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Screening and Characterization of Nitrite Degrading *Lactobacillus plantarum* in Chinese Traditional Pickles

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Abstract

A Gram-positive, non-spore, round ended, straight rod Lactic acid bacteria were screened. The strain was screened out from the traditional pickle jar in Yutang soy sauce garden of Jining. In order to degrade the nitrate content in the fermentation process of traditional pickles and improve the quality of pickles, it is necessary to screen out nitrite degrading strains from pickles, and preliminarily locate nitrite reductase, and find out the most suitable pH, temperature and culture time to degrade nitrite. *Lactobacillus plantarum* was screened by MRS medium in advance. After 48 hours of culture in a shaking table with *Bacillus subtilis*, the cell components were separated by centrifugation, wall breaking and other operations. After 20 hours at 30˚C, the content of nitrite in each component was determined by the naphthalene ethylenediamine hydrochloride method (NIR). The culture conditions were as follows: inoculation amount 3%, 6%, 9%, 12%, 15%; salinity 2%, 4%, 6%, 8%, 10%; temperature 15˚C, 20˚C, 25˚C, 30˚C, 35˚C for 20 h. The results showed that the best degradation effect of nitrite was obtained under the conditions of 9% inoculum, salinity 5% and 30˚C. Under the conditions of 9% inoculum, 5% salinity and 30˚C for 5 h, 10 h, 20 h, 48 h, 66 h and 78 h, the results showed that the degradation amount gradually increased with the extension of time, and gradually maintained a stable state. *Lactobacillus plantarum* JBA-3 is a new type of lactic acid bacteria which can degrade nitrite and produce nitrite reductase.

Keywords

Lactobacillus Plantarum, Soy Sauce Pickles, Nitrite Degradation

1. Introduction

Nitrite is a class 2A carcinogen released by the World Health Organization for

international cancer research. Nitrite is widely found in food, especially in pickled vegetables [1]. Nitrite will be produced in the process of vegetable pickling. When the human body ingests a certain amount of nitrite, a series of health problems can be induced. At present, the production method of pickle and pickle is relatively backward, the hygiene is not up to standard, the bacterial pollution is serious, and the content of nitrite is high [2]. Nitrite content has become a "stumbling block" to the progress and development of pickle industry. Lactobacillus is one of the most common bacteria in nature, which exists in pickled vegetables [3]. *Lactobacillus plantarum* can degrade nitrite in pickles. It is of great significance for people's production and life to screen *Lactobacillus plantarum* strains with a strong ability to degrade nitrite. Jining pickle is famous for its good taste and high quality. The purpose of this study was to extract *Lactobacillus plantarum* with strong nitrite degradation ability from Jining pickles [4] and provide the reference for selecting suitable nitrite degrading *Lactobacillus plantarum* in the process of pickle production [5].

Nitrite is a potential carcinogen, which is produced in the process of vegetable fermentation, which brings potential problems to food safety. Intake of large amounts of nitrite can lead to methemoglobinemia and acute poisoning [6] [7]. Under appropriate conditions, nitrite reacts with amine, the product of protein decomposition, to form N-nitroso compounds. At present, more than 100 kinds of N-nitroso compounds have been synthesized, of which 80% are strong carcinogens in animals [8]. However, nitrite is also widely used in the meat industry to prevent the growth of Clostridium botulinum, and is also used as a colorant. Therefore, limiting nitrite in food is the focus of food safety research [9]. It was found that inoculating lactic acid bacteria could inhibit the accumulation of high concentration nitrite in pickle fermentation [10]. It has also been reported that more than 10 different Lactobacillus strains have been inoculated, including *Lactobacillus brevis*, *Lactobacillus fermentans*, *Lactobacillus acidophilus* and *Lactobacillus plantarum*, which can effectively reduce the concentration of nitrite during the fermentation of pickles and meat [11] [12] [13]. Our previous studies have shown that inoculation with *Lactobacillus plantarum* JBA-3 can significantly reduce the nitrite concentration in fermented pickles. However, little is known about the pathway and mechanism of nitrite degradation by lactic acid bacteria, the key enzymes involved in the process, and the subcellular localization of the enzyme. The effect of *Lactobacillus plantarum* JBA-3 on nitrite degradation during pickle fermentation and its influencing factors were studied. At the same time, the activity of NIR in different cell parts was detected to determine the subcellular localization of NIR.

2. Methods

2.1. Medium

Mrs solid medium [14] [15]: glucose 10 g, agar 20 g, peptone 5 g, KH_2PO_4 2 g, yeast extract 2G, calcium carbonate powder 15 g, distilled water 1000 mL, pH

7.0. Liquid medium: glucose 10 g, peptone 5 g, KH_2PO_4 2 g, yeast extract 2G, distilled water 1000 mL, pH 7.0.

2.2. Isolation of *Lactobacillus plantarum*

1 g of pickle is randomly cut into a mortar, added with 1 mL normal saline, fully ground, and left for 15 minutes, then put into the ultra clean table. The pickle samples of Yutang soy sauce were diluted with normal saline according to the concentration gradient of 10^{-1} - 10^{-3}: Six EP tubes were prepared, numbered 1, 2, 3, 4, 5 and 6 on the tube rack, and the bacterial solution of different concentrations was prepared in EP [16]. Colony observation: under the microscope, the direct microscopic examination was carried out first. Gram staining was performed before microscopic examination. The strains with large calcium dissolving circle were selected from the plate and purified repeatedly on Mrs solid medium. *Lactobacillus plantarum* was screened and inoculated into liquid medium.

2.3. Screening of Nitrite Degrading *Lactobacillus plantarum*

According to the method of naphthalene ethylenediamine hydrochloride, the nitrite degradation ability of *Lactobacillus plantarum* was measured. The selected *Lactobacillus plantarum* was inoculated into Mrs liquid medium containing the appropriate amount of $NaNO_2$, and blank control was made at the same time. The content of $NaNO_2$ in fermentation broth was detected after 24 hours of incubation in constant temperature incubator [17].

2.4. Physiological and Biochemical Identification of Nitrite Degrading *Lactobacillus plantarum*

Lactobacillus plantarum, which was screened in 2.3, was activated for 2 generations and inoculated into Mrs agar medium. After 24 hours of culture, the morphology of the strain was observed. The physiological and biochemical characteristics of *Lactobacillus plantarum* were identified according to "Berger's Bacteria Identification Manual" and "plant lactobacillus classification and identification and experimental methods" [18]. The carbon source utilization test was conducted by biochemical identification tube.

2.5. Molecular Biological Identification of Nitrite Degrading *Lactobacillus plantarum*

According to the 16S rDNA gene sequence, a universal primer 27F was designed: 5'-agagttttgatcctggctcag-3', 1492r: 5'-cggttacttgttacgcactt-3'. The whole genome of nitrite degrading *Lactobacillus plantarum* was extracted and amplified by 16S rRNA bacteria with primers. The amplification system was 25ul PCR reaction system. The amplification reaction conditions were as follows: pre denaturation at 94˚C for 3 min, denaturation at 94˚C for 30 s, annealing at 52˚C for 1.5 min, extending for 2 min and 30 s at 72˚C for 35 cycles and extending for 10 min at 72˚C. Blast was used for homology comparison. PCR products were sent to Shenggong Bioengineering (Shanghai) Co., Ltd. for sequencing. The sequence

was compared by blast in GenBank and identified to species [19].

2.6. Phylogenetic Tree

The PCR product was purified and sequenced as described previously [20]. BLAST analysis for 16S rRNA gene sequences was done on National Center for Biotechnology Information (NCBI) database. The sequence was multiply aligned with the selected sequences of type strains obtained from GenBank/EMBL/DDBJ databases by using Clustal W version 1.81. A phylogenetic tree was reconstructed using MEGA5.0 software, based on the neighbour-joining, maximum-parsimony and maximum likelihood methods.

2.7. Parameter Optimization by Response Surface Methodology

In this study, RSM was used to identify the effects of optimum parameters of four variables, Inoculation quantity (A), salinity (B), temperature (C), and Nitrite degradation rate (D), on the yield of Nitrite degradation. To estimate the model coefficients, a three-factor-three-level Box Benhnken experiment was performed (29 runs), and the order of the experiments was randomized, analysis of the experimental design data and the calculation of the predicted responses were computed by using SPSS Statistics 22.0. Additional confirmatory experiments were subsequently conducted to verify the reliability of the statistical experimental design [21].

2.8. Pickle Fermentation

Lactobacillus plantarum JBA-3 was inoculated into sterilized MRS medium and incubated at 30˚C for 24 hours without shaking to produce seed solution. Then, 5% of the seed liquid was added to the liquid MRS medium to bring the bacteria to 10^7 - 10^8 CFU/mL, and then incubated at 30˚C for 24 hours without shaking to produce a secondary seed solution [22]. The fermentation process of pickles is carried out according to the following procedures: fresh cucumber cleaning, drying, segmentation, blanching. Add 5% secondary seed liquid and soy sauce. Fermentation was carried out at 37˚C in sealed bottles for 120 hours. Samples of fermented pickles were collected regularly under aseptic conditions to detect the concentration of nitrite [23]. Fermentation is also carried out under sterile conditions. In order to obtain the best growth and anaerobic fermentation of lactic acid bacteria, pickles were not exposed to the air in a large number of secondary seed liquid. The control group (CK_0) was fermented with conventional pickles and was not inoculated with *Lactobacillus plantarum* JBA-3.

3. Results

3.1. Isolation of Lactobacillus Plantarum

The supernatant of ground pickle was extracted and prepared into different concentrations of bacterial solution in EP tube. The higher the dilution concentration was, the higher the number of colonies was detected (**Table 1**). Morphological

Table 1. Number of colonies corresponding to different dilution.

Dilution	10^{-4}	10^{-5}	10^{-6}	10^{-7}
CFU	ND	421	69	21

ND: Not Detected.

observation was carried out to observe the morphology of the better growing colonies. After Gram staining, *Lactobacillus plantarum* was dyed blue purple, which proved that *Lactobacillus plantarum* was Gram-positive (**Figure 1**). The strains with large calcium dissolving circle were selected from the plate and purified repeatedly on Mrs solid medium. Finally, the *Lactobacillus plantarum* JBA-3 strains with the largest calcium dissolving circle were selected. More than 300 strains were isolated, and one strain with high nitrite degradation activity was found, and inoculated into liquid medium to screen *Lactobacillus plantarum*.

3.2. Phylogenetic Tree

Neighbour-joining tree based on an almost complete 16S rRNA gene sequence (1492 nt) showing the relationships among strain *Lactobacillus plantarum* JBA-3 and related type strains (**Figure 2**). Phylogenetic tree was constructed by using related strains of Lactobacillus. Asterisks (*) and hashes (#) indicate that the corresponding nodes were also found in trees generated with the maximum-likelihood and maximum-parsimony algorithms, respectively. The numbers at branch nodes indicate bootstrap percentages derived from 1000 replications; only values > 50% are shown. Bar, 0.01 substitutions per single nucleotide position.

3.3. Response Surface Analysis

The response surface 3D map (**Figure 3**) showed the combined effects of different factors on the response (Nitrite degradation rate). The experimental data were fitted to equation, with the following optimal operating parameters: (a): Inoculation quantity, 9.13%; (b): salinity, 5.41%; (c): temperature, 30.19˚C; (d): Nitrite degradation rate, 91.47%.

3.4. Dynamic Change of Nitrite Concentration in Pickle Fermentation

The nitrite concentration of fermented pickles after different fermentation time is shown in **Figure 4**. The content of nitrite in fermented pickles without *Lactobacillus plantarum* JBA-3 18.35 mg/Kg and 51.26 mg/Kg, which decreased to 1.75 mg/Kg and 0.89 mg/Kg after 24 h and 48 h fermentation by *Lactobacillus plantarum* JBA-3. The content of nitrite after 72 hours of fermentation is higher than that of after 48 hours from **Figure 4**. Compared with the conventional fermentation, the nitrite concentration in the fermentation system with *Lactobacillus plantarum* JBA-3 was significantly lower ($P < 0.01$). The nitrite concentration

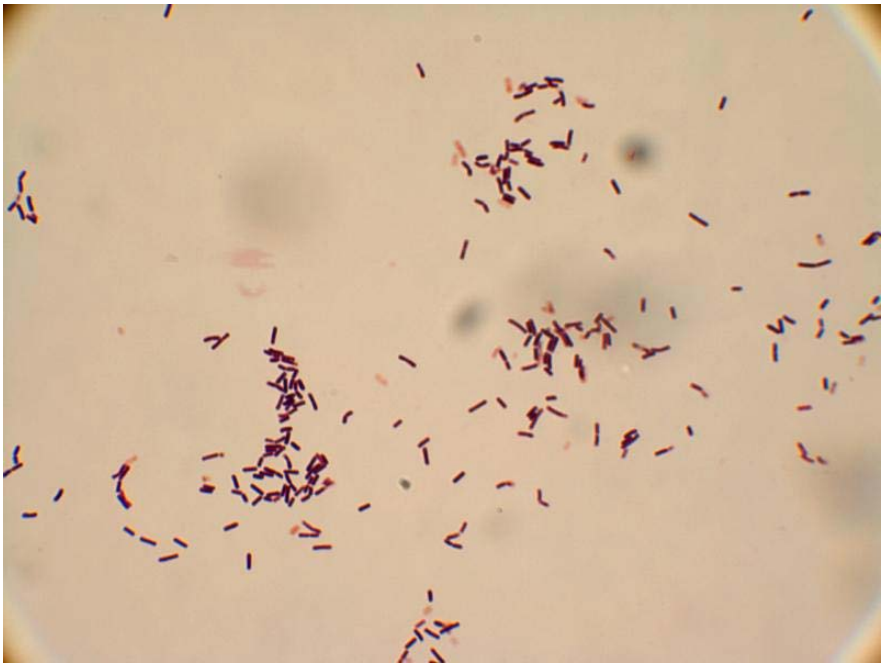

Figure 1. General optical micrograph of cells of strain *Lactobacillus plantarum* JBA-3 grown in Mrs broth at 30˚C for 24 h (400×). Morphological observation was carried out to observe the morphology of the better growing colonies. After Gram staining, *Lactobacillus plantarum* was dyed blue purple, which proved that *Lactobacillus plantarum* was Gram-positive.

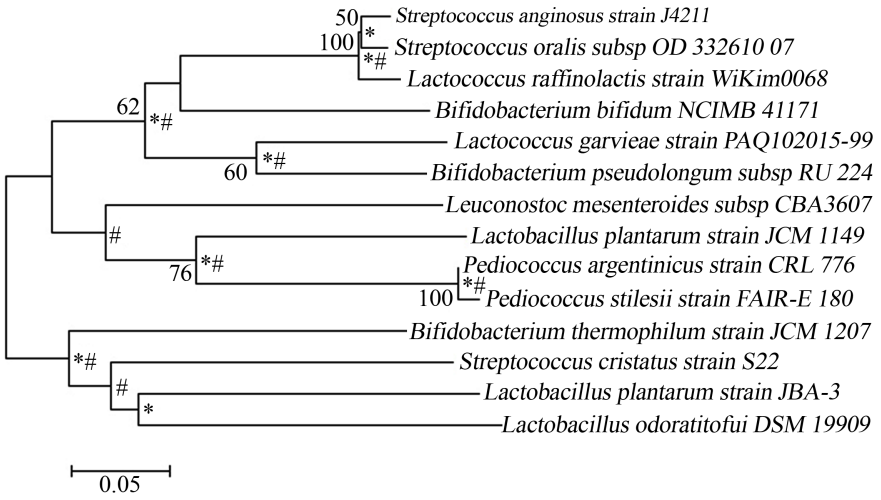

Figure 2. Neighbour-joining tree based on an almost complete 16S rRNA gene sequence (1492 nt) showing the relationships among strain *Lactobacillus plantarum* JBA-3 and related type strains. Asterisks (*) and hashes (#) indicate that the corresponding nodes were also found in trees generated with the maximum-likelihood and maximum-parsimony algorithms, respectively.

in fermented pickles without *Lactobacillus plantarum* JBA-3 increased first and then decreased, and the highest concentration reached 51.26 mg/Kg after 48 h fermentation, which was consistent with the previous report. Therefore, *Lactobacillus plantarum* JBA-3 could significantly inhibit the abnormal accumulation

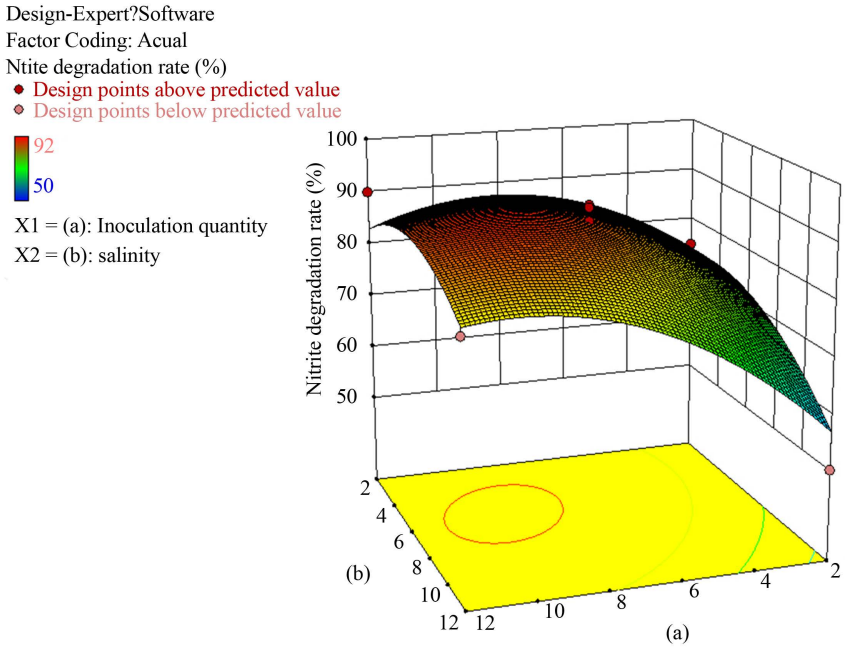

Figure 3. The 3D response surface of nitrite degradation rate affected by Inoculation quantity, salinity, and temperature. Inoculation quantity (%) (a), salinity (%) (b), temperature, nitrite degradation rate, on the yield of Nitrite degradation. To estimate the model coefficients, a three-factor-three-level Box Benhnken experiment was performed (29 runs).

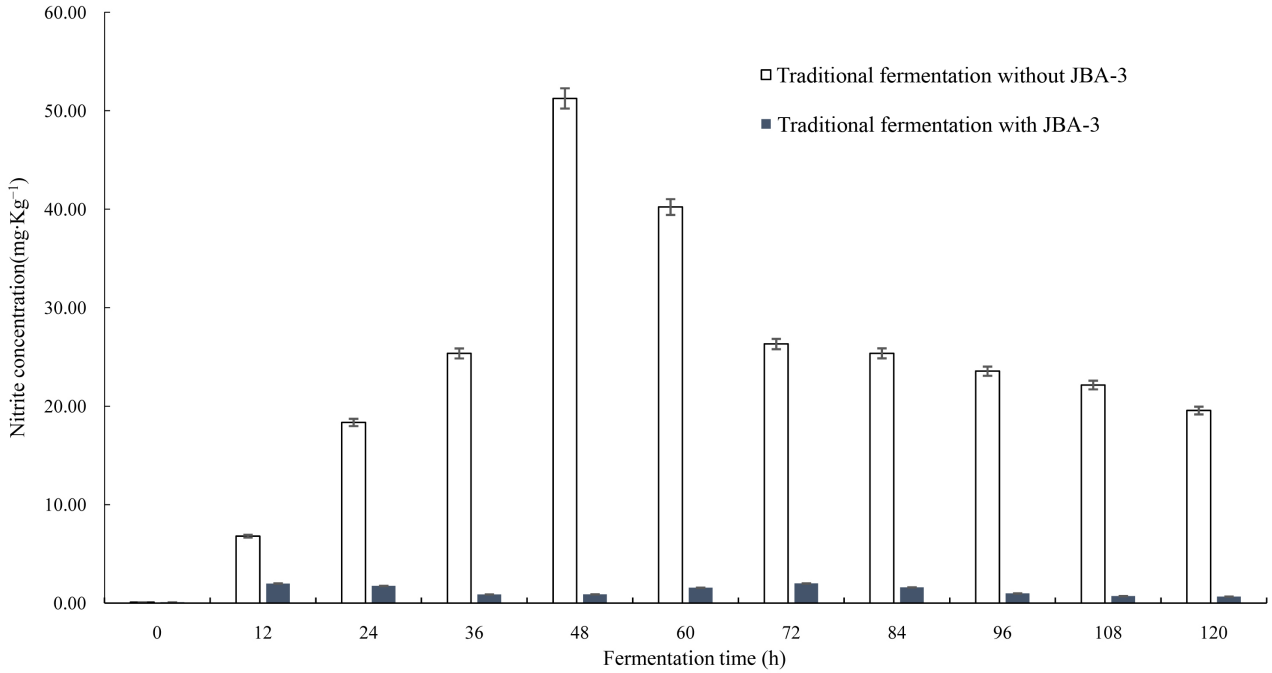

Figure 4. Nitrite concentration during the fermentation of traditional pickles with and without *Lactobacillus plantarum* JBA-3.

of nitrite during the fermentation of pickles. For a long time, high concentration of nitrite in food has been a research hotspot in the field of food safety. The potential reason of nitrite accumulation in Chinese pickles was studied. The results

showed that nitrate accumulated in the process of bacterial fermentation, and lactic acid fermentation reduced nitrate concentration. High concentration of nitrite was maintained for a long time. The abnormal accumulation of nitrite in pickles was related to the following factors: 1) The number of coliforms was higher than that of the control group; 2) The concentration of soluble nitrogen compounds was higher than that of the control group; 3) The buffering capacity of the control group was higher than that of the control group. These results suggest that the abnormal accumulation is caused by the long-term survival of coliforms that promote nitrate reduction. *Lactobacillus delbrueckiicidca* 133 inhibited the nitrate reductase activity of *Escherichia coli*. The nitrate reductase activity was closely related to the number of viable cells of *Lactobacillus*, rather than low pH value. This indicates that some substances are directly transferred from *Lactobacillus* to *Escherichia colis*.

4. Discussion

The most suitable pH value of lactic acid bacteria was obtained from this experiment. According to the method of naphthalene ethylenediamine hydrochloride in China National Quality Standard (GB/T 5009.33-2003 determination of nitrite and nitrate in food), *Lactobacillus plantarum* JBA-3 with strong nitrite degradation ability was selected. *Lactobacillus plantarum* JBA-3 was extracted from pickles in Jining area. It was proved that the nitrite content in the pickles produced by the lactic acid bacteria may be low and the harm to human body is low. The selected *Lactobacillus plantarum* JBA-3 can improve the taste and flavor of pickles, reduce the content of nitrite, reduce the pickling time and improve the economic benefits. *Lactobacillus plantarum* JBA-3 can also be used to produce other aspects of life, such as the production of yogurt, medicine and so on.

Lactobacillus plantarum JBA-3 is active in the temperature range of 15 - 40, can degrade sodium nitrite, has the ability to decompose D-glucose and malic acid, and can secrete amylase, arginase and urease (**Table 2**). The degradation amount of nitrite in Periplasmic NiR was 1.15 mg/L and the enzyme activity was 15.5 U. The degradation of nitrite in Cytoplasmic NiR was 98.66 mg/L and the enzyme activity was 1327.6 U (**Table 3**). The enzyme activity of periplasmic NIR was determined by culturing bacteria at 30˚C for 20 h, and then the culture medium was centrifuged at 8000 rpm for 20 min. The supernatant was taken for enzyme activity determination.

The method for determining the enzyme activity of nitrosamic NIR was as follows: firstly, the bacteria were cultured at 30˚C for 20 h, the culture medium was centrifuged at 8000 rpm for 20 min, and then the precipitate was diluted and put into two 2 ml EP tubes respectively. After crushing for 20 min, the crushing solution was centrifuged at 10,000 rpm for 20 min. Finally, the supernatant was taken to determine the enzyme activity of nitrite reductase.

Lactobacillus plantarum JBA-3, which can degrade nitrite, is not the dominant strain in the traditional process of pickle fermentation. In the traditional

Table 2. Differential characteristics of strain JBA-3 Andrelated species of the lactobacillus.

Characteristic	*Lactobacillus plantarum* JBA-3	*Lactobacillus brevis* ATCC367	*Pediococcus cerevisiae* NCIMB12174
Temperature for growth (˚C)	15 - 40	10 - 42	4 - 37
pH for growth	5 - 8	5.5 - 9	5.5 - 8
Nitrate reduction	+	-	-
Gelatin hydrolysis	-	+	-
PET hydrolysis	-	-	+
Assimilation of:			
L-Arabinose	-	+	-
D-Glucose	+	-	+
Capric acid	-	-	+
Malic acid	+	+	+
Trisodium citrate	-	+	-
D-Mannitol	-	-	+
Enzyme activities:			
amylase	+	+	-
hippuricase	-	+	-
Arginase	+	+	+
urease	+	+	-
Isolation source	Soy sauce pickles	Cheese	Fermented food

Strains: 1, *Lactobacillus plantarum* JBA-3; 2, *Lactobacillus brevis*ATCC367; 3, *Pediococcus cerevisiae*NCIMB12174. All phenotypic characteristics were determined in this study. +: Positive; -: Negative.

Table 3. The location of nitrite reductase in JBA-3.

	Periplasmic NiR	Cytoplasmic NiR	CK
$NaNO_2$ degradation (mg/L)	1.15	15.5	0.0012
Enzyme activity (U)	98.66	1327.6	-

Data were presented as the mean standard deviation (S.D.). The degradation amount of nitrite in Periplasmic NiR was 1.15 mg/L and the enzyme activity was 15.5 U. The degradation of nitrite in Cytoplasmic NiR was 98.66 mg/L and the enzyme activity was 1327.6 U.

fermentation of pickles, *Lactobacillus plantarum* JBA-3 was not enriched to control the accumulation of nitrite in pickles. In our improved production process, we can increase the number of *Lactobacillus plantarum* JBA-3 ahead of time. In the process of pickle fermentation, we can establish the fermentation production

system of *Lactobacillus plantarum* JBA-3 which can degrade nitrite as the dominant strain. In the whole process of pickle production, the synthesis and accumulation of nitrite can be controlled to make the pickle safer.

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Authors' Contributions

J. J., N. L., W. J. W., R. N. X. performed experiments. Y. Q. and C. Z. Z. analysed data. F. W. provided project administration and funding. J. J. and N. L. wrote the manuscript.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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