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Comparative studies on antibiotic resistance in *Lactobacillus casei* and *Lactobacillus plantarum*

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ABSTRACT

The levels of resistance in 17 *Lactobacillus casei* isolates and 15 *Lactobacillus plantarum* isolates to 10 antibiotics were determined using a standardized macrodilution method and the presence/absence of 20 genes implicated in antibiotic resistance in these isolates was determined by polymerase chain reaction (PCR) using gene-specific primers; 11 isolates possessed one or more of these genes but they were not always associated with phenotypic resistance. *L. plantarum* isolates had the widest spectrum of MIC values for streptomycin ranging from 16 to 512 μg/mL. In particular, two isolates of *L. plantarum* IMAU60045 and IMAU80091 both possessed *aadA* and *ant(6)* genes implicated in resistance to streptomycin but varied in their tolerance to streptomycin as evidenced by their minimum inhibitory concentration (MIC) of 16 and 256 μg/mL, respectively. Selection of high streptomycin resistance of *L. plantarum* IMAU60045 was performed over a 30 day period using serial passage with regular increases in streptomycin concentration to reflect the changes in resistance levels. Final MIC value of 16,384 μg/mL was recorded which was 1024-fold higher than the original parental isolate. Furthermore, associated variable degrees of increase in the MIC value for gentamicin, kanamycin and neomycin illustrated that, under the challenge of streptomycin, cross-resistance to other structurally related antibiotics of the same class developed. The relative quantity of gene expression (RQ) for the streptomycin resistance gene *aadA* was 3.35 times greater after passage in increasing concentrations of streptomycin than the original parental isolate. This was greater than the increase in the RQ value for the streptomycin resistance gene *ant(6)* after passage.

1. Introduction

Lactic acid bacteria (LAB) are a diverse clade of Gram-positive bacteria that share the ability to produce lactic acid as an end product of carbohydrate fermentation; they are used widely in food production and preservation (Lahtinen et al., 2011). Of all the genera of LAB, *Lactobacillus* is the most economically important for human nutrition and for its probiotic properties (Tannock, 2005). There have been numerous studies on the commercial production and use of *Lactobacillus*-based probiotics but, because of their 'generally recognized as safe' (GRAS) status, basic toxicological and safety evaluations have been more limited (García-Fruitós, 2012).

Conjugation is the mechanism by which DNA (including resistance genes) can be transferred between different genera of bacteria (Thomas and Nielsen, 2005). While intrinsic resistance, such as resistance to vancomycin, is considered highly unlikely to spread horizontally in this way (Mathur and Singh, 2005), acquired resistance mediated by the acquisition of additional genes is known to spread laterally between bacteria (Ammor, Flórez, & Mayo, 2007). In fact horizontal or lateral transfer of resistance genes by conjugative plasmids or transposons, is common between bacteria in the gastrointestinal tract with the potential to occur between innocuous species and harmful pathogens. If beneficial *Lactobacillus* species have antibiotic resistance genes, then it is possible that those genes could be transferred to other microbiota, including

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pathogens, in the same niche (gastrointestinal tract); this has implications for the safe use of *Lactobacillus* species in the food industry (Scott, 2002; Van Reenen & Dicks, 2011).

Consequently, to determine the level of risk, it is necessary to identify whether any commonly used isolates of *Lactobacillus* species have phenotypic resistance to antibiotics and, if so, the level of resistance and the identity of the genes conferring it. Standardized methods have been developed to determine levels of antibiotic resistance and, as they allow comparisons of results between laboratories, they are recommended by a number of international agencies including the Clinical and Laboratory Standards Institute (CLSI), International Organization for Standardization (ISO), European Food Safety Authority (EFSA) and the International Dairy Federation (IDF). These methods allow the determination of Minimum Inhibitory Concentrations (MICs) defined as the lowest concentration of an antibiotic that will inhibit the visible growth of a microbe after overnight incubation; using defined cut-off values for experimentally determined MICs, LAB can be categorized as 'susceptible' or 'resistant' to each antibiotic tested (Andrews, 2001).

PCR with gene-specific primers can be used to detect the presence of genes known to contribute to antibiotic resistance (e.g. Ouoba et al., 2008) but, to verify exactly which of these genes (or even other genes) are actively contributing to the expression of resistance to a given antibiotic, it is necessary to select for phenotypically resistant isolates using antibiotic challenge, and compare their gene expression profiles with those of the original non-resistant parental isolate. Such research into the potential evolution of resistance in LAB is possible in the laboratory because of their rapid generation time; bacteria can be passaged for a predetermined time in media with a known antibiotic concentration to select for resistance mutants and then the expression of target genes compared with the original isolate. This is essential for the development of strategies for managing resistance. Recently, The evolution of antibiotic resistance in pathogens has been widely studied due to its clear threat to human health (Lee et al., 2010; Toprak et al., 2010; Wong et al., 2012); however, such research on lactic acid bacteria has been largely neglected.

The objective of this study was to quantify current levels of antibiotic resistance in isolates of *Lactobacillus casei* and *Lactobacillus plantarum* from China using a standardized macrodilution method to determine MICs for each antibiotic, and PCR on total genomic DNA to detect the presence/absence of genes with the potential to confer antibiotic resistance. Furthermore, potential roles of streptomycin resistance genes actively contributing to resistance were identified in an experimental isolate of *L. plantarum* by a combined use of selection of high streptomycin resistance of the isolate and quantitative real-time PCR.

2. Materials and methods

2.1. Bacterial isolates and culture conditions

We used 17 isolates of *L. casei* and 15 isolates of *L. plantarum* from different food sources and from different regions of China and Mongolia (Table 1). The *L. casei* isolates studied were all from the Tibet Autonomous Region of China and isolated from either fermented cow's milk or fermented yak's milk. The *L. plantarum* isolates had more diverse origins, coming from five Autonomous Regions/Provinces in China or from Mongolia; they were isolated from sauerkraut and a range of fermented dairy products (Table 1). *Lactobacillus paracasei* isolate ATCC334 and *L. plantarum* isolate ATCC14917 were also used as quality control reference isolates to validate the precision and accuracy of the susceptibility testing procedure and the performance of the reagents used (antibiotics, culture medium, solvents) according to ISO10932/IDF223 guidelines (IDF and ISO, 2010). All isolates were lyophilized in a cryoprotectant and stored at 4 °C in the culture collection of the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University and had been identified by partial 16s rDNA sequencing analysis (Liu et al., 2010; Sun et al., 2010).

Each isolate was activated in sterile (121 °C for 7 min) 10% (w/v) skimmed milk fortified with 1% (w/v) yeast extract. Following activation each isolate was propagated further in sterile (121 °C for 15 min) de man, Rogosa, Sharpe broth (M.R.S. broth; OXOID CM0359) or on M.R.S. agar (OXOID, CM0361) prior to evaluation.

Table 1
Isolates of *Lactobacillus casei* and *Lactobacillus plantarum* used in this study.

Species	Strain	Region	Source
Lactobacillus casei	IMAU60006	Dongjiao, Tibet	Fermented cow's milk
	IMAU60015	Dongjiao, Tibet	Fermented cow's milk
	IMAU60017	Dongjiao, Tibet	Fermented cow's milk
	IMAU60023	Niangdui, Tibet	Fermented cow's milk
	IMAU60032	Chongzi, Tibet	Fermented cow's milk
	IMAU60062	Jiangdang, Tibet	Fermented cow's milk
	IMAU60063	Jiangdang, Tibet	Fermented cow's milk
	IMAU60074	Luoma, Tibet	Fermented yak's milk (Kurut)
	IMAU60097	Sangxiong, Tibet	Fermented yak's milk
	IMAU60103	Sangxiong, Tibet	Fermented yak's milk
	IMAU60108	Sangxiong, Tibet	Fermented yak's milk
	IMAU60126	Longren, Tibet	Fermented yak's milk
	IMAU60127	Longren, Tibet	Fermented yak's milk
	IMAU60136	Longren, Tibet	Fermented yak's milk
	IMAU60160	Namucuo, Tibet	Fermented yak's milk
	IMAU60161	Namucuo, Tibet	Fermented yak's milk
	IMAU60165	Namucuo, Tibet	Fermented yak's milk
Lactobacillus plantarum	IMAU60042	Bazha, Tibet	Fermented cow's milk
	IMAU60045	Bazha, Tibet	Fermented cow's milk
	IMAU60051	Bazha, Tibet	Fermented cow's milk
	IMAU30043	Yili, Xinjiang	Fermented mare's milk (Koumis)
	IMAU30116	Yili, Xinjiang	Fermented mare's milk
	IMAU10014	Baiyinxile, Inner Mongolia	Fermented mare's milk
	IMAU10015	Baiyinxile, Inner Mongolia	Fermented mare's milk
	IMAU10016	Baiyinxile, Inner Mongolia	Fermented mare's milk
	IMAU40072	Tianjun, Qinghai	Fermented yak's milk
	IMAU40089	Gangcha, Qinghai	Fermented yak's milk
	IMAU80002	Xinjin, Sichuan	Sauerkraut
	IMAU80007	Suchang, Sichuan	Sauerkraut
	IMAU80091	Ganxi, Sichuan	Sauerkraut
	IMAU20014	Arixiyatusumu, Mongolia	Fermented cow's milk
	IMAU20697	Ulanbator, Mongolia	Fermented mare's milk

Table 2
Desired final concentration range for each antibiotic to be used to determine MIC values and the solvent used to dissolve each antibiotic.

Antibiotic	Range (μg/mL)	Solvent
Ampicillin	0.03125–64	Phosphate buffer, pH 6.0
Chloramphenicol	0.125–256	95% Ethanol
Ciprofloxacin	0.25–256	LSM
Erythromycin	0.0625–16	Glacial acetic acid
Gentamicin	0.25–256	LSM
Kanamycin	2–2048	LSM
Neomycin	0.25–512	LSM
Streptomycin	0.5–1024	LSM
Tetracycline	0.125–128	LSM
Vancomycin	0.125–512	LSM