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Antimicrobial resistance of *Lactobacillus* spp. from fermented foods and human gut

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ABSTRACT

A total of 122 *Lactobacillus* strains were isolated from household-prepared or commercial yogurts and pickles in Sichuan, among which 15 representative strains, along with another 37 strains isolated from human guts, were analyzed for their antimicrobial resistance (AMR) to 17 clinically significant antimicrobials applied in China by agar dilution method. The correlation of AMRs in *Lactobacillus plantarum* from fermented foods and human gut was examined. The AMRs varied with *Lactobacillus* species. For *L. plantarum*, the distributions of minimum inhibitory concentration to 12 antimicrobials were correlated between food and gut source ($P < 0.05$), and the percentages of resistant strains from foods and gut were also similar ($P > 0.05$) except to norfloxacin, where the AMR was more severe in gut than in foods ($P < 0.05$), while all 14 high-level vancomycin-resistant strains were from gut. Illustrated by L. *plantarum*, the severity of resistance to most antimicrobials in lactobacilli from fermented foods and human gut gradually converged, with gut ones taking the leading role. Accordingly, AMR in human gut lactobacilli was more likely the consequence of clinical medication instead of acquired from fermented foods. *Lactobacillus* strains widely utilized in fermented foods would unlikely aggravate their AMR in human guts.

1. Introduction

Antimicrobial resistance (AMR) has become a serious health threat (WHO, 2014). Today, the annual death rate associated with resistant bacteria is estimated to be 700,000; by 2050, AMR could cause as many as 10 million deaths per year unless effective policies are implemented to stop the spread of AMR. Moreover, the rise of AMR infections could cost the world up to 100 trillion US dollars by 2050 (O'Neill, 2016).

Lactic acid bacteria (LABs) are extensively utilized in the food industry (Schjorring & Krogfelt, 2011). They are commensal probiotics among human gut microflora and have acquired the "generally recognized as safe" (GRAS) status. However, LABs may also serve as a reservoir of mobile AMR genes that can be transferred to pathogenic or opportunistic pathogenic bacteria in the food chain and the gastrointestinal (GI) tract (Danielsen & Wind, 2003; Toomey, Bolton, & Fanning, 2010).

Lactobacillus is the largest genus of LABs (da Silva Sabo, Vitolo, González, & Oliveira, 2014). Intrinsic resistance of *Lactobacillus* strains to a variety of antimicrobials has been reported (Abriouel et al., 2015; Sharma, Tomar, Goswami, Sangwan, & Singh, 2014). Such antimicrobial tolerance presents a minimal potential risk of horizontal spread and can be introduced into food chain (EFSA, 2012). The mobile AMR gene transfer from *Lactobacillus* strains has gained considerable attention, as AMR and AMR gene transfer from foodborne lactobacilli have been observed in a number of studies (Devirgiliis, Zinno, & Perozzi, 2013; Feld et al., 2008; Jacobsen et al., 2007; Klare et al., 2007; Mathur & Singh, 2005; Muñoz, Benomar, Lerma, Gálvez, & Abriouel, 2014). AMR genes of *Lactobacillus* spp. are found to be transferred to other bacteria *in vitro* by filter mating or in gnotobiotic animal models (Danielsen, 2002; Feld et al., 2008; Jacobsen et al., 2007; Nawaz et al., 2011;

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Toomey et al., 2010). The failure of AMR gene transfer in more complex gastrointestinal environments indicates the interference from indigenous gut microbiota of the hosts (Egervärn, Lindmark, Olsson, & Roos, 2010; Feld et al., 2008). However, it is still not clear whether foodborne lactobacilli would aggravate the AMR of bacteria in the GI tract of humans. The correlation of AMR of *Lactobacillus* strains from fermented foods and human gut could provide a circumstantial evidence to this question.

For their recognized health benefits, yogurts consumption is increasing worldwide in recent decades, with lactobacilli being used in the major starter culture by dairy industry (van Reenen & Dicks, 2011). Sichuan pickles are traditional fermented foods recorded in the Chinese classic work "Important Arts for People's Welfare" and consumed on a daily basis by locals for about 3000 years in Sichuan province, China (Chen, Xia, Zhang, Li, & Yu, 2010). They are traditionally homemade in many households using natural lactic acid fermentation, with the dominant strains being lactobacilli (Ao, Zhang, Shi, & Zhang, 2011). Because typically lactobacilli are ingested at a very high dosage in Sichuan, this region in China is a preferable location to study the AMR correlation between food and gut strains.

In order to establish the potential role of *Lactobacillus* strains from fermented foods in AMR transfer to the microflora in human gut, *Lactobacillus* strains were isolated from main fermented foods, including yogurts and Sichuan pickles, and the gut strains of healthy human volunteers in Sichuan province. All the strains were examined for their AMR against clinically significant antimicrobials used in China and finally, the correlation of the two bacterial sources was evaluated.

2. Materials and methods

2.1. Bacterial strains

A total of 37 gut strains of *Lactobacillus* were isolated from fecal samples of healthy 20–60 years old adult men and women (nonpregnant and nonlactating) donors who satisfied the following conditions: i) free of digestive diseases and not on any antimicrobial in the last month; ii) with a generally healthy cardiovascular, cerebrovascular, liver, kidney, and hemopoietic system; iii) devoid of endocrine disorders; and vi) no psychosis. These gut strains were preserved by the Department of Medical Examination, West China School of Public Health, Sichuan University (Chen et al., 2007). Ethical approval was obtained from the Internal Ethical Review Board of the West China Hospital, Sichuan University.

The foodborne strains were isolated from 11 pickle samples (10 homemade and one commercial), and five yogurt samples of five popular commercial brands collected in Sichuan region. Ten-fold dilutions of pickle juice and yogurts were made in physiological saline. Liquid de Man, Rogosa and Sharpe (MRS, Oxoid Ltd., Basingstoke, Hampshire, UK) agar was poured into the plates and mixed with the dilutions. After the medium had solidified, the inverted plates were incubated at 30 °C (pickles samples) or 37 °C (yogurt samples) for 48 h in aerobic conditions. The colonies were randomly picked from MRS plates containing 30–300 colonies and streaked on the MRS agar repeatedly for purification at 37 °C. Gram-positive rods were stored at −20 °C in 30% (v/v) glycerol for further confirmation.

Total genomic DNA was extracted from cultures incubated at 30 °C or 37 °C for 48 h using EZNA™ Bacterial DNA Kit (Omega, Bio-Tek, Norcross, GA. USA). The 16S rRNA genes of the isolates were amplified using universal primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-TACGGCTACCTTGTTACGACTT-3′) with PCR master mix (TaKaRa Bio-Co., Shiga, Japan). The reaction conditions were as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min, and then a final extension 72 °C for 10 min. The PCR products of interest were isolated from the agarose gel using a Gel Extraction Kit (Omega, Bio-Tek, Norcross, GA. USA) and sequenced by Life Technologies Corporation.

Phylogenetic trees based on 16S rRNA sequences were constructed using neighbour-joining (NJ), and maximum likelihood (ML) methods along with 1000 bootstrapping replicates using MEGA 6.06 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). *L. plantarum* (LC177235) (Doi et al., 2013), *L. plantarum* subsp. plantarum (AB831182) (Li et al., 2015), *L. plantarum* (AB601179) (Tohno et al., 2012), *L. plantarum* (KP230424) (Kang, Shin, Kim, & So, 2016.), *L. fermentum* (CP002033) (Jiménez et al., 2010), *L. fermentum* (DQ779203) (Woo et al., 2007), *L. delbrueckii* subsp. bulgaricus (CP002341) (Sun et al., 2011), *L. alimentarius* (GU125458) (Yu et al., 2012), *L. acidophilus* (CP010432) (Iartchouk, Kozyavkin, Karamychev, & Slesarev, 2015) were used as the reference strains.

2.2. Antimicrobial susceptibility testing

Seventeen antimicrobial agents commonly used in antimicrobial therapy in China were included in this study, encompassing nearly all important classes including β-lactams (penicillin G, ampicillin, oxacillin, cefoxitin, ceftriaxone, meropenem and imipenem), macrolides (erythromycin), lincosamides (clindamycin), fluoroquinolones (norfloxacin, ciprofloxacin and levofloxacin), glycopeptides (teicoplanin and vancomycin), aminoglycosides (gentamicin), folate pathway inhibitors (trimethoprim), and tetracycline (Tao, Zhang, Xu, & Wu, 2012; Zhang, Ying, Pan, Liu, & Zhao, 2015). All antimicrobial agents were obtained from Sigma-Aldrich (St. Louis, MO, USA). The minimum inhibitory concentration (MIC) was determined for each strain by agar dilution method (CLSI, 2012) using the standardized LAB susceptibility test medium (LSM) agar formulation recommended by ISO 10932/IDF 223 (ISO 10932/IDF 233, 2010), essentially consisting of a mixture of Iso-Sensitest agar (Oxoid) (90%) and MRS agar (Oxoid) (10%) adjusted to pH 6.7 (Klare et al., 2005). The cation-adjusted Mueller-Hinton broth (CAMHB) with lysed horse blood suggested by CLSI (CLSI, 2010) was not used in this study because some *Lactobacillus* strains grew weakly or even failed to grow on CAMHB medium (Klare et al., 2005; Mayrhofer et al., 2014). The antimicrobials concentrations in plates ranged from 0.125 to 256 μg/mL. For MIC determination, diluted inocula of the strains with 0.9% NaCl solution was set to a turbidity of the 0.5 McFarland standard. The inoculated saline was diluted by 1:10 in LSM broth. An aliquot of each inoculum on the agar surface with multipoint inoculation apparatus (SAKUMA MIT-P60 type, Sakuma Ltd., Japan) reached a final concentration of 10^4 CFU/spot before the plates were incubated at 37 °C in aerobic conditions for 16–20 h. The MIC was defined as the lowest concentration of the antimicrobial agent that completely inhibits the growth of the tested strain, disregarding a single colony or a faint haze caused by the inoculums. Each test was performed in triplicate.

2.3. Data processing and statistical analysis

Data analysis was performed in SPSS software for windows (version 19.0, SPSS Inc., USA). The χ^2 test was employed to assess the population differences between resistant strains belonging to *Lactobacillus. plantarum* vs. *Lactobacillus fermentum* or those from fermented foods vs. human gut, as well as their specific ratios at a given MIC. Correlations between MIC distributions for *L. plantarum* strains from gut ($n = 27$) and foods ($n = 9$) were analyzed by using the Spearman's rank correlation coefficient. The adopted level of significance and high significance were 5% ($P < 0.05$) and 1%