflammation in Chronic Obstructive Pulmonary Disease (COPD)<sup>[18,19]</sup>.

Other literature suggests a close relationship between Potentially Pathogenic Micro-organisms (PPMs) and airway inflammation during the transition from stable COPD to acute exacerbations<sup>[20]</sup>. In respiratory samples from patients with bronchial asthma, the proportion of various respiratory pathogens, such as *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* is higher than in the normal population<sup>[21,22]</sup>. Additionally, the diversity of bacterial communities in the lungs of patients with cystic fibrosis is significantly lower compared to healthy individuals<sup>[23]</sup>. These studies demonstrate that numerous respiratory diseases are closely related to changes in the respiratory microbiota. However, there are few reports on the respiratory microbiota of tuberculosis patients. This article focuses on patients with open pulmonary tuberculosis, revealing their respiratory microbiota status and initially exploring the correlation between lower respiratory tract microorganisms and the development of tuberculosis. It is hoped that this can provide a new reference for the clinical treatment of tuberculosis.

## 2. Materials and methods

In this experiment, two sets of samples were collected. For the healthy control group, bronchial mucosal scrapings from five lung segments on both sides were collected via fiberoptic bronchoscopy. For the tuberculosis group, bronchial mucosal scrapings were collected through fiberoptic bronchoscopy, along with throat swab samples. Patients in the tuberculosis group were required to have a positive sputum smear and a positive Xpert-MTB test.

### 2.1. Selection of research subjects

The health status of healthy volunteers and patients was evaluated through medical history inquiries (recorded in medical records), physical examinations, and necessary laboratory tests. After evaluation and screening, healthy volunteers were excluded if they met any of the following criteria:

- (1) Used antibiotics or probiotics outside the hospital in the month before the visit;
- (2) Had a history or current pulmonary infection, including cured tuberculosis, acute cardiac insufficiency, or diseases causing acute cough;

- (3) Had chronic lung diseases such as chronic bronchitis, COPD, cystic fibrosis (CF), pulmonary interstitial fibrosis, bronchial asthma, chronic cardiac insufficiency, or diseases causing chronic cough;
- (4) Had genetic metabolic diseases such as diabetes, hyperthyroidism, or congenital muscular dystrophy.

Exclusion criteria for tuberculosis patients were:

- (1) Used antibiotics or probiotics outside the hospital in the month before the visit;
- (2) Had other lung infections besides tuberculosis;
- (3) Had tuberculosis combined with chronic lung diseases;
- (4) Had tuberculosis combined with other genetic metabolic diseases.

After strict screening based on the above exclusion criteria, the study finally selected 11 healthy volunteers, including 8 males and 3 females, aged between 30 and 70 years old, and 20 tuberculosis patients, including 8 males and 12 females, aged between 16 and 75 years old. After analysis, the age groups of the two sets of subjects were relatively close, and there were basically no other diseases that could affect the microecology.

## **2.2. Methods**

This study relied on second-generation sequencing technology to perform high-throughput sequencing targeting the V3–V4 variable region of bacterial 16S rDNA. The two sets of samples were analyzed, and then a comparative analysis of the bacterial community results obtained from sequencing was conducted to explore the relationship between tuberculosis and human microecology.

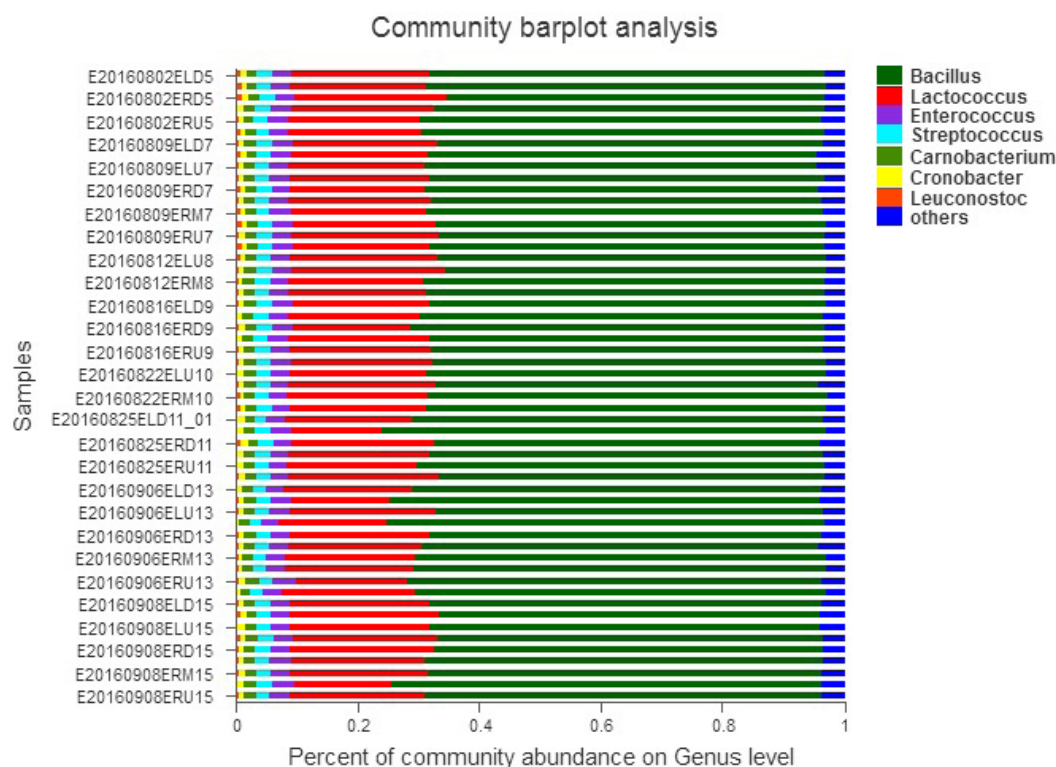
## **2.3. Statistical analysis**

In this experiment, QIIME software was used to adjust the distance difference between samples based on importance, detect the beta diversity of respiratory bacteria, and analyze differences between groups. Two-dimensional or three-dimensional coordinates were created to identify important biomarkers. Special data was estimated using bias-corrected unimodal embedding, and hierarchical clustering analysis, principal coordinate analysis, and other analytical charts were obtained through R language analysis. Differences were analyzed using the *t*-test and nonparametric tests. A *P*-value < 0.05 indicated a significant difference, and the false discovery rate *q*-value was calculated for the *P*-value.

## **3. Results**

### **3.1. Detection results of lower respiratory tract bacteria at the genus level in healthy populations**

The dominant colonizing bacteria in the lower respiratory tract of healthy populations in our province include *Bacillus*, *Lactococcus*, *Enterococcus*, and *Streptococcus*. The composition of the bacterial flora in the left and right lungs, as well as the upper and lower lobes of the lungs, of healthy individuals in our study indicated that there were no significant differences among all lung segments of the left and right lungs in healthy individuals from the same region (**Figure 1**).



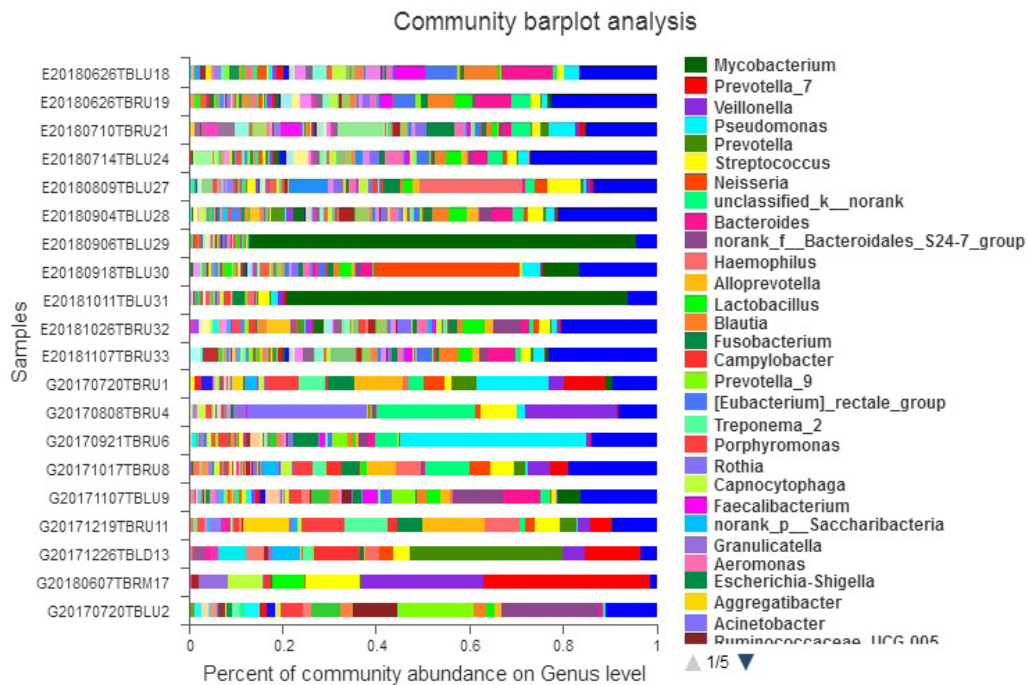
**Figure 1.** Bar chart showing the abundance of bacterial flora at the genus level in the lower respiratory tract of healthy individuals.

### 3.2. Detection results of lower respiratory tract bacteria at the genus level in tuberculosis patients

By comparing the composition of respiratory flora between healthy individuals and tuberculosis patients, it was found that the species and abundance of respiratory bacteria in tuberculosis patients increased significantly. Moreover, the respiratory tract of tuberculosis patients had its unique dominant bacterium, *Prevotella*, which is an anaerobic conditional pathogen that is difficult to cultivate effectively using traditional methods. Additionally, the abundance of other anaerobic bacteria in the respiratory tract of tuberculosis patients also increased, suggesting that the growth of anaerobic bacteria, predominantly *Prevotella*, may be closely related to the occurrence and development of tuberculosis.

After drawing the research conclusions, a review of patient case data revealed significant differences in the abundance of dominant bacteria among patients who received antibiotic treatment before bronchoscopy. Meanwhile, gender factors may also affect the distribution of dominant bacteria. Comparing the differences in bacterial colonies among various tuberculosis patients, it was discovered that some patients had a significantly higher proportion of *Mycobacterium tuberculosis*, resulting in a substantial decrease in the abundance of newly emerging dominant bacteria. This could be caused by the amount of tuberculous infection or by certain factors inhibiting the growth of dominant bacteria, thereby promoting the proliferation of *Mycobacterium tuberculosis* (**Figure 2**).

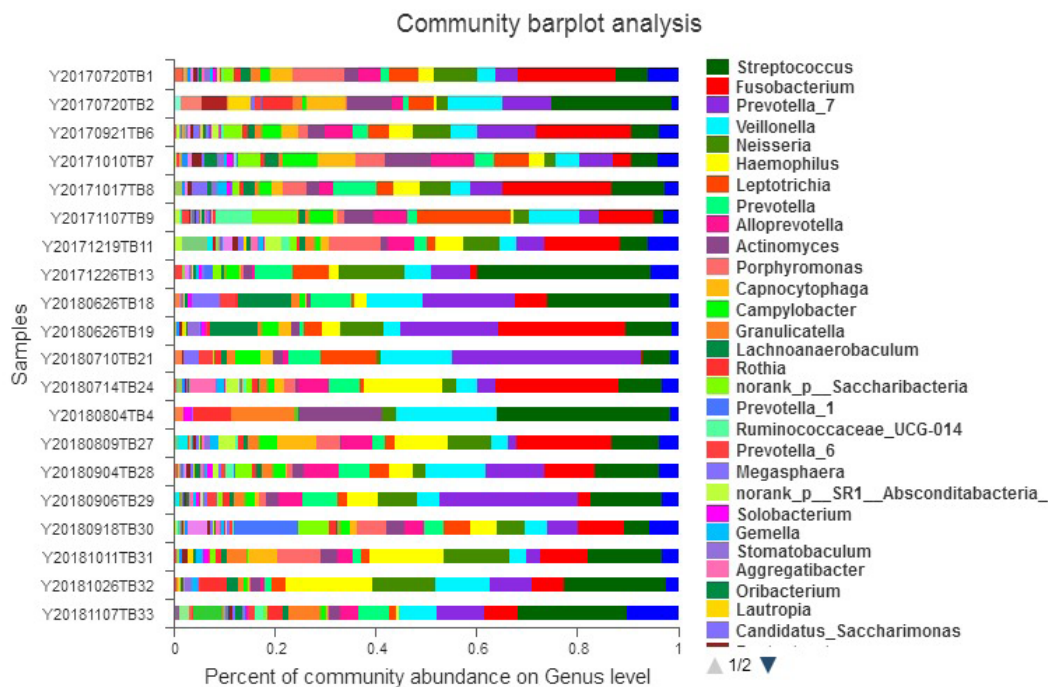




**Figure 2.** Bar chart showing the abundance of bacterial flora at the genus level in the lower respiratory tract of tuberculosis patients.

### 3.3. Detection results of upper respiratory tract bacteria at the genus level in tuberculosis patients

The dominant bacteria in the upper respiratory tract flora of tuberculosis patients include Prevotella, Streptococcaceae, Fusobacteriaceae, and Veillonellaceae. Among them, Streptococcaceae and Veillonellaceae are common colonizing bacteria in the upper respiratory tract. The abundance of Streptococci and Fusobacterium is relatively high. Prevotella is represented by four genera. Although the proportion of each genus varies, their overall proportion is still significantly higher than other bacteria (Figure 3).



**Figure 3.** Bar chart showing the abundance of bacterial flora at the genus level in the upper respiratory tract of tuberculosis patients.

## 4. Discussion

The results of this study show that, when comparing samples from healthy volunteers with those from tuberculosis patients, there is a significant increase in the diversity and abundance of respiratory bacteria in tuberculosis patients. However, there are some overlapping bacteria in the two groups of samples, which the study believes are likely to exist as background bacteria and have a smaller impact on disease development. It is worth focusing on other bacteria in the samples of tuberculosis patients that are distinct from healthy individuals. These bacteria may rapidly proliferate based on the original foundation after the disease occurs, suggesting that when the human body is infected with tuberculosis, the respiratory microecology not only increases *Mycobacterium tuberculosis* but also breaks the original balance, leading to more active bacteria. During the occurrence, development, and prognosis of tuberculosis, various bacteria may have a significant or minor role, especially the dominant flora with higher abundance, which should be given extra attention as they are likely to play a key role in the disease process.

Tuberculosis is a respiratory infectious disease. However, there are significant differences in the prognosis of individuals infected with *Mycobacterium tuberculosis*. Some people do not develop the disease after infection and only discover a history of infection during physical examinations. This indicates that the onset of tuberculosis may be closely related to changes in the body's microecology, besides being associated with *Mycobacterium tuberculosis* infection. Therefore, if other treatment methods can be used to maintain the lung microecology of patients consistent with that of healthy individuals, it may be possible to improve the prognosis of tuberculosis patients. Of course, this requires continuous follow-up to observe changes in bacteria. If a bacterium is identified as a pathogenic bacterium causing the disease, a large amount of literature needs to be reviewed to explore effective and feasible countermeasures.

## 5. Conclusion

There are significant differences in respiratory microorganisms between tuberculosis patients and healthy individuals. These unique bacteria are likely to be closely related to the occurrence and development of tuberculosis. Additionally, bacterial abundance may also be associated with antibiotic use. In subsequent studies, the study will focus on analyzing the specific mechanisms of these unique bacteria in the process of tuberculosis, while exploring the intrinsic relationship between antibiotic dosage, duration, and changes in bacterial abundance. The study hope to provide a scientific basis for tuberculosis prevention and treatment strategies.

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## Disclosure statement

The authors declare no conflict of interest.

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# Exploration of the Role of Sulfur Bacteria in the Sulfur Cycle of Bottom Sediments in Aquaculture Ponds

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**Abstract:** As an important place for aquaculture, the water quality and ecological environment of aquaculture ponds are crucial for the growth and health of aquaculture organisms. The sulfur cycle, as an essential link in pond ecosystems, has a significant impact on water purification and the survival of aquaculture organisms. This article delves into the role of sulfur bacteria in the sulfur cycle in aquaculture ponds, including their promotion of sulfide oxidation, organic matter degradation, and provision of nutrients and energy for aquaculture organisms. Through comprehensive analysis, this article reveals the positive impact of sulfur bacteria on water purification in aquaculture ponds and the growth of aquaculture organisms, and proposes application strategies to optimize the living environment and increase the number of sulfur bacteria. This article also looks forward to the potential applications of sulfur bacteria in genetic manipulation, biological leaching, and environmental remediation, providing new ideas and methods for the ecological management of aquaculture ponds.

**Keywords:** Sulfur bacteria; Aquaculture pond; Sulfur cycle; Water purification

**Online publication:** June 28, 2025

## 1. Introduction

With the rapid development of aquaculture, water quality and ecological environment issues in aquaculture ponds are becoming increasingly prominent. Sulfides, as a common harmful substance in aquaculture ponds, pose a serious threat to the growth and health of aquaculture organisms. The sulfur cycle, as a key process in pond ecosystems, plays an important role in the transformation and removal of sulfides. As the main participants in the sulfur cycle, sulfur bacteria have gradually attracted the attention of researchers in terms of their physiological characteristics and ecological functions. By systematically analyzing the distribution characteristics, physiological mechanisms, and interactions with other microorganisms of sulfur bacteria in aquaculture ponds, this article will reveal the important role of sulfur bacteria in promoting water purification, improving the growth and health levels of aquaculture organisms, and other aspects. This article will also explore the practical application strategies of sulfur bacteria in the sulfur cycle of aquaculture ponds, as well as their potential application value in other fields, providing new perspectives and ideas for the research and application of sulfur bacteria.



## **2. Overview of sulfur bacteria**

### **2.1. Classification and characteristics of sulfur bacteria**

#### **2.1.1. Survival environment and reproductive conditions**

Sulfur bacteria live in diverse environments, ranging from deep-sea sediments to freshwater lakes, from hydrothermal vents to cold spring sediments. They are usually able to survive in extreme environments such as high salinity, high temperature, low oxygen, or anaerobic conditions. The reproductive conditions of sulfur bacteria also vary depending on their species, and they all require suitable pH values, temperature, light, and sufficient sulfides or other sulfur-containing compounds as energy and nutrients. In some cases, sulfur bacteria can also form symbiotic relationships with other microorganisms to jointly utilize resources in the environment <sup>[1]</sup>.

#### **2.1.2. Photosynthesis and energy acquisition**

For sulfur bacteria capable of photosynthesis, photosynthesis is their main pathway for obtaining energy. These bacteria contain special photosynthetic pigments that can absorb light energy and convert it into chemical energy. During photosynthesis, sulfur bacteria use sulfides or thiosulfates as electron donors to convert light energy into energy substances such as ATP (adenosine triphosphate) and NADPH (nicotinamide adenine dinucleotide phosphate) through a series of enzymatic reactions. These energy substances are then used for bacterial growth, metabolism, and maintenance of their life activities. For sulfur bacteria that cannot carry out photosynthesis, they obtain energy through chemical processes, using sulfur-containing compounds such as sulfides as electron donors for respiration <sup>[2]</sup>.

### **2.2. The importance of sulfur bacteria in the sulfur cycle**

As a key microbial community in the sulfur cycle, sulfur bacteria play a crucial role by oxidizing sulfides, reducing sulfates, and decomposing organic sulfur. Firstly, sulfur bacteria can utilize sulfides as an energy source for growth and metabolic activities, during which sulfides are oxidized to sulfates or other sulfur-containing compounds. This process provides energy and nutrients for sulfur bacteria themselves, while also promoting the removal of sulfides and the generation of sulfates in the environment. Some sulfur bacteria can also reduce sulfates to sulfides, a process that occurs in marine sediments, freshwater lakes, and soil environments <sup>[3]</sup>. The reduction of sulfates provides electron acceptors and energy sources for sulfur bacteria and promotes the cycling and reuse of sulfates in the environment. Sulfur bacteria also participate in the decomposition and synthesis of organic sulfur. They can decompose organic sulfur compounds to release inorganic sulfur forms such as sulfides or sulfates and convert inorganic sulfur forms into organic sulfur forms and store them in living organisms <sup>[4]</sup>.

## **3. Mechanism of sulfur cycling in bottom sediment of aquaculture ponds**

### **3.1. Sources and forms of sulfur in sediment**

The sulfur element in the sediment of aquaculture ponds mainly comes from two sources: natural sulfur sources and anthropogenic sulfur sources. Natural sulfur sources mainly include sulfur elements brought about by processes such as rock weathering, groundwater carrying, and atmospheric deposition. These sulfur elements exist in the sediment in inorganic sulfur forms (such as sulfides, sulfates, etc.) or organic sulfur forms (such as sulfur-containing organic compounds), while anthropogenic sulfur sources mainly include sulfur elements from sulfur-containing feed, drugs, and excrement from aquaculture organisms added during the breeding process <sup>[5]</sup>.

The sulfur element in sediment exists in various forms, including inorganic sulfur (such as sulfides, sulfates, elemental sulfur, etc.) and organic sulfur (such as sulfur-containing amino acids, sulfur-containing proteins, etc.). These different forms of sulfur elements undergo a series of transformation processes in sediments, such as oxidation, reduction, methylation, and demethylation. These conversion processes are influenced by various factors, such as temperature, pH value, redox potential, and microbial activity <sup>[6]</sup>. Microbial activity is one of the key factors affecting the transformation

of sulfur forms in sediment. Sulfur bacteria, as an important microbial community in sediment, play a crucial role in the sulfur cycle by oxidizing sulfides, reducing sulfates, and decomposing organic sulfur <sup>[7]</sup>.

### 3.2. The role of sulfur bacteria in sediment sulfur cycling

#### 3.2.1. Oxidation of sulfides and formation of sulfates

- (1) The process of sulfur bacteria oxidizing sulfides: Sulfur bacteria can use sulfides as an energy source for growth and metabolic activities. During this process, sulfides are oxidized to sulfates or other sulfur-containing compounds through a series of enzymatic reactions. This oxidation process usually occurs on the cell membrane or cytoplasm of sulfur bacteria, accompanied by electron transfer and energy release. The rate and efficiency of sulfide oxidation by sulfur bacteria are influenced by various factors, such as sulfide concentration, temperature, pH value, and redox potential <sup>[8]</sup>.
- (2) The impact of sulfate on pond ecology: Sulfate is one of the main products of sulfur bacteria oxidizing sulfides, and it plays multiple roles in aquaculture pond ecosystems. Firstly, sulfates can act as electron acceptors to participate in redox reactions in sediment, promoting the decomposition and mineralization of organic matter. Sulfate can also be absorbed and utilized as a nutrient by aquaculture organisms, promoting their growth and development. However, excessive sulfate concentration may also have adverse effects on aquaculture organisms, such as causing osmotic pressure imbalance and affecting metabolic processes <sup>[9]</sup>.

#### 3.2.2. Decomposition and conversion of organic sulfur

- (1) Microbial decomposition pathways of organic sulfur: Organic sulfur is one of the important forms of sulfur in sediment, which includes complex organic compounds such as sulfur-containing amino acids, sulfur-containing proteins, and sulfur-containing polysaccharides <sup>[10]</sup>. These organic sulfur compounds undergo decomposition and transformation processes under the action of microorganisms, releasing inorganic sulfur forms such as sulfides or sulfates. Sulfur bacteria, as an important microbial community in sediment, can decompose various organic sulfur compounds and release inorganic sulfur forms <sup>[11]</sup>.
- (2) The role of sulfur bacteria in the process: Sulfur bacteria play a crucial role in the decomposition and conversion of organic sulfur. They can use their own enzyme system to decompose complex organic sulfur compounds and convert them into easily usable inorganic sulfur forms. This process provides energy and nutrients for sulfur bacteria, while also promoting the cycling and reuse of organic sulfur in sediment. Sulfur bacteria can also indirectly affect the decomposition rate and conversion direction of organic sulfur by regulating environmental factors such as redox potential and pH value in sediment, affecting the activity of other microorganisms <sup>[12]</sup>.

### 3.3. The interaction between sulfur bacteria and other microorganisms

There are complex interactions between sulfur bacteria and anaerobic bacteria in the sediment of aquaculture ponds. Sulfur bacteria and anaerobic bacteria compete for limited energy and nutrients, such as sulfides and organic matter, in sediment. This competitive relationship may lead to changes in the quantitative balance between the two, thereby affecting the sulfur cycling process in sediment. There may also be a symbiotic relationship between sulfur bacteria and anaerobic bacteria <sup>[13]</sup>. In some cases, anaerobic bacteria can use the sulfate produced by sulfur bacteria oxidizing sulfides as an electron acceptor for respiration, while sulfur bacteria can obtain energy and nutrients from the metabolic products of anaerobic bacteria <sup>[14]</sup>.

As a vital microbial community in sediment, the presence and activity of sulfur bacteria have a significant impact on the microbial community structure of ponds. Firstly, sulfur bacteria alter the form and distribution of sulfur in sediment through processes such as oxidation of sulfides and decomposition of organic sulfur, thereby affecting the survival and activity of other microorganisms. The activity of sulfur bacteria can also alter environmental factors such as redox potential and pH value in sediment, thereby affecting the activity of other microorganisms. Sulfur bacteria can also form complex interaction networks with other microorganisms, maintaining the stability and diversity of pond microbial communities

through competition, symbiosis, and predation <sup>[15]</sup>.

## **4. The impact of sulfur bacteria on the environment of aquaculture ponds**

### **4.1. Water quality regulation and purification function**

In aquaculture ponds, nutrients such as phosphorus and nitrogen are essential for the growth of aquaculture organisms, but excessive concentrations can lead to water quality deterioration, causing problems such as the overgrowth of algae and eutrophication of water bodies <sup>[16]</sup>. Sulfur bacteria can convert nutrients such as phosphorus and nitrogen in water into other forms through a series of biochemical reactions, thereby reducing their concentration and mitigating their impact on water quality. For example, sulfur bacteria can use sulfides as electron donors to reduce nitrate to ammonia nitrogen or nitrogen, thereby reducing the nitrogen content in water. They can also remove phosphate and other phosphorus elements from water through adsorption, precipitation, and other processes <sup>[17]</sup>.

The organic matter in aquaculture ponds mainly comes from the excrement, residual feed, and dead bodies of aquaculture organisms. These organic compounds decompose in water, producing a large amount of harmful substances such as ammonia nitrogen and sulfides, which seriously affect water quality and the health of aquaculture organisms. Sulfur bacteria play an important role in the degradation of organic matter. They can use their own enzyme system to decompose organic matter, converting it into inorganic or low-molecular-weight organic matter, thereby reducing the organic content in the water <sup>[18]</sup>.

### **4.2. Promotion of the growth and health of aquatic organisms**

During the process of oxidizing sulfides and decomposing organic matter, sulfur bacteria release some beneficial nutrients and energy for aquaculture organisms, such as their ability to oxidize sulfides into sulfates, providing sulfur elements for aquaculture organisms. They can also decompose organic matter and release nutrients such as ammonia nitrogen and phosphate, which can be absorbed and utilized by aquaculture organisms. Sulfur bacteria can provide nutrients and energy for aquaculture organisms and enhance their immunity by regulating the microbial community structure in water bodies. Research has shown that sulfur bacteria can inhibit the growth and reproduction of some pathogenic microorganisms, thereby reducing the incidence of diseases in aquaculture organisms <sup>[19]</sup>.

### **4.3. Application strategy of sulfur bacteria in aquaculture ponds**

To fully utilize the role of sulfur bacteria in aquaculture ponds, effective measures need to be taken to increase their population. On the one hand, adding sulfur-containing organic or inorganic substances to the pond can provide sufficient energy and nutrients for sulfur bacteria, promoting their growth and reproduction. On the other hand, bacterial strains can also be introduced from other sulfur-rich environments, such as deep-sea sediments, hydrothermal vents, etc., to enrich the sulfur bacterial community in aquaculture ponds. In addition to increasing the number of sulfur bacteria, it is also necessary to optimize their living environment to improve their activity and efficiency. It is important to reasonably control the water quality parameters of the pond, such as temperature, pH value, dissolved oxygen, etc., to maintain them within a suitable range for the growth and activity of sulfur bacteria. It is also necessary to strengthen the management and maintenance of ponds, regularly clean sediment, replace water bodies, etc., in order to reduce the accumulation of harmful substances and the inhibitory effect on sulfur bacteria <sup>[20]</sup>.

## **5. Conclusion**

This article comprehensively analyzed the role and impact of sulfur bacteria in the sulfur cycle of aquaculture ponds, revealing their important role in promoting water purification, improving the growth and health levels of aquaculture

organisms, and so on. By optimizing the living environment of sulfur bacteria and increasing their numbers, the ecological and economic benefits of aquaculture ponds can be further enhanced. The potential applications of sulfur bacteria in genetic manipulation, biological leaching, and environmental remediation also provide broad space for their future research and application. This study provides new ideas and methods for the ecological management of aquaculture ponds and opens up new directions for the research and application of sulfur bacteria.

## Disclosure statement

The author declares no conflict of interest.

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# Identification and Analysis of Preservation Conditions for Soil-Isolated *Priestia megaterium* Subspecies

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**Abstract:** This study aimed to isolate and identify a subspecies of *Priestia megaterium* with potential probiotic properties from soil and evaluate its cryopreservation conditions. The bacterium was isolated using dilution and plate spreading methods, and identified through 16S rDNA sequencing and physiological and biochemical experiments. Different cryoprotectants (glycerol, DMSO, and starch) were also assessed. The results showed that the strain is a Gram-positive spore-forming bacterium, with 8% DMSO providing the best protective effect, glycerol showing a relatively poor effect, and starch having a detrimental impact. This study provides a theoretical basis for the development and application of probiotics.

**Keywords:** Microbes; Probiotics; Protease; Bacillus; Freezing

**Online publication:** June 28, 2025

## 1. Introduction

Proteases have significant application value in various industries, including food processing, leather treatment, and detergent manufacturing<sup>[1-3]</sup>. However, traditional proteases extracted from plants or animals suffer from high production costs, low yield, and lengthy production cycles, gradually becoming insufficient for meeting industrial demands. Microbial-derived proteases have attracted considerable research attention due to their advantages such as high-efficiency production, low cost, and ease of operation<sup>[4,5]</sup>. In probiotic research, microorganisms with proteolytic abilities have also drawn wide attention<sup>[6-9]</sup>. Screening and isolating such microorganisms have significant application potential.

For the long-term preservation of microorganisms, selecting appropriate storage conditions is crucial. Cryopreservation technology has become a commonly used preservation method due to its effectiveness in reducing cell damage and maintaining microbial viability. Compared to other preservation methods, cryopreservation is simpler to operate and can preserve the biological characteristics of microorganisms for an extended period<sup>[10]</sup>.

In this study, a protease-producing strain, *Priestia megaterium*, was isolated and identified from soil. Its survival rates under different cryopreservation conditions were evaluated, aiming to provide references for industrial and pharmaceutical applications.

## **2. Materials and methods**

### **2.1. Materials and reagents**

Glucose and magnesium sulfate were purchased from Sinopharm Chemical Reagent Co., Ltd. Yeast extract was purchased from Servicebio, and casein was obtained from Biosharp. Skim milk powder (brand: BTNature) originated from New Zealand. Potassium dihydrogen phosphate was purchased from Tianjin Kaitong Chemical Reagent Co., Ltd. Agar was acquired from BioFroxx, and bacterial culture dishes were purchased from Beijing Labgic Technology Co., Ltd.

### **2.2. Instruments and equipment**

Biochemical incubator: Jintan Medical Instrument Factory; constant-temperature shaker incubator: Shanghai Xinmiao Medical Equipment Manufacturing Co., Ltd.; autoclave: Zhiwei (Xiamen) Instrument Co., Ltd.; double-person single-sided clean bench: Suzhou Purification Equipment Co., Ltd.; refrigerator: Hefei Meiling Co., Ltd.

### **2.3. Experimental methods**

#### **2.3.1. Protease identification medium**

The medium contained 0.25% glucose, 0.5% yeast extract, 0.5% casein, 0.5% skim milk powder, 0.1% potassium dihydrogen phosphate, 0.04% magnesium sulfate, and 1.5% agar, prepared with deionized water and adjusted to pH 7.4. The medium was sterilized at 108 °C for 30 min by autoclaving, poured into plates at approximately 60 °C, and used once solidified.

#### **2.3.2. Preparation of bacterial cryopreservation solutions**

Solutions containing 50% glycerol and 50% glycerol + 2% potato starch were prepared with glycerol, potato starch, and deionized water, autoclaved at 108 °C for 30 min before use. Solutions of 24%, 16%, and 8% DMSO were prepared by mixing sterile liquid medium (1% tryptone, 1% NaCl, 0.5% yeast extract, 0.5% glucose, pH 7.4, with deionized water) directly with DMSO without further sterilization. For preparing 16% DMSO solutions with starch, 8.4 mL of liquid medium was placed in Erlenmeyer flasks, mixed with 0.1 g, 0.2 g, or 0.3 g potato starch, sterilized at 108 °C for 30 min, cooled, then mixed thoroughly with 1.6 mL DMSO to obtain 16% DMSO solutions with 1%, 2%, or 3% starch.

#### **2.3.3. Dilution and spread-plate method**

Approximately 1 g of soil samples randomly collected from the field were homogenized in 1 mL of sterile water. A 100 µL suspension was diluted 10-fold repeatedly up to 10<sup>6</sup> times by transferring into 900 µL sterile water. Subsequently, 50 µL aliquots from dilutions of 10<sup>4</sup>, 10<sup>5</sup>, and 10<sup>6</sup> were plated onto solid protease identification medium containing 15–20 mL agar. The samples were evenly spread using sterilized glass spreaders and incubated invertedly at 37 °C in a biochemical incubator for 24–48 h. Colonies were counted, and bacteria capable of protein hydrolysis were isolated by the formation of clear zones. These colonies underwent streak cultivation for single colonies, followed by a second protease screening on identification medium to confirm protease production (clear zone formation). Positive isolates were preserved.

#### **2.3.4. Gram staining**

Microscope slides were cleaned with alcohol and dried. One drop of saline was placed on a slide, inoculated with colonies grown for 48 h via streak cultivation, evenly dispersed, smeared, and gently heated over an alcohol flame to inactivate and fix. One or two drops of crystal violet were applied for 1 min and rinsed gently with deionized water. After slight drying, Lugol's iodine solution was added for 1 min and gently rinsed with deionized water. Decolorization was performed by gently rinsing with 95% alcohol until no more violet color was released, followed by washing with deionized water. After drying, safranin was added to counterstain for 30 sec, gently rinsed again with deionized water, and dried. Microscopic observation and photography were performed using a 100 × oil immersion lens.

### 2.3.5. Spore staining

One to two drops of sterile water were added to a small test tube, inoculated with bacterial colonies using a sterile loop, and mixed thoroughly. Then, 2–3 drops of 5% malachite green solution were added. The test tube was heated in a boiling water bath for 15–20 min. From the bottom of the tube, 2–30 µL bacterial suspension was spread onto a slide, air-dried, and fixed over an alcohol flame. Excess stain was gently rinsed off with deionized water until no green color was released. One drop of safranin was added for counterstaining for 5 min, washed with water, blotted dry with absorbent paper, and air-dried. Spore staining results were examined and photographed under a 100 × oil immersion microscope.

### 2.3.6. Strain identification

#### (1) 16S rDNA sequencing

A single colony of the purified bacterial strain was inoculated into LB liquid medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) and incubated to obtain a pure bacterial culture. The fermentation broth was then sent to Sangon Biotech (Shanghai) Co., Ltd. for 16S rDNA sequencing, using the following primers:

27F 5'-AGAGTTTGATCCTGGCTCAGGATGA-3'

1492R 5'-TACGGCTACCTTGTTACGACTTAGC-3'

#### (2) Homology analysis

The sequencing results were aligned using the Standard Nucleotide BLAST on the NCBI website to assess the homology between the 16S rDNA nucleotide sequence of the strain and those of known bacterial species.

#### (3) Physiological and biochemical tests

Physiological and biochemical tests, including catalase reaction and methylene blue reduction, were carried out according to the methods described in Bergey's Manual of Determinative Bacteriology (8th edition) and the Manual for Systematic Identification of Common Bacteria.

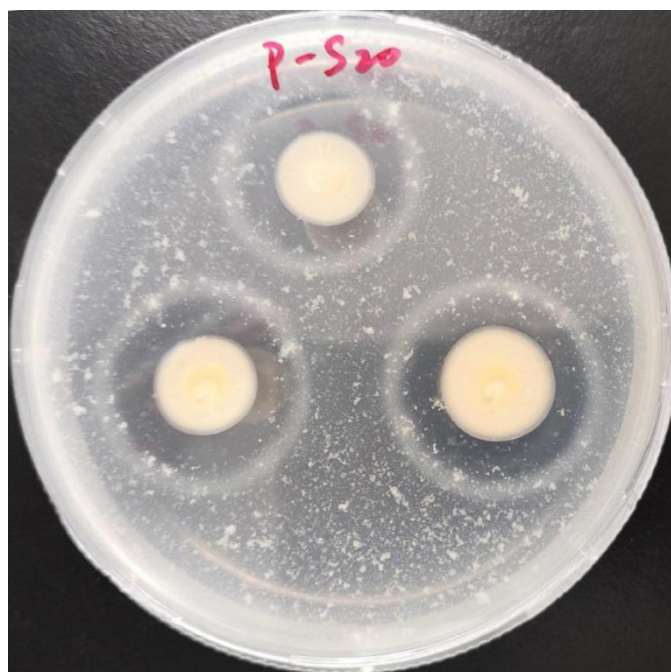
### 2.3.7. Study on cryopreservation conditions

Bacterial strains were retrieved from storage and inoculated into liquid culture medium (containing 1% tryptone, 1% NaCl, 0.5% yeast extract, 0.5% glucose, prepared with deionized water, pH adjusted to 7.4), then incubated at 37 °C for 24 hours with shaking at 200 rpm. Viable counts were obtained by the dilution plate method. Aliquots of bacterial suspension were mixed at a 1:1 ratio with different cryoprotectants including 50% sterile glycerol, 16% DMSO, 50% sterile glycerol plus 2% starch, 24%, 16%, or 8% DMSO alone, or combinations of 16% DMSO plus 3%, 2%, or 1% starch, reaching a final volume of 1 mL. Samples were frozen at −20 °C, and viability was assessed by the dilution plate method after 1 day, 2 days, and 7 days to determine bacterial survival rates.

## 3. Results and analysis

### 3.1. Colony morphology and protein degradation capability

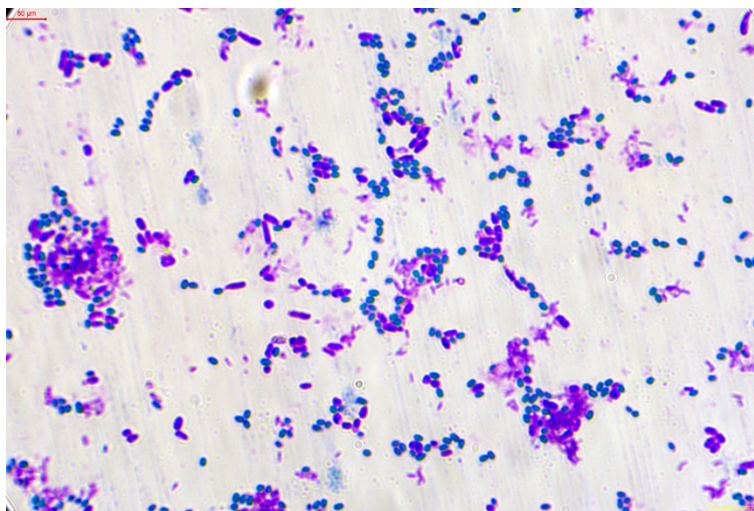
Bacterial isolates from soil samples formed individual colonies on selective media supplemented with casein and skim milk (protein sources). Colonies appeared creamy-yellow in colour, had smooth edges, moist surfaces, and were easily picked. The average colony diameter was 1.55 cm. A clear zone indicating protein degradation formed around the colonies, averaging 2.55 cm in diameter (**Figure 1**). The ratio between the clear zone diameter and colony diameter was 1.65. The bacterial isolate was designated as strain QG-P-S20.



**Figure 1.** Colony morphology and protein degradation transparent circle of bacterial strain QG-P-S20.

### 3.2. Gram staining and spore staining

After Gram staining and observation under an oil immersion lens, strain QG-P-S20 was identified as a Gram-positive bacillus. Spore staining revealed the presence of large central endospores within the bacterial cells (**Figure 2**), rather than terminal spores. The spores appeared green, while the vegetative cells were stained red. These results indicate that QG-P-S20 is a spore-forming bacillus.



**Figure 2.** Spore staining of bacterial strain QG-P-S20.

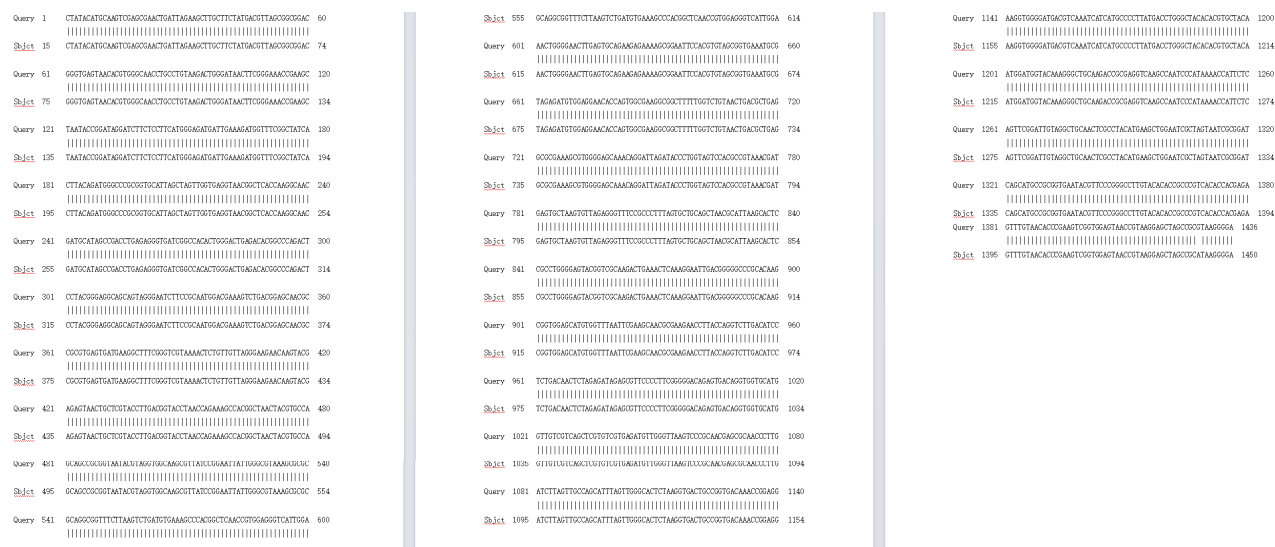
### 3.3. Strain identification

#### 3.3.1. Sequence determination

The nucleotide sequence of the 16S rDNA gene of strain QG-P-S20 was obtained through sequencing, and is as follows:

GGGGGGGGGGCTATACATGCAAGTCGAGCGAACTGATTAGAAGCTTGCTTCTATGACGTTAGCGGCGG  
ACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGGGAAACCGAAGCTAATACCGG





the photograph, the colony on the right displays bubble formation due to the reaction with hydrogen peroxide, whereas the colony on the left, which was not treated with 3% hydrogen peroxide, shows no bubble formation (**Figure 4**).

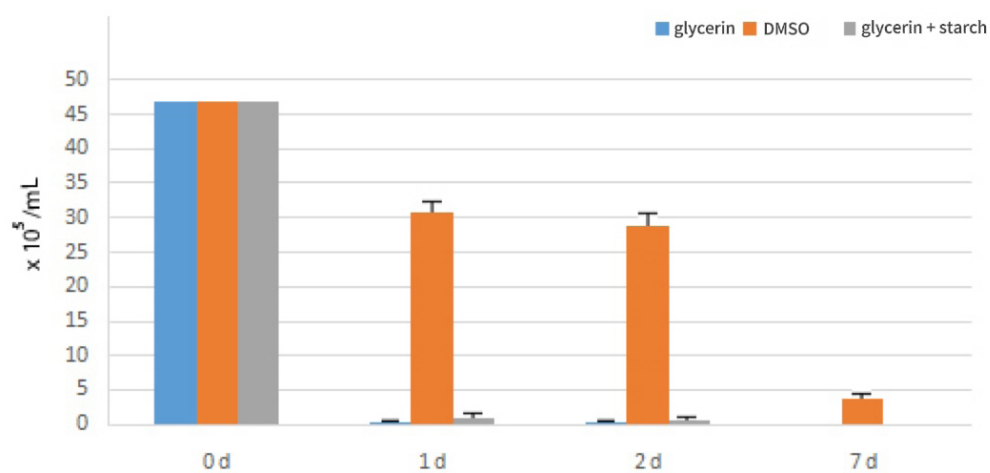


**Figure 4.** Reaction of catalase.

### 3.4. Analysis of cryopreservation conditions

#### 3.4.1. Effect of different chemical reagents on bacterial cryopreservation

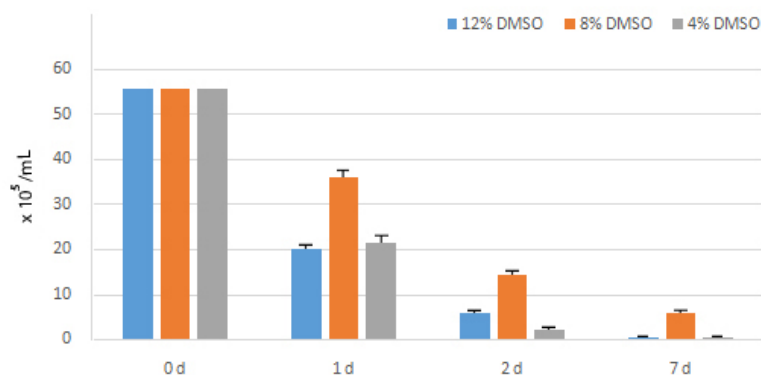
As shown in **Figure 5**, the experimental results indicate that dimethyl sulfoxide (DMSO) provided the best protective effect for the spore-forming bacterium P-S20. After freezing at  $-20^{\circ}\text{C}$  for one day, the survival rate reached 65.5%, and even after one week of storage, 8.1% of the bacterial cells remained viable. In contrast, glycerol and starch offered less effective protection for bacterial cells under the same conditions.



**Figure 5.** Effects of glycerol, dimethyl sulphoxide, and starch on freezing of bacterial strain QG-P-S20.

### 3.4.2. Effect of different DMSO concentrations on bacterial cryopreservation

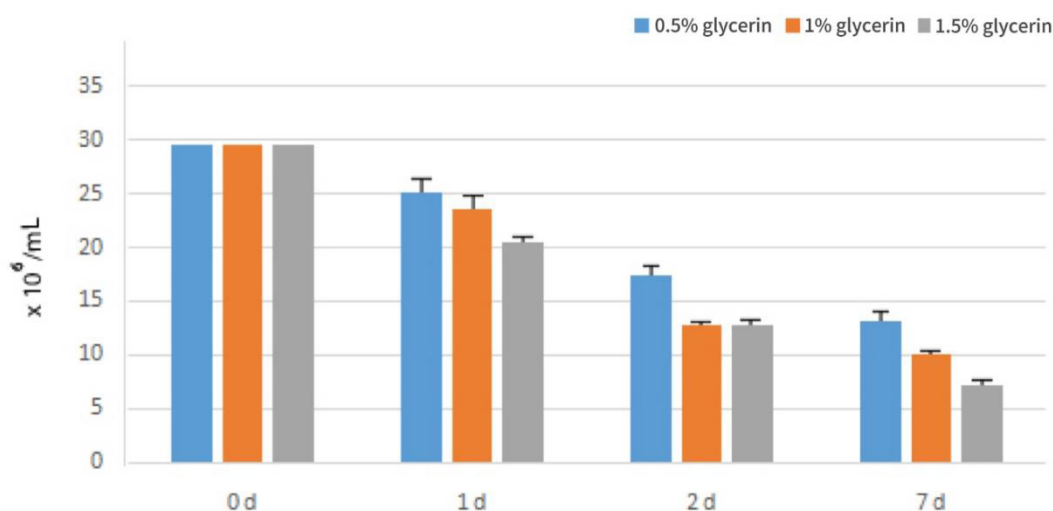
As shown in **Figure 6**, 8% DMSO was sufficient to effectively protect bacterial cells from damage caused by low temperature and freezing. A concentration around 4% provided slightly weaker protection. However, concentrations higher than 8% were detrimental to bacterial viability, as high levels of DMSO can destabilize bacterial cell membranes, leading to cell lysis and death.



**Figure 6.** Effects of different concentrations of DMSO on the freezing of bacterial strain QG-P-S20.

### 3.4.3. Effect of starch on bacterial cryopreservation

At a concentration of 8% DMSO, the addition of starch had a detrimental effect on the cryopreservation of bacterial cells, as shown in **Figure 7**. The presence of starch significantly reduced the survival rate of the bacteria. Therefore, the use of starch or similar substances is not recommended in bacterial cryopreservation solutions.



**Figure 7.** Effects of different concentrations of starch on the freezing of bacterial strain QG-P-S20.

## 4. Discussion and conclusion

In this study, a Gram-positive spore-forming bacterium was isolated from soil. Based on 16S rDNA sequencing and BLAST analysis, the strain was identified as a subspecies of *Priestia megaterium*. Under cryopreservation conditions, 8% DMSO provided the most effective protection for the strain, while glycerol showed a limited protective effect, and starch was detrimental to bacterial survival.



The strain demonstrated efficient casein degradation, indicating high protease activity. By optimizing culture medium composition, fermentation processes, or applying physical, chemical, or biological mutagenesis, as well as genetic engineering approaches, it is possible to obtain strains with enhanced protease production or activity.

This study provides a new perspective for the screening and application of protein-degrading bacteria and lays a foundation for future industrial applications. Further research should explore the performance of this strain under various industrial conditions and conduct large-scale production trials to evaluate its feasibility and economic potential for commercialization.

## Disclosure statement

The author declares no conflict of interest.

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# Gene Editor: Innovative Use of Zebrafish Models in Inflammatory Bowel Disease Research

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**Abstract:** Inflammatory bowel disease (IBD) is a multifactorially induced complex disease, and the most technologically mature animals for IBD disease modelling are mainly mice. However, compared with rodents, zebrafish are more efficient and convenient, with their short growth cycle, high reproductive capacity, small size, light weight, embryonic transparency, and high homology with human intestines, which makes it easy to perform observation of continuous pathological changes and high-throughput drug screening studies. With the development of sequencing technology, gene editing of sterile zebrafish using CRISPR technology plays an important role in revealing in-depth the colony-immune interactions and the pathogenesis of IBD.

**Keywords:** Inflammatory bowel disease; Zebrafish; Animal models; Innovative applications; Gene editing

**Online publication:** June 28, 2025

## 1. Introduction

Inflammatory bowel disease (IBD), a chronic immune disease involving the intestinal tract and comprising two subtypes, ulcerative colitis and Crohn's disease, has been referred to as the "green cancer" because of its recurrent, incurable, and disabling features<sup>[1]</sup>. Although the pathogenesis of IBD has not been fully elucidated, genetic susceptibility, immune dysregulation, and flora interactions are the main contributing factors. Animal models play a key role in resolving IBD pathogenesis and drug development, but traditional mouse models (e.g., TNBS, DSS-induced models) have limitations such as large differences in immune systems, high mortality rates, and difficulties in gene editing, which make it difficult to satisfy the demand for dynamic observation of chronic intestinal mucosal injury. Zebrafish is becoming a novel model organism for IBD research under its advantages of embryonic transparency, rapid intestinal development (completion of intestinal system construction 5 days after fertilization), and 75% homologous genes with humans. The highly conserved nature of its intestinal structure and immune system (key pathways such as TLR4/NF- $\kappa$ B are homologous to humans), combined with the specific gene mutation model constructed by CRISPR technology, can accurately simulate the pathological process of IBD. Through real-time *in vivo* imaging, researchers can visualize the dynamic changes of inflammatory factors and the migration of immune cells, providing a unique platform for analyzing the pathogenesis of IBD and high-throughput drug screening. In this paper, we systematically describe the innovative application strategies of the zebrafish model in IBD research, which provides a basis for the selection of model organisms at different stages of research.

## 2. Homology analysis of the zebrafish and human intestinal tracts

Zebrafish and humans belong to the same vertebrate class, and their growth and development processes and the structure of the tissue systems and humans have a high degree of similarity, and the homology of genes with humans is more than 70%. The intestinal tract of zebrafish is located at the bottom of the abdominal cavity, and its structure is similar to that of human beings, divided into three sections, namely, the foregut, the midgut and the hindgut, and the intestinal wall is composed of mucous, muscular and plasma membranes from the inside to the outside, respectively. Under light and electron microscopy, it can be observed that the zebrafish intestinal mucosa has a well-developed and obvious mechanical barrier, chemical barrier, and immune barrier, which together form the structural basis of the zebrafish intestinal mucosal barrier system<sup>[2]</sup>. For example, the presence of tight junction proteins distributed in bands between zebrafish intestinal mucosal epithelial cells fuses adjacent cell membranes, closes cellular gaps, and is part of the mechanical barrier of zebrafish intestinal tissues. The abundance of cup cells in the zebrafish intestinal mucosal layer suggests that they may secrete immune-related factors involved in the intestinal mucosal immune barrier, and zebrafish have immune cells such as lymphocytes, macrophages, granulocytes, and dendritic cells<sup>[3]</sup>. The presence of these structures in its body proves its high scientific value as a research model for human intestinal system diseases.

Among the genes homologous to humans in zebrafish, there are many genes related to IBD, such as autophagy genes and genes related to inflammatory signaling pathways. Autophagy, as one of the adaptive catabolic and energy-generating pathways, is a process of self-degradation and recycling of damaged biomolecules and cytoplasmic organelles in cells, and plays an important role in inflammatory bowel disease. And autophagy-related genes such as ATG10 are highly conserved from yeast to human<sup>[4]</sup>.

## 3. CRISPR technology to construct zebrafish IBD-related gene mutation models

Based on the breakthrough of zebrafish genome sequencing technology and the deepening of functional genomics research, researchers have systematically established a genetic engineering model system for zebrafish inflammatory bowel disease (IBD). Different from the traditional chemical-induced model, the gene editing model can more realistically simulate the molecular pathogenesis of IBD by precisely regulating the expression of specific genes. Hwang *et al.*<sup>[5]</sup> successfully applied CRISPR/Cas9 technology in zebrafish for the first time, which optimized the composite system of microinjection of Cas9 mRNA and single-stranded guide RNA (sgRNA) to make the gene insertion/deletion/deletion. system, resulting in an insertion/deletion (indel) mutation efficiency of  $78.6 \pm 5.2\%$ . Follow-up studies confirmed that when the Cas9:sgRNA ratio was controlled in the 1:2 to 1:4 interval, the off-target effect could be significantly reduced while maintaining an effective editing rate of more than 92%<sup>[6]</sup>.

In terms of technological innovation, by fusing mCherry fluorescently labeled Cas9 protein with biotin-modified sgRNA at the 5' end (Biotin-TEG-sgRNA), the researchers realized two-color fluorescence in situ tracing of endogenous gene transcription products (**Figure 1**). This technological breakthrough enabled the mRNA dynamic monitoring cycle to be shortened from the conventional 72 hours to 24 hours, which greatly enhanced the experimental throughput. In the field of vector design, the CRISPR/Cas9 expression system constructed based on tissue-specific promoters (e.g., the intestinal epithelial-specific *fabp2* promoter) can realize spatiotemporal-specific editing targeting intestinal cells, with a cell-selectivity index (CSI) of 9.3–12.6, which is significantly better than the conventional pan-expression system<sup>[7]</sup>.

A study using CRISPR/Cas9 gene editing technology to construct an ATG10 gene mutation model against the zebrafish autophagy gene showed that ATG10 gene defects led to intestinal barrier breakdown in zebrafish, with a decrease in mucus and an increase in apoptosis of epithelial cells. In addition, when inflammatory factors were detected using enzyme immunoassay, it was found that the expression levels of tumor necrosis factor (TNF- $\alpha$ ) and interleukin (IL- $\beta$ ) in the intestinal tract of zebrafish with mutation of ATG10 gene were abnormal, which indicated that there was a close correlation between ATG10 gene and intestinal inflammation.

## 4. Application of gene-edited zebrafish in colony-immunity interaction studies

In recent years, with the establishment of the sterile zebrafish model and the in-depth integration of gene editing technology, researchers have been able to systematically analyze the regulation mechanism of the immune system by bacterial colonization at the molecular, cellular and individual levels. The construction of the sterile zebrafish model serves as the basis for the study of colony-host interactions, and its core technologies include the decontamination of embryos, the maintenance of aseptic culture systems, and the precise implementation of bacterial colonization experiments. By treating the embryo surface with antibiotics and adopting ultraviolet sterilization technology, combined with the confined environment of a sterile isolator, aseptic culture of zebrafish from embryo to adult stage can be realized. Some studies have now revealed the direct role of certain bacterial species for zebrafish in the gut. For example, the mucin-degrading bacterium *Akkermansia muciniphila* can be directly involved in the regulation of host barrier function and immune response, and can repair broken intestinal barriers to a certain extent, suggesting that it may be a potential target for the treatment of inflammatory bowel disease<sup>[8]</sup>. This also reveals the key regulatory role of the flora on immune development.

In bacterial colonization experiments, researchers can use single-strain colonization or colony transplantation strategies. For example, colonization of sterile transgenic zebrafish with a commensal microbiota activated NF- $\kappa$ B and led to upregulation of its target genes in the GI tract and extraintestinal tissues<sup>[9]</sup>. The spatial distribution of bacteria in the gut can be visually tracked by colonizing sterile zebrafish with single bacteria of *Escherichia coli* labeled with green fluorescent protein (GFP). This controlled colonization strategy provides a unique model for resolving the causal relationship between colony composition and immune phenotype.

The combination of the unique transparent properties of zebrafish embryos and fluorescent labeling technology has enabled real-time visualization of colony-host interactions. Confocal microscopy and light-sheet fluorescence microscopy allow researchers to dynamically track bacterial colonization processes in three dimensions. For example, using transgenic zebrafish strains, e.g., TgBAC (mpx:GFP) labeled neutrophils, the dynamics of immune cell recruitment after colonization of a specific strain can be observed in real time<sup>[10]</sup>.

Zebrafish gene editing technology has been shown to be effective in achieving gene knockouts, and there are several transgenic zebrafish lines available that can be used to study the interactions between the gut microbiota-gut-brain axis. It has been shown that zebrafish and mammals have similar host responses to gut colonization by microbiota, and subsequent realization of some of the zebrafish research results in humans has become possible.

## 5. Translational medicine value and challenges of zebrafish modelling

With its short growth cycle, strong fecundity and miniaturization, zebrafish provides a unique advantage for building a high-throughput drug screening platform. The researchers utilized their large-scale culture system, combined with automated microscopic imaging technology, to significantly improve the efficiency of initial screening of anti-inflammatory drugs, and to establish the foundation of compound libraries for IBD therapeutic research and development.

In terms of disease model construction, the zebrafish gene editing system (microinjection completed within 6 hours after fertilization) enables rapid recapitulation of human disease-causing mutations. For example, in the neuroblastoma model, the outgrowth rate of MYCN/ALK double transgenic lines was elevated to 83% (WT < 5%) and the response threshold to the third-generation ALK inhibitor, Lorlatinib, was reduced by 10-fold (IC<sub>50</sub> = 12 nM vs. 120 nM)<sup>[11]</sup>, which establishes an accurate prediction system for preclinical drug efficacy assessment.

Although CRISPR-Cas9 technology has achieved more than 85% germline editing efficiency, its application still faces challenges: the stochastic nature of homologous recombination repair (HDR) efficiency constrains the success rate of precise point mutations; the aseptic feeding system needs to continuously maintain a positive-pressure isolation environment with a 0.22  $\mu$ m membrane, which accounts for 23–35% of the total laboratory budget; the potential ecological risks of gene-drive technology need to be controlled by physical/biological containment strategies; and the potential ecological risks of gene-drive technology need to be controlled by physical/biological containment strategies. The potential



ecological risks of the gene drive technology need to be controlled by a dual physical/biological containment strategy.

## 6. Conclusion

The unique biological characteristics of zebrafish, such as small size, strong reproductive ability, rapid development, short growth cycle, good optical transparency, and high homology with human genes, make zebrafish an ideal animal model for the study of the pathogenesis and treatment of IBD, which is of high application value in high-throughput drug screening, disease modeling, and basic biological research. The application of gene editing technology in the construction of zebrafish disease models enables researchers to precisely manipulate target genes and monitor the onset and progression of IBD and its regulation in vivo in real time, providing scientists with a dynamic and efficient experimental platform. In addition, the integration of multidisciplinary technologies provides unprecedented opportunities for IBD research. The integration of bioinformatics, systems biology, materials science, and clinical medicine not only promotes the development of precision therapies for IBD, but also provides multidimensional support for the development of individualized intervention strategies. Through the integrated application of technologies such as big data analysis, high-throughput screening and precision medicine, researchers can more accurately identify the disease subtypes of IBD patients and design personalized disease models for specific patient groups in order to find the best treatment options.

## Disclosure statement

The author declares no conflict of interest.

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# Research Progress on the Mechanisms of Drug Resistance in Common Clinical Microorganisms

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**Abstract:** The popularity of antibacterial drugs has made microbial drug resistance a thorny problem in global public health. In clinical practice, the phenomenon of resistance to antibacterial drugs by common microorganisms, such as bacteria and fungi, is becoming increasingly common. This article focuses on the main molecular mechanisms by which these microorganisms develop drug resistance. It conducts in-depth analyses from multiple aspects such as changes in drug targets, overexpression by drug efflux pumps, horizontal transfer of drug resistance genes, and biofilm formation. Based on the review of the latest progress in drug resistance research, it explores future research directions. It is hoped that it can provide a practical and feasible theoretical basis for clinical anti-infection treatment and prevention and control of drug resistance.

**Keywords:** Microorganisms; Drug resistance mechanism; Antibacterial drugs; Research progress

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## 1. Introduction

Microbial drug resistance refers to the phenomenon where microorganisms develop resistance to originally effective antibacterial drugs. In recent years, due to the irrational use of antibacterial drugs, multidrug-resistant strains and “superbugs” have emerged continuously, posing extremely severe challenges to clinical anti-infection treatment<sup>[1]</sup>. The World Health Organization has listed antibiotic resistance as one of the top ten public health threats worldwide<sup>[2]</sup>. In-depth research on the mechanism of microbial drug resistance is of great significance for developing new antibacterial drugs and optimizing existing treatment regimens<sup>[3–5]</sup>. Microorganisms are widely distributed in nature and come in a variety of types, including bacteria, fungi, viruses etc. In the long-term survival competition, microorganisms have been constantly evolving to adapt to environmental changes, and developing resistance to antibacterial drugs is one of their adaptation strategies<sup>[6]</sup>. Only by understanding the formation mechanism of microbial drug resistance can we formulate more effective prevention and control measures and improve the success rate of clinical anti-infection treatment.

## 2. The main mechanism of microbial drug resistance

### 2.1. Changes in the target sites of antibacterial drugs

Microorganisms often cause structural changes in drug targets through pathways such as gene mutations or gene transfer,

thereby reducing the binding ability of drugs to the targets. Take *Staphylococcus aureus* as an example. It can change the structure of penicillin-binding protein (PBP), thereby developing resistance to  $\beta$ -lactam drugs <sup>[7]</sup>. Under normal circumstances,  $\beta$ -lactam drugs can specifically bind to PBP, inhibit the synthesis of bacterial cell walls, and thereby exert a bactericidal effect. However, when the PBP structure of *Staphylococcus aureus* changes,  $\beta$ -lactam drugs cannot effectively bind to it, allowing the bacteria to continue synthesizing the cell wall and thereby developing drug resistance.

## **2.2. Production of drug-degrading or modified enzymes**

Microorganisms can secrete specific enzymes to degrade or modify antibacterial drugs, rendering them inactive.  $\beta$ -lactamase can hydrolyze the  $\beta$ -lactam ring of  $\beta$  lactam antibiotics, which is the main reason why Gram-negative bacteria are resistant to penicillin drugs. The antibacterial activity of  $\beta$ -lactam antibiotics depends on the integrity of the  $\beta$ -lactam ring. After the  $\beta$ -lactamase hydrolyzes the  $\beta$ -lactam ring, the drug loses its antibacterial activity, and Gram-negative bacteria develop resistance to penicillin drugs. Carbapenase can hydrolyze carbapenem drugs. Carbapenem drugs are a kind of broad-spectrum and highly efficient antibacterial drugs, which have good antibacterial activity against a variety of drug-resistant bacteria. However, with the emergence of carbapenase, the resistance of bacteria to carbapenem drugs has gradually increased, bringing great difficulties to clinical treatment.

## **2.3. Drug efflux pump system**

Microorganisms can actively excrete drugs out of cells by overexpressing efflux pump proteins. The AcrAB-TolC system in Gram-negative bacteria can excrete various antibiotics <sup>[8]</sup>. This system is composed of the inner membrane protein AcrB, the outer membrane protein TolC and the membrane fusion protein AcrA. They work synergistically to pump various antibiotics that enter the bacterial cells out of the cells, reduce the intracellular drug concentration, and cause the bacteria to develop drug resistance.

## **2.4. Changes in cell membrane permeability**

Microorganisms can reduce drug influx by altering the expression or structure of membrane pore proteins. The deletion of the ompK35/36 well protein in *Klebsiella pneumoniae* can lead to its resistance to carbapenem drugs. The ompK35/36 well protein is an important channel on the outer membrane of *Klebsiella pneumoniae*. Carbapenem drugs enter the bacterial cells through the well protein to exert antibacterial effects <sup>[9]</sup>. When ompK35/36 well proteins are missing, carbapenem drugs have difficulty entering cells, and bacteria develop resistance to them.

## **2.5. Biofilm formation**

Microorganisms secrete extracellular polymers to form biofilms with multiple protective effects. Biofilms, as physical barriers, can restrict the penetration of drugs. The extracellular polymers in the biofilm form a complex network structure. When drug molecules pass through the biofilm, they are hindered and have difficulty reaching the surface of bacterial cells, thereby reducing the bactericidal effect of the drugs. The cell metabolism within the biofilm is slow, and the sensitivity to antibacterial drugs decreases <sup>[10]</sup>. Within the biofilm, the growth rate and metabolic activity of bacteria are relatively low, and they are in a relatively dormant state. Bacteria in this state have a decreased sensitivity to many antibacterial drugs because antibacterial drugs are usually more effective against bacteria that are growing vigorously and metabolically active.

## **2.6. Horizontal transfer of drug resistance genes**

Microorganisms spread drug resistance genes through mobile genetic elements such as plasmids and transposons. Plasmid-mediated drug resistance gene transfer is the main route of drug resistance transmission among Gram-negative bacteria. Plasmids are circular double-stranded DNA molecules that can replicate autonomously and carry multiple drug resistance genes. Gram-negative bacteria can transfer plasmids to other bacteria through conjugation, transformation and other



means, enabling the recipient bacteria to acquire drug resistance.

### **2.7. Activation of metabolic bypass**

Microorganisms can bypass the sites of drug action by altering metabolic pathways. Sulfonamide-resistant bacteria obtain folic acid through exogenous sources. The mechanism of action of sulfonamide drugs is to inhibit the synthesis of folic acid in bacteria. However, sulfonamide-resistant bacteria can take up ready-made folic acid from the environment, thereby bypassing the effect of sulfonamide drugs and developing resistance to them. Some bacteria can activate alternative enzyme systems to maintain basic life activities when antibacterial drugs inhibit their normal enzyme systems<sup>[11]</sup>. In this case, the bacteria continue their metabolic activities to survive, thereby demonstrating resistance to the antibacterial drug. This metabolic bypass activation mechanism enables bacteria to adapt to the environment by adjusting metabolic pathways when facing the stress of antibacterial drugs, thereby enhancing their survival ability.

## **3. Research progress on special drug resistance mechanisms**

### **3.1. Post-translational modification of proteins and drug resistance**

In recent years, the key role of post-translational modifications of proteins in the formation of drug resistance has gradually attracted academic attention. Take *Escherichia coli* as an example. YjgM crotonyltransferase can modify the transcription factor PmrA, thereby up-regulating the lipopolysaccharide modification gene, causing the bacteria to develop resistance to polymyxin. Under normal conditions, the expression regulation of lipopolysaccharide modification genes by PmrA is maintained at a specific level. However, when YjgM crotonyltransferase acts on PmrA, the activity of PmrA changes and can bind more tightly to the promoter regions of the phosphoethylamine transferase EptA and the glycosyltransferase PmrK/ArnT genes, promoting the expression of these genes. This series of changes eventually led to an increase in the lipid A modification level of the bacterial outer membrane lipopolysaccharide (LPS), a change in the outer membrane structure, making it difficult for polymyxin to bind to it, thereby generating resistance.

### **3.2. The regulatory effect of synonymous mutations on drug resistance**

For a long time, the traditional view holds that synonymous mutations do not affect protein functions. However, the latest research has found<sup>[12]</sup> that synonymous mutations of the *hisD* gene (522 G > A and 972 C > T) can regulate bacterial resistance to fluoroquinolone drugs by affecting the translation rate and protein conformation. Under normal circumstances, the expression products of the *hisD* gene play an important role in the bacterial metabolic process and are closely related to the resistance of fluoroquinolone antibiotics<sup>[13–15]</sup>. When the *hisD* gene undergoes the above synonymous mutations, although the amino acid sequence of the encoded protein remains unchanged, the secondary structure of the mRNA may change, affecting the movement speed of the ribosome on the mRNA, that is, the translation rate. The change in translation rate further affects the folding process of proteins, resulting in conformational changes of HisD proteins. After this conformational change, the HisD protein loses its normal regulatory function for downstream genes, such as being unable to up-regulate the expression of *sbmC* and *umuD* genes, thereby affecting the mutation frequency of the *gyrA* gene and ultimately altering the bacteria's resistance to fluoroquinolone antibiotics.

### **3.3. Interaction between bacteriophages and bacterial drug resistance**

There is a potential association between the defense mechanism of bacteria against bacteriophages and antibiotic resistance. Bacteria mainly resist phage infection through the CRISPR-Cas system, restriction modification system, etc. Among them, the CRISPR-Cas system, as an important component of bacterial adaptive immunity, is capable of recognizing and cutting the DNA of invading bacteriophages to protect bacteria from infection. The restriction modification system resists phage invasion by methylating its own DNA and simultaneously recognizing and cutting unmethylated exogenous DNA, including phage DNA. On the other hand, bacteriophages have also evolved corresponding strategies in order to

successfully infect bacteria. Some bacteriophages can encode specific antagonistic proteins, which can act on the defense proteins of bacteria. For instance, by phosphorylation modification, the activity of bacterial defense proteins can be altered, interfering with the defense mechanisms of bacteria, thereby enabling bacteriophages to successfully infect bacteria<sup>[16]</sup>. During this process, the physiological state of bacteria may change, thereby affecting their sensitivity to antibiotics.

#### **4. Prospects of new anti-drug resistance strategies**

Researchers are actively exploring effective strategies to deal with the drug resistance situation. Given its increasingly severe current situation, these explorations are extremely urgent. In the field of new antibiotic research and development, the lasso peptide antibiotic lariatocidin has demonstrated unique advantages. It acts on brand-new sites on the ribosome that have not yet been reached by traditional antibiotics, making it difficult for bacteria to rapidly develop resistance to them through common genetic mutation methods. By interfering with the synthesis process of bacterial proteins, lariatocidin has opened up a new path for the clinical treatment of drug-resistant bacterial infections, effectively compensating for the frequent occurrence of bacterial resistance caused by the extensive use of traditional antibiotics.

Phage therapy is to treat infections by taking advantage of the specific infection characteristics of phages against drug-resistant bacteria. Because bacteriophages have a high degree of host specificity, they can precisely identify and invade specific types of bacteria. During the treatment process, bacteriophages can directly attack drug-resistant bacteria and achieve the therapeutic goal by lysing bacterial cells. Research has found<sup>[17]</sup> that some antagonistic proteins encoded by bacteriophages can interfere with the defense mechanism of bacteria and enhance the infection ability of bacteriophages. The combined use of these antagonistic proteins with bacteriophages or the expression of antagonistic proteins in bacteriophages through genetic engineering modification is expected to further enhance the therapeutic effect<sup>[18]</sup>.

The combination medication strategy is a multi-target treatment plan designed for the complex and diverse drug resistance mechanisms of microorganisms. A single drug is often difficult to deal with complex drug resistance situations. However, the combined use of antibacterial drugs that act on different targets can simultaneously target multiple drug resistance mechanisms of bacteria and reduce the possibility of bacteria developing drug resistance. For instance, the combined use of drugs that inhibit the synthesis of bacterial cell walls and those that inhibit protein synthesis can attack bacteria from multiple perspectives, significantly enhancing the bactericidal effect.

#### **5. Conclusion**

The mechanism of microbial drug resistance is extremely complex and diverse in form. Different mechanisms often collaborate and act together, thereby triggering a high level of drug resistance. In recent years, with continuous breakthroughs in disciplines such as omics technology, structural biology, and synthetic biology, our understanding of drug resistance mechanisms has also been continuously deepened. In future research, the deep integration of basic research and clinical application is extremely urgent. Basic research should continuously explore the brand-new mechanisms of microbial resistance to lay a solid theoretical foundation for the development of new anti-infective drugs and strategies. Clinical application requires the timely transformation of basic research results into practical and feasible treatment plans, and the verification and optimization of new therapies through clinical practice. Meanwhile, regulating the use of antibacterial drugs is of great significance. Relevant departments should strengthen the supervision of the use of antibacterial drugs, enhance the awareness of clinical doctors and the public to use antibacterial drugs rationally, reduce unnecessary medication behaviors, and prevent the emergence and spread of drug resistance from the root. Only by taking multiple measures and jointly addressing the challenge of drug resistance can the continuous research on the mechanism of microbial drug resistance lay a solid foundation for clinical anti-infective treatment, which is of immeasurable value for ensuring public health security.

## Disclosure statement

The author declares no conflict of interest.

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# Transcriptome Sequencing Analysis of Nasopharyngeal Carcinoma 5-8F Cells Induced by Timosaponin A-III

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**Abstract:** *Objective:* To explore the differential gene expression of nasopharyngeal carcinoma cells induced by Timosaponin A-III and conduct biological information analysis. *Methods:* Total RNA was extracted from nasopharyngeal carcinoma cells treated with Timosaponin A-III for transcriptome sequencing using Illumina HiSeq 2500. Differentially expressed genes (DEGs) were screened by DESeq2, and their functions and signaling pathways were explored using GO enrichment analysis and KEGG pathway analysis. *Results:* Differential gene expression analysis revealed 3858 DEGs, including 1077 upregulated and 2781 downregulated genes. The heatmap and volcano plot showed significant changes in the gene expression profile. GO analysis indicated that the DEGs were enriched in biological processes such as regulation of apoptosis, autophagy, lipid metabolism, and redox processes, involving cellular components like cell membrane, cytoplasm, and mitochondria, as well as molecular functions including protein binding and protein kinase activity. KEGG pathway analysis revealed significant enrichment in signaling pathways such as apoptosis, p53, and TNF. *Discussion:* Timosaponin A-III may exert antitumor effects by activating apoptosis pathways and the p53 pathway. The interaction of these pathways mediates the inhibitory effect on nasopharyngeal carcinoma cells. *Conclusion:* This study reveals that Timosaponin A-III inhibits nasopharyngeal carcinoma cells through multi-gene and multi-pathway regulation, providing a theoretical basis for further experiments.

**Keywords:** Nasopharyngeal carcinoma; Timosaponin A-III; Transcriptome sequencing

**Online publication:** June 28, 2025

## 1. Research background and significance

Nasopharyngeal carcinoma (NPC) is a malignant tumor originating from the epithelial cells of the nasopharynx, closely related to the Epstein-Barr virus (EBV) <sup>[1]</sup>. It has significant regional clustering and a higher incidence rate in southern China, such as the Guangdong and Guangxi regions <sup>[2]</sup>. Due to its concealed early symptoms, about 70% of patients have already developed lymph node or distant metastasis at the time of diagnosis, posing significant challenges to treatment and seriously threatening patients' lives and health. There is an urgent need to analyze its metastasis mechanism and develop targeted treatment options in clinical practice <sup>[3]</sup>. Currently, the treatment of nasopharyngeal carcinoma mainly includes radiotherapy, chemotherapy, and surgical treatment. However, these treatment methods have certain limitations.



For example, radiotherapy may cause radiation injury, chemotherapy may cause severe adverse reactions, and surgical treatment is limited by tumor location and staging<sup>[4]</sup>. Therefore, finding new treatment methods and drugs has become an important direction in nasopharyngeal carcinoma research. In this study, we used the nasopharyngeal carcinoma cell line 5-8F as the research object and employed transcriptome sequencing technology to explore the changes in gene expression profiles of 5-8F cells induced by Timosaponin A-III. We screened differentially expressed genes and analyzed their functions, aiming to lay a foundation for understanding the antitumor mechanism of Timosaponin A-III and provide a theoretical basis for developing new drugs and methods for the treatment of nasopharyngeal carcinoma.

## 2. Materials and methods

### 2.1. Materials

The human highly metastatic nasopharyngeal carcinoma cell line 5-8F (purchased from Shanghai Fuheng Biotechnology Co., Ltd.) was isolated from lung metastases of a 58-year-old Chinese male patient with poorly differentiated squamous cell carcinoma of the nasopharynx in 1980. It was subcloned through passage in nude mice to obtain a highly metastatic subclone. ATCC standardized cell resources and supporting experimental protocols were provided to ensure the stability of cell quality and characteristics. Timosaponin A-III (purity  $\geq 98\%$ , CAS number: 41059-79-4) was purchased from Chengdu Desite Biotechnology Co., Ltd., with a molecular formula of  $C_{39}H_{64}O_{13}$  and a molecular weight of 740.9177. RPMI 1640 medium (Gibco, USA), high-quality fetal bovine serum (FBS, Gibco, USA), 0.25% trypsin-EDTA digestion solution (Gibco, USA), TRIzol reagent (Invitrogen, USA), RNAsimple Total RNA kit (Tiangen Biotech Co., Ltd.), PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa Bioengineering Co., Ltd.), and NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, USA) were used. Equipment included a CO<sub>2</sub> incubator (Thermo Fisher Scientific, USA), a clean bench (Suzhou Purification Equipment Co., Ltd.), a high-speed frozen centrifuge (Eppendorf, Germany), a microplate reader (Bio-Rad, USA), a real-time fluorescent quantitative PCR instrument (Applied Biosystems, USA), and an Illumina HiSeq 2500 sequencing platform (Illumina, USA).

### 2.2. Cell culture

The 5-8F cells were thawed and inoculated into a T25 flask containing RPMI 1640 complete medium (with 10% FBS) and cultured in a 37°C, 5% CO<sub>2</sub> incubator. When the cell density reached 80–90%, they were digested with 0.25% trypsin-EDTA for 2–3 minutes and passaged at a ratio of 1:3 to 1:5, twice a week. The experiment was divided into a control group and a Timosaponin A-III treatment group. The treatment group was treated with 7.5  $\mu$ M Timosaponin A-III solution for 24 hours, while the control group received an equal amount of DMSO solvent. The experiment was repeated three times.

### 2.3. Transcriptome sequencing analysis

Total RNA was extracted from cells using TRIzol reagent and further purified using the RNAsimple Total RNA kit to remove impurities and genomic DNA contamination. RNA concentration was determined using a Nanodrop 2000 spectrophotometer, ensuring an A<sub>260</sub>/A<sub>280</sub> ratio between 1.8 and 2.2. RNA integrity was checked using an Agilent 2100 Bioanalyzer, and RNA samples with a RIN value greater than 7.0 were used for subsequent experiments.

mRNA was reverse transcribed into cDNA using the PrimeScript RT reagent Kit with gDNA Eraser. Library construction was then performed using the NEBNext Ultra RNA Library Prep Kit for Illumina, including steps such as end repair, A-tailing, ligation of sequencing adapters, and PCR amplification. The constructed libraries were quantified using a Qubit 2.0 fluorometer, and the fragment size distribution was checked using an Agilent 2100 Bioanalyzer to ensure library quality.

Qualified libraries were sequenced on the Illumina HiSeq 2500 platform with paired-end 150 bp reads. During sequencing, standard operating procedures were followed, and sequencing data quality was monitored in real-time to ensure accurate and reliable data output. Raw sequencing data were obtained.

## 2.4. Differential gene screening and analysis

Gene expression levels were calculated using StringTie software and normalized using the FPKM (Fragments Per Kilobase of exon per Million reads mapped) method to obtain a gene expression matrix. Differential gene screening was performed using DESeq2 software, with a threshold of  $|\log_2FC| \geq 1$  and  $\text{padj} < 0.05$  to determine differentially expressed genes between the Timosaponin A-III treatment group and the control group. GO (Gene Ontology) enrichment analysis and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis were performed on the differentially expressed genes using the clusterProfiler package.

## 3. Results

### 3.1. Analysis of differentially expressed genes in the transcriptome

Transcriptome sequencing yielded a total of 45,435,638 and 46,317,472 reads for the control group and the treatment group, respectively. After filtering, the proportions of clean reads were 97.18% and 97.03%, respectively. A total of 58,825 genes were detected in this transcriptome sequencing. According to the differential expression screening criteria, 3,858 differentially expressed genes were obtained, including 1,077 upregulated genes and 2,781 downregulated genes. Heatmaps (Figure 1A) and volcano plots (Figure 1B) are shown in Figure 1.

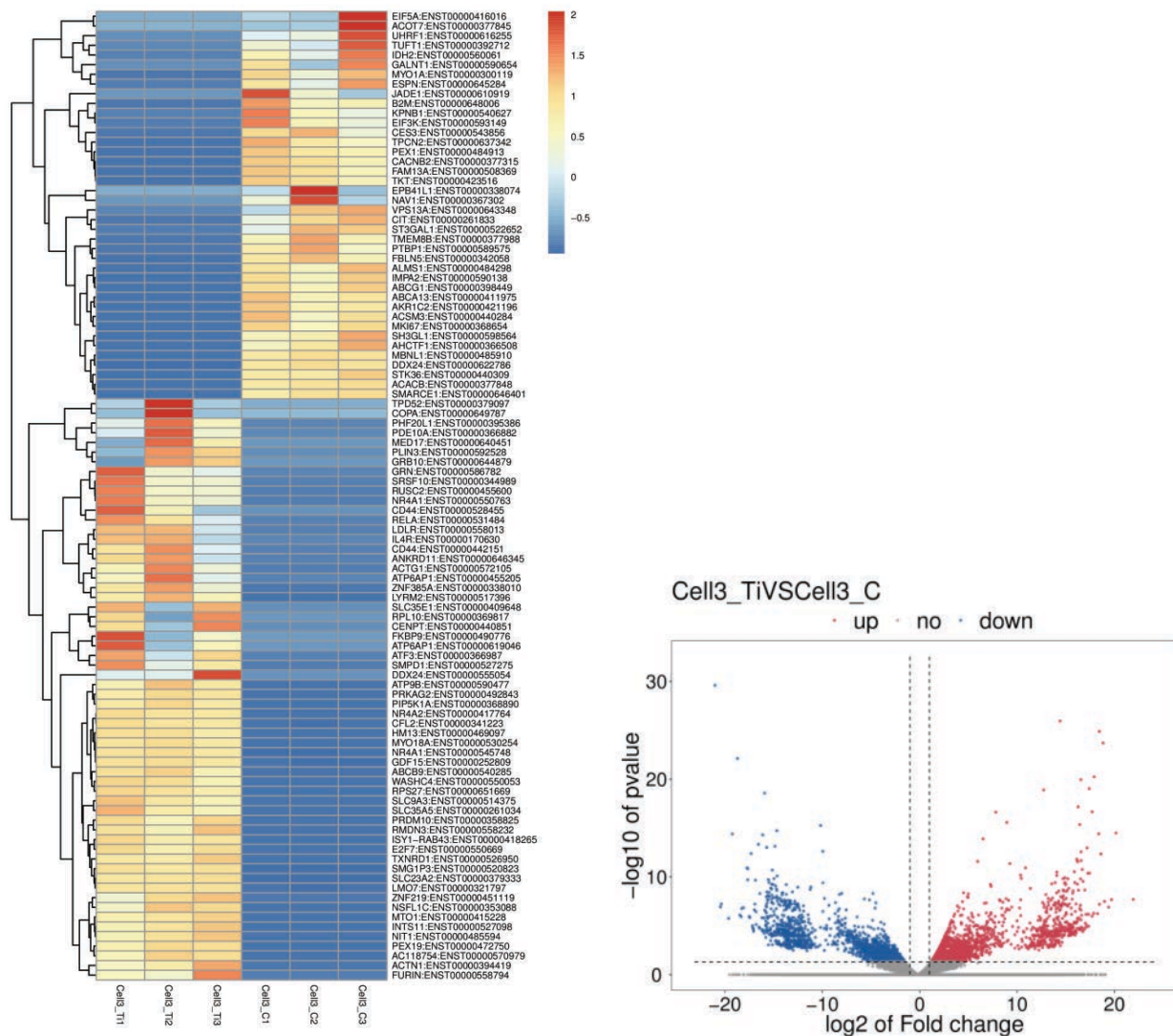
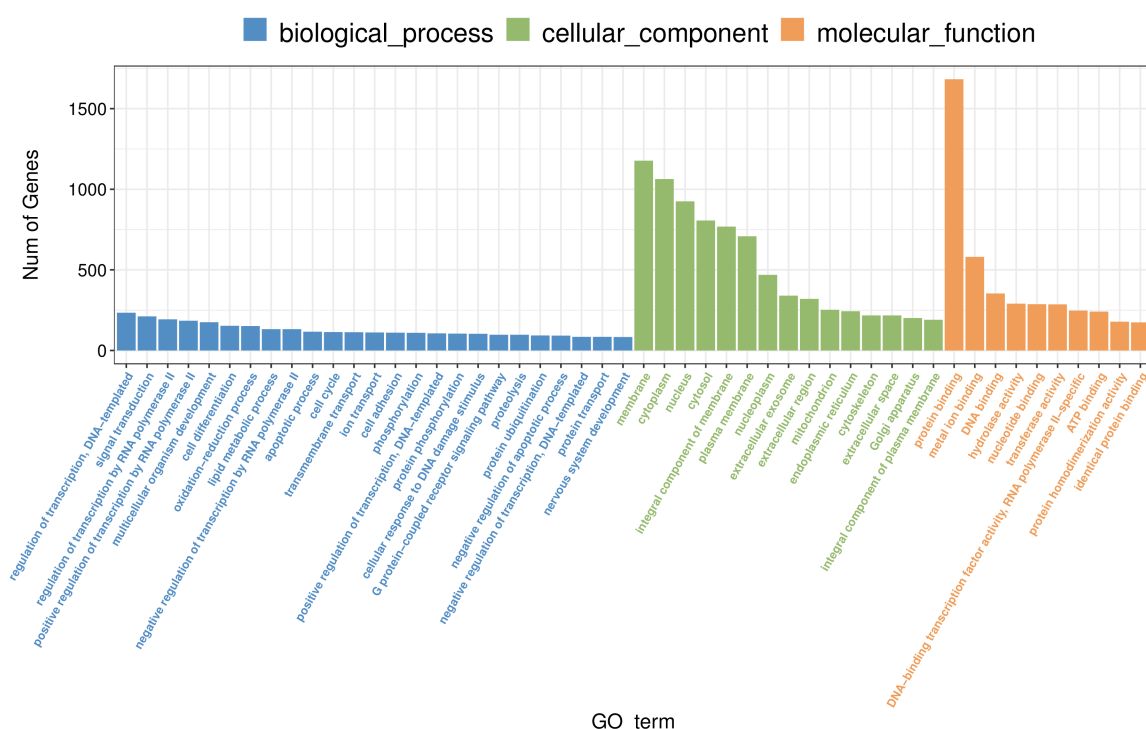


Figure 1. Heatmap (1A) and Volcano plot (1B) of differentially expressed genes.

### 3.2. Functional enrichment analysis of genes

To gain a deeper understanding of the functions of differentially expressed genes, GO enrichment analysis and KEGG pathway enrichment analysis were performed (**Figure 2** and **Figure 3**). The results of GO enrichment analysis showed that in biological processes, the differentially expressed genes were mainly enriched in biological processes such as regulation of apoptosis, autophagy, lipid metabolic process, and oxidation-reduction process. For example, during the regulation of apoptosis, multiple apoptosis-related genes such as BAX and CASP3 were upregulated<sup>[5]</sup>, suggesting that Timosaponin A-III may inhibit the growth of nasopharyngeal carcinoma cells by activating the apoptotic pathway. In terms of molecular function, the differentially expressed genes were mainly enriched in molecular functions such as protein binding, protein kinase binding, and oxidoreductase activity. Among them, changes in genes related to protein kinase activity may affect the activation of intracellular signaling pathways, thereby regulating the biological behavior of cells. In terms of cellular components, the differentially expressed genes were mainly enriched in cellular structures such as the cell membrane, cytoplasm, and mitochondrial matrix, indicating that the effects of Timosaponin A-III on 5-8F cells involve multiple cellular levels.



**Figure 2.** Bar chart of GO enrichment analysis.

The results of KEGG pathway enrichment analysis indicated that the differentially expressed genes were significantly enriched in multiple important signaling pathways, such as the apoptosis signaling pathway, p53 signaling pathway, and TNF signaling pathway. In the apoptosis signaling pathway, multiple key genes such as BCL-2 and IAP were upregulated, while BAX, BAK, and others were downregulated. Abnormal activation of this pathway is closely related to the proliferation, survival, and metastasis of tumor cells<sup>[6,7]</sup>. Inhibition of this pathway by Timosaponin A-III may be one of the important mechanisms by which it exerts its anti-tumor effects. In the p53 signaling pathway, the p53 gene and its downstream target genes such as PUMA and NOXA were upregulated. As an important tumor suppressor gene, p53



induces apoptosis by activating apoptosis-related genes<sup>[8]</sup>, which is consistent with the results of apoptosis regulation in the GO enrichment analysis.

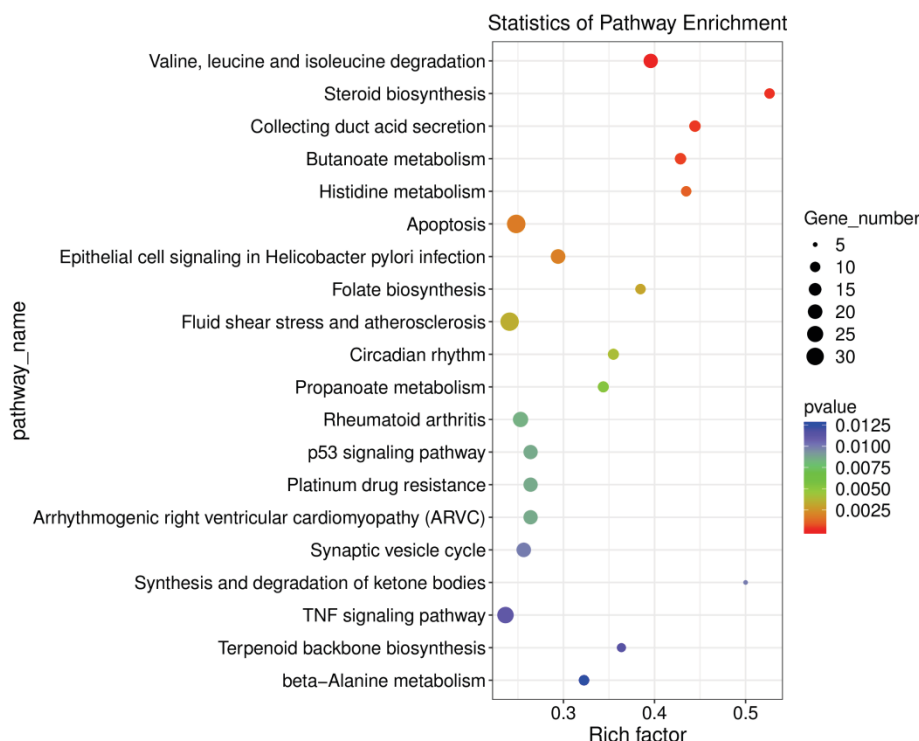


Figure 3. Bubble chart of KEGG pathway enrichment analysis.

## 4. Discussion

In this study, transcriptome sequencing analysis comprehensively revealed significant changes in gene expression in 5-8F cells treated with Timosaponin A-III. Among the differentially expressed genes, multiple genes closely related to tumor development and progression showed altered expression. For example, the upregulation of apoptosis-related genes such as BAX and CASP3 suggests that Timosaponin A-III may induce apoptosis in nasopharyngeal carcinoma cells by activating the endogenous apoptotic pathway, promoting the release of cytochrome C from mitochondria, and activating the Caspase cascade reaction. Downregulation of cell cycle-related genes such as CCND1 and CDK4 indicates that Timosaponin A-III may arrest the cell cycle in the G1 phase, inhibiting the transition from G1 to S phase and thus suppressing cell proliferation. These changes in gene expression provide important clues for a deeper understanding of the molecular mechanism of Timosaponin A-III's anti-nasopharyngeal carcinoma activity.

The results of KEGG pathway enrichment analysis showed significant changes in multiple key signaling pathways in 5-8F cells treated with Timosaponin A-III. These pathways play crucial roles in tumor initiation, development, and metastasis.

The apoptotic signaling pathway is one of the important signal transduction pathways in cells, and its abnormal activation is closely related to the proliferation, survival, migration, and invasion of tumor cells. In this study, Timosaponin A-III treatment led to changes in the expression of key genes such as BAX and CASP3 in the apoptotic signaling pathway, indicating that Timosaponin A-III may exert its antitumor effect by affecting this pathway and blocking the transmission of cell proliferation and survival signals.

The p53 signaling pathway, as an important tumor suppressor pathway, plays a key role in maintaining genomic stability, inducing apoptosis, and cell cycle arrest. Upregulation of p53 and its downstream target genes such as PUMA

and NOXA after Timosaponin A-III treatment suggests that Timosaponin A-III may activate the p53 signaling pathway, inducing apoptosis and cell cycle arrest, thereby inhibiting the growth of nasopharyngeal carcinoma cells <sup>[9]</sup>. Mutations or functional deletions of the p53 gene are common in various tumors, leading to evasion of apoptosis and uncontrolled proliferation of tumor cells <sup>[10–12]</sup>. Therefore, activating the p53 signaling pathway is one of the important strategies for tumor treatment.

These key signaling pathways may have complex interactions and regulatory networks that mediate the inhibitory effect of Timosaponin A-III on nasopharyngeal carcinoma cells. Further investigation of the interactions between these signaling pathways will help to fully reveal the molecular mechanism of Timosaponin A-III's anti-nasopharyngeal carcinoma activity, providing a more solid theoretical foundation for developing new treatment strategies.

## 5. Conclusion

In this study, transcriptome sequencing analysis of nasopharyngeal carcinoma cells 5-8F induced by Timosaponin A-III revealed that Timosaponin A-III can significantly alter the gene expression profile of 5-8F cells. The differentially expressed genes are mainly enriched in biological processes such as apoptosis regulation, cell cycle regulation, and signal transduction, as well as key signaling pathways such as MAPK and p53. These results suggest that Timosaponin A-III may exert its anti-nasopharyngeal carcinoma effect through multiple mechanisms, such as activating apoptotic pathways and inhibiting key signaling pathways. This study provides an important theoretical basis for further exploring the molecular mechanism of Timosaponin A-III's anti-nasopharyngeal carcinoma activity and lays a foundation for developing new treatment strategies based on Timosaponin A-III for nasopharyngeal carcinoma.

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## Disclosure statement

The author declares no conflict of interest.

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