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Psychrotrophic bacterial populations in Chinese raw dairy milk

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Four-hundred and eighty isolates isolated from raw milk samples collected from different farms in China were subjected to random amplified polymorphic DNA analysis and 16S rRNA sequencing for clustering and identification, respectively. All isolates comprised 24 genera and 74 species in total. *Pseudomonas* was the most prevalent genus, representing 58.8% of all isolates, followed by *Acinetobacter* (13.3%), *Flavobacterium* (6.0%) and *Sphingobacterium* (4.2%). Among the 25 species belonging to *Pseudomonas*, *Pseudomonas fluorescens* (15.8%) proved to be the most dominant species, followed by *Pseudomonas fragi* (7.1%) and *Pseudomonas psychrophila* (5.4%). Out of these isolates, 12.3% have not been reported in previous studies, highlighting that many psychrotrophic bacteria are rather underexplored in raw milk. In the future, a detailed investigation will be required to characterize these bacterial isolates based on their potential to deteriorate the quality of dairy products.

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1. Introduction

Raw milk serves as an ideal medium for the growth of a broad spectrum of bacteria due to its high nutritional value and can be easily contaminated mainly with bacteria during handling, transportation and processing (Yoon, Lee, & Choi, 2016; Claeys et al., 2013; Kalmus, Kramarenko, Roasto, Meremäe, & Viltrop, 2015). In order to slow down the growth of bacteria, a cold chain system is widely used in the dairy industry. However, psychrotrophic bacteria can still grow at low temperatures and become the predominant microflora. Psychrotrophic bacteria are defined as the microorganisms which have the ability to multiply below 7 °C, although their optimal growth temperatures are above 15 °C (Sørhaug & Stepaniak, 1997). In Chinese dairy processing, it's a common practice to keep raw milk at refrigerated temperature for up to 2 days before processing which exacerbates the possibility of microbial growth and affect product quality.

The diversity of psychrotrophic bacteria in raw milk has been studied (Decimo, Morandi, Silvetti, & Brasca, 2014; Vithanage et al., 2016). Psychrotrophic bacteria in raw milk are mainly Gram-negative genera, namely, *Pseudomonas*, *Acinetobacter*, *Aeromonas*, *Serratia* and *Flavobacterium*. Among the Gram-positive genera, *Microbacterium*, *Staphylococcus* and *Carnobacterium* have also been frequently reported (Ercolini, Russo, Ferrocino, & Villani, 2009; Vithanage et al., 2016).

Psychrotrophic bacteria can produce heat stable proteases and lipases which can survive after pasteurization or ultra-high temperature (UHT) treatment (Baur et al., 2015). These enzymes are responsible for quality defects in dairy products. For instance, proteases limit the shelf life of milk by increasing the viscosity and developing bitter flavor (Champagne et al., 1994; Claeys et al., 2013). Similarly, lipolytic activity of lipases result in the development of rancidity in milk (Bekker et al., 2016). In addition, the proclivity of psychrotrophic bacteria to adhere to stainless steel surface during raw milk storage and transportation leads to the development of biofilms, which serve as persistent source of microbial contamination as bacteria grow within biofilms and produce spoilage enzymes (Teh et al., 2011). The primary need to control psychrotrophic bacteria in raw milk is to prevent problems with dairy product quality.

The random amplified polymorphic DNA (RAPD) technique has advantages over other genotyping methods such as the ability to use low quantity of DNA for amplification, less time and labor and

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no requirement for any prior information on the DNA sequence (Dini-Andreote et al., 2009; Thomas, Divya, Chandrika, & Paulton, 2009). This technique is widely used to categorise bacteria into groups and has been reported to analyse microbiota of dairy products (Decimo et al., 2014; Sadiq et al., 2016).

China is the fourth largest producer, consumer and a major importer of milk in the world, and it is forcasted that in 2017 milk production and consumption will reach 36.5 and 37 million tons, respectively in China. (U.S. Department of Agriculture/ Foreign Agriculture Services, 2016). Quality defects in dairy products are frequently caused by psychrotrophic bacteria (Champagne et al., 1994). Dairy products produced from raw milk need to be of high quality and should meet customer and regulatory requirements. The microbiota and microbial diversity of psychrotrophic bacteria in Chinese raw milk have not been explored well. Therefore, the objective of this study is to reveal the prevalence and diversity of psychrotrophic bacteria in raw milk collected from sixteen different farms, provide a better understanding of current status of psychrotrophic bacteria in raw milk samples at the species level.

2. Materials and methods

2.1. Sample collection and selection of isolates

Sixteen raw milk samples were collected aspetically from different dairy farms in 11 cities that contribute to the industrial milk supply chain, representing different regions of China, from April to June 2016 (labelled from A to P), and kept at 4 °C during transportation.

All samples were decimally diluted in 0.1% sterile peptone water and plated onto sterile plate count agar (PCA) supplemented with 1% skim milk (Difco, USA) in triplicate followed by incubation at 7 °C for 7 days for the enumeration of psychrotrophic bacteria (Vithanage et al., 2016). Thirty colonies were randomly picked from each plate containing >30 colonies and sub-cultured onto PCA plates for three consecutive times. In total, 480 isolates were subjected to RAPD after initial grouping of the isolates based on morphology, Gram staining and catalase tests.

2.2. DNA extraction and RAPD-PCR amplification

Bacterial DNA was extracted from 1.5 mL nutrient broth cultures using the DNA extraction kit according to the manufacturer's instructions (AxyPrep Bacterial Genomic DNA Miniprep Kit, Axygen, China) and quantified by NanoDrop 2000 (Thermo Fisher Scientific, USA). The extracted DNA was stored at −20 °C until use. Isolates were clustered based on genetic similarity as determined by RAPD.

Each RAPD test was done in a volume of 25 μL containing 10xThermo buffer (Takara, Japan), 2.5 mM $MgCl_2$, 200 mM dNTP (Takara, Japan), 1U Taq polymerase (Takara, Japan) and 1.5 μM M13 primer (5′-GAGGGTGGCGGTTCT-3′, Huey & Hall, 1989) and 20 ng template DNA. The amplification was carried out in a programmable thermal cycler (Bio-Rad Inc., Hercules, CA, USA) using the following protocol: an initial denaturation step at 94 °C for 5 min followed by 33 cycles with denaturation at 94 °C for 1 min, 45 °C for 2 min and 72 °C for 1min and 30 s, and a final extension step at 72 °C for 5 min. PCR amplified products were analyzed by electrophoresis on 1.5% agarose gel (Biowest Agarose, SunShineBio, Spain) in 1 × TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), stained with DNA dye GelRed Nucleic Acid Gel Stain (Biotium Hayward, CA, USA) and photographed on a UV transilluminator (ChampGel 500, SAGECREATION, Beijing, China).

2.3. RAPD analysis and identification of isolates by 16S rRNA gene sequence

All 480 RAPD fingerprints were analyzed by the Bionumerics version 6.6 software package (Applied Maths, Sint-Martens-Latem, Belgium), and the dendrogram was generated using representative profiles after clustering. The similarities and differences among the RAPD fingerprints were calculated by using the Pearson correlation and a dendrogram was generated using the unweighted pair group method with arithmetic average (UPGMA). The identity level (80%) for different genotypes discrimination is represented by a dotted line.

Each biotype was identified by 16S rRNA gene sequence analysis. In order to amplify the 16S rRNA gene, the pair of oligonucleotide primers 27F (5′-AGAGTTTGAT CCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) were used (Liu et al., 2016). The PCR had a volume of 25 μL containing 10x Thermo buffer (Takara, Japan), 2.5 mM $MgCl_2$, 200 mM dNTP (Takara, Japan), 1U Taq polymerase (Takara, Japan), 100 ng template DNA and 1.0 μM primer of each 27F and 1492R. The amplification reaction was performed under the following conditions: preliminary denaturation for 5 min at 94 °C, then 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min, and final extension at 72 °C for 10 min.

The PCR products (about 1.4 kb) were sequenced by a commercial sequencing facility (Shanghai Sangon Biotechnology Corporation, Shanghai). The 16S rRNA gene sequences were used to identify the isolates using EzTaxon (http://www.ezbiocloud.net; Yoon et al., 2017). Species were grouped and identified at a homology level of 98%.

2.4. Statistic analysis

The results obtained for psychrotrophic bacterial count (PBC) in raw milk samples were expressed as log CFU/mL, and used for the calculation of mean values, standard deviation and significance of difference between different farms using one-way ANOVA (SPSS, version 20).

3. Results

3.1. Psychrotrophic bacterial count

Sixteen raw milk samples collected from different regions were analyzed for PBC. It is a common practice to store raw milk for 2 days after immediately collected from farms. In order to explore the effect of storage at refrigerated temperature for 2 days, PBC of each raw milk sample stored for 2 days was also determined. The results are shown in Table 1. The values of PBC before any storage ranged from 3.81 to 4.27 log CFU/mL. The mean for the 16 samples was 4.1 log CFU/mL. Only 4 samples out of 16 showed value less than 4 log CFU/mL whereas, the other 12 samples had counts greater than 4 log CFU/mL. The PBC after 2 days storage was increased and reached a maximum of 4.9 log CFU/mL.

3.2. Results of gram staining and catalase tests

Among all the isolates, *Brochothrix thermosphacta*, *Carnobacterium maltaromaticum*, *Microbacterium oxydans* and *Staphylococcus epidermidis* were Gram-positive bacteria, which accounted for 1.7% of the total isolates. All the isolates were catalase-positive except *C. maltaromaticum*.

3.3. Distribution of RAPD profiles and identification of isolates

All 480 psychrotrophic bacteria from 16 raw milk samples were

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