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The Analysis of Drug Resistance of the Strains of Lactic Acid Bacteria Isolated from Yoghurt Made in China and Outside of China

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Abstract. To analysis and compare the drug resistance of *lactic acid bacteria* isolated from yoghurt made in China and outside of China. Yoghurt products were purchased from Nanchang supermarkets. The *Lactic acid bacteria* in yoghurt were isolated by MRS, MC and M17 medium. The isolated strains were identified by gram's stain, colony morphology observation and biochemistry reaction test. Drug resistance analysis of the identified strains were analyzed by K-B method. 26 kinds of *Lactic acid bacteria*, 13 strains of cocci and 13 strains of bacillus, were isolated. All strains were identified by physiology and biochemistry test. They were *Lactic acid bacteria bulgaricus*, *Lactic acid bacteria delbrueckii subsp. Bulgaricus*, *Streptococcus thermophilus*, *Bifidobacteria (Bacillus bifidus)*. Drug resistance analysis revealed that the 26 strains of *Lactic acid bacteria* showed multiple drug resistance to 13 kinds of antibiotics, such as kanamycin. And all of these *Lactic acid bacteria* were completely resistant to kanamycin, ampicillin and oxacillin. The drug resistance spectrum of China's *Lactic acid bacteria* is similar to that of foreign *Lactic acid bacteria*, but the drug resistance of China's *Lactic acid bacteria* is significantly stronger than that of foreign *Lactic acid bacteria*. The abuse of antibiotics is serious in dairy industry. We should strengthen the management of antibiotics, reduce the chance of super resistant bacteria, and maintain the food health of the general public.

Keywords. Yoghurt, *Lactic acid bacteria*, drug resistance.

1. Introduction

Yoghurt is a common drink popular among the general public. The fermentation of pure milk by *Lactic acid bacteria* is the main way to produce the yoghurt. Lactate can not only keep food well, but also promote the growth of beneficial bacteria in human intestine. However, the drug resistance of *Lactic acid bacteria* has become a universal global problem [1, 2]. Some studies have shown that different *Lactic acid bacteria* have different degrees of drug resistance, and the same *Lactic acid bacteria* have drug resistance to a variety of antibiotics [3].

At present, there are more and more researches notified the antibiotic resistance of *Lactic acid bacteria* in yoghurt in China, but few of them on extraction, identification and drug resistance detection of imported yoghurt *Lactic acid bacteria* [4-7]. The purpose of this study is to understand the drug resistance of *Lactic acid bacteria* in yoghurt in and outside of China; Different brands of yoghurt samples were purchased from different supermarkets in Nanchang. After dilution, *lactic acid bacteria* were isolated and purified. Then, identified by morphology and biochemistry. Finally, the K-B method was used for drug resistance test to analyze the difference of drug resistance between domestic and



foreign yoghurt.

2. Materials and Methods

2.1. Materials

Four China brands Yoghurts, Yili, Mengniu, Guangming and Tiantianyangguang, were bought from three large supermarkets in Nanchang. Four brands of Imported yoghourts, zottis (pr24 04 2018 eh6), Bauer (ex 15 08 2018 BF2), Emmi (2018 08 06 K7), Pascual (ED: 2019-01-01 80928v5), were purchased from Nanchang Metro supermarket. All yoghourts were refrigerated and preserved at 4 °C. Microbiological media were bought from Hangzhou Tianhe microbial Reagent Co., Ltd. MRS solid medium [8] (20180528-00): for isolation and culture of *Lactic acid bacteria bulgaricus* and *Bifidobacterium lactis*, MC solid medium (20171120-00): for isolation and culture of *Streptococcus thermophilus*, M17 solid medium (BS 1039): for isolation and culture of *Streptococcus thermophilus* and *Lactic acid bacteria acidophilus*.

Antibiotics used in this study, Vancomycin, gentamicin, oxacillin, kanamycin, polymyxin, ampicillin and other 13 kinds of drug sensitive paper (manufacturer's batch number: 20180904), were all purchased from Hangzhou Tianhe microbial Reagent Co., Ltd in Hangzhou, China.

2.2. Methods

2.2.1. Isolation and Purification of Lactic Acid Bacteria. In the sterile operation platform, 2.5 mL of the sample of yoghurt was taken from 12 purchased yoghurt sold in Nanchang City and 4 imported yoghurt in Nanchang City by using the sterile pipette gun, and placed in a conical flask filled with sterile physiological saline, which was homogenized to make a 1:10 sample diluent. Then, 1 mL of the sample diluent was drawn from the 1:10 sample diluent by using the 1 mL pipette gun and slowly injected along the wall of the tube into a 9 mL sterile physiological saline tube to make a 1:100. Of yoghurt sample dilutions. At the same time, the samples of yoghurt dilutions sold in Nanchang were numbered: I1, I2, I3, II1, II2, ii3, III1, iii2, III3, xii1, xii2, xii3. The imported yoghurt of Nanchang was numbered: Z1, Z2, Z3, B1, B2, B3, E1, E2, E3, P1, P2, P3.

First separation: pre-cooled medium about 60 °C was poured into the sterile culture dish on the super clean working table. After the MRS culture medium, MC culture medium and M17 culture medium are completely solidified, respectively use the pipette gun to suck the local yoghurt I1, I2, i3, II1, II2, III3, xii1, xii2, xii3, and the yoghurt Z1, Z2, Z3, B1 imported from Nanchang city. B2, B3, E1, E2, E3, P1, P2, P3 were placed in MRS medium, MC medium and M17 medium, coated with sterilized coating rod, then inverted in anaerobic tank (oxygen was removed by candle combustion method), and anaerobic tank was placed in a constant temperature incubator at 37 °C for 2 days.

According to morphological observation such as shape, size, color and transparency, the strains grown in the first separation are classified. Different colonies cultured in different yoghurt are marked in MRS medium, MC medium and M17 medium, and the pure single colonies of different lactic acid bacteria are separated. The marked plates are inverted in the anaerobic tank, and then the anaerobic tank is placed in the culture. After incubation at 37 °C for 1-2 days, repeat the above steps until a single colony of Lactic acid bacteria is isolated and purified.

2.2.2. Identification of Lactic Acid Bacteria.

Morphological identification: observe the morphology, texture, dryness, wetness and transparency of the isolated single colony, and Gram staining method was used to identify *Lactococcus* or *Lactic acid bacteria*.

Biochemical test: conduct biochemical identification according to the instructions of biochemical Kit (purchased from Hangzhou Tianhe Microbial Science and Technology Co., Ltd. 20152400204).

2.2.3. K-B Test. Isolated strains were coated or scribed in the culture dish suitable, and the paper

containing 13 kinds of antibiotics were respectively used in the corresponding culture dish. The *Lactic acid bacteria* was cultured at 37 °C for 2 days. in the incubator We observed and recorded the bacteriostatic circle. The results of drug sensitivity were divided into sensitive (R), intermediate (I) and resistant (s). Panlin et al. [9, 10] pointed out that the minimum mic value of *Lactococcus* to neomycin, kanamycin, streptomycin and gentamicin was 10 µg/mL. according to the latest standard judgment result of the clinical and Laboratory Standards Institute (CLSI), the drug resistance degree was judged.

3. Results

3.1. Isolation and Identification of *Lactobacillus*

20 strains of *lactic acid bacteria* were isolated from four yoghurt factories in Nanchang, and numbered 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 respectively. The results of Gram staining showed that 20 strains of *lactic acid bacteria* were all gram positive. 10 were Gram-positive bacillus, 10 were gram-positive cocci. 6 strains of *lactobacillus* were isolated from 4 imported yoghurt factories in Nanchang City, and their numbers were 21, 22, 23, 24, 25 and 26 respectively, and theirs staining results also presented purple, including 3 Gram-positive bacillus and 3 strains of gram-positive cocci. The colony of *lactic acid bacteria* on MRS medium, as shown in figure 1b. milky white round colony with rough surface and neat edge and protruding shape in the middle, as shown in figure 1d: milky white or transparent droplet colony with wet and sticky surface, neat or irregular edge and protruding shape. The colony size of all *lactobacillus* was similar, but the sensory texture was different.

3.2. Morphology

3.2.1. *Colony Morphology*. The morphology of *lactic acid bacteria* colonies was observed from the aspects of dryness or wetness, texture, shape, transparency, convex, and the flatness of the colony edge. It was found that four different forms of colonies I, II, III and IV were as shown in figure 1.

3.2.2. *Lactobacillus Staining Results*. Four different *lactic acid bacteria* (I, II, III, IV) in figure 2 were observed according to cocci or bacilli, with or without chains.

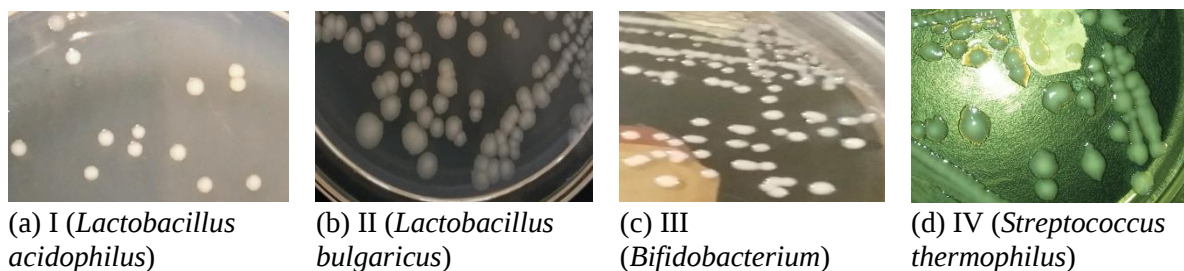
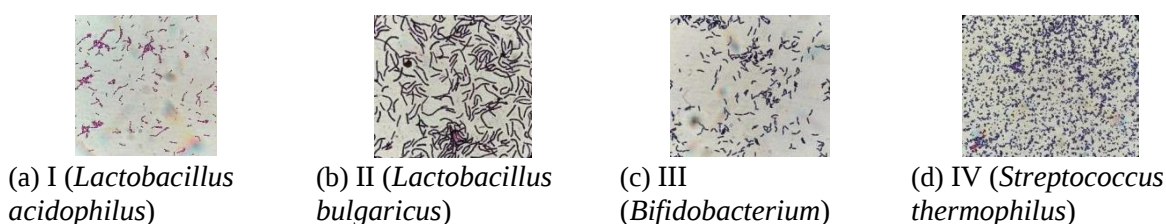
3.3. Physiological and Biochemical Tests

Biochemical identification was carried out on 26 strains of *lactic acid bacteria*. Comparing the above results with Berger's Bacterial Identification Manual (8th Edition) [8], Of all 26 strains, 7 strains are of *Lactobacillus bulgaricus* (3, 10, 11, 13, 16, 18, 20), 10 strains are of *Streptococcus thermophilus* (2, 4, 5, 7, 9, 12), respectively. No. 14, No. 15, No. 17 and No. 19, 2 strains are of *B. lactis* (No. 1 and No. 6), 1 strain is of *Lactobacillus acidophilus* (No. 8), 3 strains are of *Streptococcus thermophilus* (No. 22, No. 26 and No. 24), 1 strain is of *Lactobacillus delbrueckii subsp. Bulgaricus* (No. 21), 1 strain is of *Lactobacillus bulgaricus* (No. 25), 1 strain is of *Lactobacillus acidophilus* (No. 23).

3.4. Drug Resistance Test

The drug resistance tests were carried out on 4 strains of *Lactobacillus* (*Lactobacillus bulgaricus*, *Streptococcus thermophilus*, *Bifidobacterium lactis*, *Lactobacillus acidophilus*) and 4 strains of *Lactobacillus* (*Lactobacillus delbrueckii subsp. Bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*). The test results are showed in table 1.

It can be seen from table 1 hat all the strains of *Lactobacillus* have strong resistance to kanamycin, ampicillin and oxacillin, and high resistance to gentamicin, vancomycin and ciprofloxacin. It can be found that the resistance rate of *Lactobacillus* in China had higher resistance ability to 13 antibiotics than that of foreign Lactic acid bacteria; However, *Lactobacillus* in China were more sensitive to cephalixin, cefazolin and ciprofloxacin. While foreign strains of *lactobacillus* were more sensitive to Cefalexin, cefazolin, ciprofloxacin and carboxybenzillin [11].

**Figure 1.** The colony morphology.**Figure 2.** Gram's staining microscopy.**Table 1.** Results of susceptibility test of bacteria strains.

Antibiotics	<i>Lactobacillus bulgaricus</i>	<i>Bifidobacterium lactis</i>	<i>Lactobacillus acidophilus</i>	<i>Streptococcus thermophilus</i>	<i>Lactobacillus bulgaricus</i>	<i>Bifidobacterium lactis</i>	<i>Lactobacillus acidophilus</i>	<i>Streptococcus thermophilus</i>
CN	10	11	14	12	9	12	11	11
VA	10	14	18	13	13	14	14	14
K	12	11	11	13	13	11	13	13
CIP	23	27	24	22	20	18	21	21
CFP	23	26	26	26	16	23	23	23
KZ	19	16	24	17	15	15	29	29
CL	22	32	31	32	37	25	32	32
AMP	0	0	0	0	0	0	0	0
AK	16	15	14	12	17	14	14	16
OX	0	0	0	0	0	0	0	0
N	20	11	22	16	16	15	20	20
CAR	19	10	0	16	18	16	28	20
CT	11	7	12	0	11	0	10	9

Note: Diameter of bacteriostatic circle of strains isolated from yoghurt (mm).

4. Discussion

In this study, *Lactic acid bacteria* were isolated and identified from the yoghurt produced in Nanchang and the imported yoghurt. Four kinds of Lactic acid bacteria were isolated and identified in the yoghurt sold in Nanchang, including *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidophilus* and *Bifidobacterium lactis*. Four kinds of Lactic acid bacteria were also isolated and identified in the yoghurt, including *Lactobacillus delbrueckii*, subspecies *bulgaricus*. *Streptococcus thermophilus*, *Lactobacillus acidophilus*.

According to table 1, lactic acid bacteria at home and abroad have strong resistance to kanamycin, ampicillin and oxacillin, and high resistance to gentamicin, vancomycin and ciprofloxacin. The research

results are similar to those of Dangqiao [12], Chen David [13], Imeme Fhoula [14], Cristian Botta [15], etc. The strains of Dangqiao and David Chen are highly resistant to gentamicin, the strains of Imeme Fhoula are sensitive to ampicillin and gentamicin, moderate resistant to vancomycin, and the strains of Cristian Botta are resistant to kanamycin and gentamicin [11, 16]. It can be seen from table 1 that *lactic acid bacteria* in yoghurt at home and abroad have multiple drug resistance, and the drug resistance of domestic *lactic acid bacteria* is significantly stronger than that of foreign *lactic acid bacteria*. The drug resistance rate of domestic *Lactobacillus* and *Streptococcus thermophilus* is higher than that of foreign *Lactobacillus* and *Streptococcus thermophilus*, and the drug resistance rate of domestic *lactobacillus* is the highest. The drug resistance rate of *Lactobacillus delbrueckii* was 53.8 (7/13) in foreign countries, the total drug resistance rate of domestic *lactobacillus* was 48.1% (25/52), the total sensitivity rate was 36.5% (19/52), the total drug resistance rate of foreign *lactobacillus* was 40.4% (21/52), and the total sensitivity rate was 40.4% (21/52). It was found that the drug resistance rate of foreign *lactobacillus* was lower than that of China. The drug resistance rate of internal *Lactobacillus* and foreign *lactobacillus* is higher than that of domestic *lactobacillus*, so it can be concluded that the drug resistance of domestic *lactobacillus* is stronger than that of foreign *lactobacillus*, that is to say, the inhibitory effect of antibiotics on foreign *lactobacillus* is significantly stronger than that of domestic *lactobacillus*, which indicates that the abuse of antibiotics in animal husbandry in China is more serious than that in foreign animal husbandry, resulting in the improvement of intestinal function of *Lactobacillus* in China is weaker than that in foreign countries' *Acid bacteria*.

In addition, the resistance of *Lactobacillus* to oxacillin, ampicillin, kanamycin, vancomycin and gentamicin was the strongest, which was similar to the resistance of the strains to amoxicillin, ampicillin, gentamicin, erythromycin and vancomycin. Africans and Europeans are resistant to vancomycin, kanamycin, tetracycline, neomycin and myxomycin. Therefore, in order to prevent the spread of antibiotic resistance, the European Food Safety Agency introduced live bacteria into food or feed [17]. In Liu Lu's [18] research, 8 kinds of bacteria are resistant to vancomycin. Vancomycin is a glycopeptide antibiotic. By inhibiting the synthesis of peptidoglycan, it has a strong bactericidal effect on Gram-positive bacteria, and vancomycin resistance in this experiment is also strong. The final results show that: the lactic acid bacteria isolated from yoghurt have multiple resistance to many kinds of antibiotics, but the inhibitory effect of domestic antibiotics is weaker than that of foreign ones, which leads to the stronger resistance of intestinal probiotics in Chinese citizens, more prone to produce superbacteria, which is not conducive to people's health and social stability. The detection of resistance and safety evaluation of *lactic acid bacteria* in China compared with foreign countries need to be done and strengthened.

Some studies have shown that the drug resistance of bacteria in China is 30% higher than the world average [19]. From the results of this study, it is also obvious that the drug resistance of domestic *lactic acid bacteria* is higher than that of foreign *lactic acid bacteria*, and there are two reasons for the drug resistance of bacteria [19]: Quilodran Vega emphasized the performance of probiotics on pigs and the survival of pigs. Therefore, it is suggested to develop alternative control methods of antibiotics in feed to protect animals. Due to the unreasonable use of antibiotics, especially aminoglycosides, on the farm, the laboratory often obtains the bifunctional genes with resistance [6]. Other researchers detected the corresponding resistance genes from the isolated *Lactobacillus*, which proved that the strains without resistance phenotype may also carry the resistance genes. Studies have shown that the resistance genes of some *lactobacillus* can be found in *Lactobacillus*. The drug resistance of *lactobacillus* can be extended by mutual transmission [20]. Therefore, we should pay attention to the legal use of antibiotics in China's animal husbandry, the safety of dairy products, strict standards for the use of antibiotics, and a reasonable rest period, so as to reduce the resistance of bacteria in China to the international level.

5. Conclusion

Lactobacilli is one of the main probiotics in human body. Now, the abuse of antibiotics leads to the formation of a large number of super resistant *lactobacilli*, and yogurt made of super resistant *lactobacilli* will induce the formation of super resistant bacteria in the intestine, which seriously affects the balance

of intestinal flora, further affects the immune system of human beings, and leads to the decline of human immunity. So we must strengthen the use and management of antibiotics in animal husbandry. Avoid the formation of superbugs.

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