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Characterization and *in vitro* properties of *Lactobacillus plantarum* and *Leuconostoc mesenteroides* for probiotic potential and nitrite degradation

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ABSTRACT: In the present work, the nitrite degradation activity and probiotic potential of heterofermentative *Leuconostoc mesenteroides* (LM) and homofermentative *Lactobacillus plantarum* (LP) were demonstrated. The potential probiotic properties of these bacteria were determined *in vitro* based on their antimicrobial activity, antibiotic resistance, aggregation properties, hydrophobicity, survival under simulated gastrointestinal tract conditions, and hemolytic activity. The results suggested that these two lactic acid bacteria (LAB) species possess hydrophobicity as they exhibited microbial adhesion to xylene, chloroform, and ethyl acetate. The two strains also showed general resistance to simulated gastric and intestinal conditions. The LM was resistant to erythromycin, chloramphenicol, norfloxacin, and vancomycin, while the LP showed resistance to clindamycin, tetracycline, erythromycin, norfloxacin, and vancomycin. Both LAB were efficient antimicrobials toward *Escherichia coli* and *Staphylococcus aureus* based on their inhibition zones. Furthermore, these LAB showed good tolerance toward nitrite, and displayed α- and γ-hemolysis. These results suggest that LM and LP are promising probiotic candidates.

KEYWORDS: probiotics, gastro-intestinal resistance, antibiotic susceptibility, nitrite degradation

INTRODUCTION

Probiotics are generally defined as a live microorganisms which, when administered in adequate amounts confer a health benefit on the host[1]. LAB, one of the known probiotic microorganisms, have a widely use in various fermented products, such as pickles, bean paste, natto, miso, jiaosu. These microbial groups possess many host-associated functions including anti-cholesterol activity, anti-oxidant activity and so on[2–4]. Currently, LAB strains possessing various functions are applied in the global commercial production of probiotic fermented foods, but demand remains for further bio-functional products as well as increased implementation and diversification[5]. Therefore, it is necessary to select new strains with certain functional properties to respond to the emerging consumer demand.

LAB is divided into heterofermentative strains and homofermentative strains according to the type of fermentation they employ. Among these, heterofermentative LAB such as *L. mesenteroides*, which mainly produce CO_2, organic acid, and flavor substances as metabolic products, are only predominant during the early stages of fermentation and are responsible for the high sensory quality of fermented products[6]. The subsequent stages of fermentation are dominated by homofermentative LAB such as *L. plantarum*, which produces mostly acids. Fermentation can be controlled to improve product quality and prolong the product shelf life with the help of these two different types of LAB (homofermentative and heterofermentative). In recent studies[7–9], the dominance of *Lactobacillus* and *Leuconostoc* spp. during enset fermentation was reported and these LAB are likely important because they can contribute to the sensory characteristics and preservative effects[10]. It is reported that the main benefits of using microorganisms in fermented products are: (1) extending shelf life; (2) improve nutritional value; (3) enhance organoleptic food quality; and (4) improve food safety by inhibiting pathogens or by decomposing toxic compounds such as nitrite[11].

Nitrite is the precursor of N-nitroso compounds. As we know, the augment of nitrite content is an unavoidable problem during vegetable fermentation[12]. Nitrite may be involved in N-nitrosamine formation by N-nitrosation reactions with dietary-derived amines in the stomach[13]. It is reported that nitrite leads to potential risks of suffering from some diseases such as methemoglobinemia and gastric cancer by long-term ingestion of food containing nitrite[14]. Kim et al[15,16] reported that the N-nitrosodimethylamine levels produced by LAB can be reduced by decreasing the level of precursors such as nitrite and biogenic amines, as well as through direct depletion or degradation of nitrite in De Man, Rogosa, and Sharpe (MRS) broth containing N-nitrosodimethylamine or $NaNO_2$, respectively. Thus, LAB has been one of the efficient strategies to degrade nitrite.

The objective of the current study was to investigate the probiotic potential of these two strains and properties of nitrite degradation in order to be used as starter culture.

MATERIALS AND METHODS

Bacterial cultures and media

L. mesenteroides (LM) and *L. plantarum* (LP) from Qingdao Agricultural University were grown in MRS broth (Beijing Land Bridge Technology Co., Ltd.) at 30 °C for 24 h and pathogenic bacteria (*E. coli* CICC 24189 and *S. aureus* CICC 10384) were cultured in nutrient broth (Beijing Solarbio Science & Technology Co., Ltd) at 37 °C for 24 h.

Antagonistic activities

Antagonistic activities against pathogens (*E. coli* and *S. aureus*) were tested by the agar-well diffusion method[17]. Briefly, the LABs were collected by centrifugation (2107 × *g*, 10 min, 4 °C) and then filtered by 0.22 μm filter membrane to obtain cell-free supernatant (CFS). The pathogens were grown in nutrient broth at 37 °C for 24 h. The concentration of the pathogens was adjusted to approximately 106 CFU/ml and then incorporated into LB medium (Beijing Land Bridge Technology CO., Ltd.). Each CFS was transferred into Oxford cup which was put on the surface of the agar, respectively. After incubated at 37 °C for 24 h, the diameter of the inhibition zone was measured. Both LABs against *E. coli* CICC 24189, *S. aureus* CICC 10384 were performed in duplicate.

Antibiotic resistance

Antibiotic resistance for LAB was determined as described previously[18,19]. Exactly, the following 8 antimicrobial agents (Shanghai Ryon Biological Technology Co., Ltd) were used: ampicillin, chloramphenicol, streptomycin, tetracycline, erythromycin, clindamycin, norfloxacin, and vancomycin. Antibiotics were diluted serially in appropriate differently diluents. The antibiotics in MRS broth were adjusted, ranging from 0.5 to 1024 μg/ml with two times concentration gradient. MRS broth containing different concentrations of antibiotics was added into each well. The LAB strains grown overnight at 37 °C were approximately diluted to 0.6 at OD600, equivalent to the McFarland standard 0.5, and then prepared to each well of the microtiter plates. After incubation for 24 h at 37 °C, minimal inhibitory concentration (MIC) was obtained in triplicate in accordance with OD600.

Tolerance to simulated gastric and intestinal conditions

All methods were based on those described previously[19] with a slight modification. Simulated gastric condition was prepared by suspending 3 g/l pepsin (Shanghai Aladdin Bio-Chem Technology Co., Ltd) in a sterile 0.5% NaCl (w/v) solution. 1 M HCl was used to adjust to pH 3.5. 1 g/l pancreatin (Shanghai Aladdin Bio-Chem Technology Co., Ltd) and 0.15 or 0.3% bile salts (Shanghai Aladdin Bio-Chem Technology Co., Ltd, w/v) were suspended in a sterile 0.5% NaCl (w/v) solution. Then 1 M NaOH was used to adjust to pH 8.0. Then it was filtered through 0.22 μm filter membrane to obtain simulated intestinal juice. Following, the overnight culture of LAB strains was harvested by centrifugation (10 000 × *g*, 5 min, 4 °C), then washed twice and resuspended in phosphate buffered saline (PBS, pH 7.4). Each 200 μl bacterial suspension was incorporated into 1 ml simulated gastric juice together with 300 μl sterile 0.5% NaCl (w/v) solution. The mixture was cultured for 120 min at 37 °C. Then, survivor cells from gastric juice were harvested by centrifugation (6000 × *g*, 5 min, 4 °C), then washed twice and resuspended in phosphate buffered saline (PBS, pH 7.4). Similarly, each 200 μl bacterial suspension was incorporated into 1 ml simulated intestinal juice together with 300 μl sterile 0.5% NaCl (w/v) solution. The mixture was cultured for 120 min at 37 °C. Viable counts were determined by plating a serial dilution on MRS agar plates.

Aggregation property

Autoaggregation and coaggregation assays were performed according to Zuo et al[20]. The overnight culture of LAB was harvested by centrifugation (6000 × *g*, 10 min), then washed twice and resuspended in PBS and adjusted OD600 = 1.0. And

0.1 ml of upper suspension was mixed with 1.9 ml PBS and OD600 was measured after incubation at 37 °C for 2 h. Percent autoaggregation was expressed as 1− (OD600 of upper suspension/OD600 of total bacterial suspension) × 100.

Bacterial suspensions were prepared as described above. LAB bacterial suspensions (1 ml) were mixed with 1 ml *E. coli* CICC 24189 bacterial suspensions and OD600 was determined immediately (designated A_0). After incubation at 37 °C for 2 h, OD600 was determined again (designated as A_t). Percent co-aggregation was expressed as $(A_0 - A_t)/A_0 \times 100$.

Bacterial adhesion to hydrocarbons

The cell surface hydrophobicity test was measured based on the previous work[21].

Hemolytic activity

Fresh LABs were streaked on Columbia agar plates, containing 5% (w/v) human blood (Michopoulos S.A., Athens, Greece), and incubated for 48 h at 30 °C according to preceding study[22].

Tolerance capability on nitrite

The tolerance capability on nitrite assays were determined with the N- (1-naphthyl)-ethylenediamine dihydrochloride spectrophotometric method in the light of previous study[23]. Percent degradation was expressed as $(OD_i - OD_t)/OD_i \times 100$, where OD_t represents absorbance at time t and OD_i ($OD_{0.2}$, $OD_{0.4}$, $OD_{0.6}$, $OD_{0.8}$, $OD_{1.0}$) represents absorbance at 0.2, 0.4, 0.6, 0.8, 1.0 mg/ml nitrite.

Statistical analysis

All experiments were performed in triplicate. The results are presented as mean ± SD. The data of tolerance capability on nitrite was performed using OriginPro 9.1 software (OriginLab Corp., US).

RESULTS

Antagonistic activities and antibiotic resistance

In this study, two LABs were tested for antimicrobial activity toward *E. coli* and *S. aureus*. As indicated in Table 1, the CFS of the selected LABs expressed a clear inhibition zone (LM, 1.74 and 2.60 mm; LP, 1.65 and 2.65 mm) against the two indicators, respectively.

LAB is considered to be antibiotic-resistant when it shows a MIC value higher than the MIC breakpoints established by the European Food Safety Authority (EFSA). As shown in Table 1, neither of the LAB revealed sensitivity toward ampicillin or streptomycin. However, the LM was resistant toward erythromycin, chloramphenicol, norfloxacin, and vancomycin, while the LP showed resistance toward clindamycin, tetracycline, erythromycin, norfloxacin, and vancomycin.

Tolerance to simulated gastrointestinal conditions

Probiotics can be used to restore microbial homeostasis in the gut. Important criteria for the selection of probiotics are that following ingestion, they must maintain cellular integrity and they must retain their beneficial metabolic functions[24]. During passage through the gastrointestinal tract, probiotic cells face hostile, antimicrobial conditions, namely low pH and the presence of bile salts, pepsin or pancreatin. Here, we tested the tolerance of the two LABs toward simulated gastric and intestinal conditions (Table 2). The secretion resembling gastric juice had a profound effect on the viability of the LAB. The survival rate of the strains in the simulated gastric conditions varied from more than 6–18% over a period of 2 h. The physiological concentration of human bile ranges from 0.1–0.3%, and the survival rate of the LABs under simulated intestinal conditions with 0.15% and 0.3% bile salts varied from 0.6 ± 0.7% to 11.4 ± 0.7% after 2 h (Table 2). The low survival of the two selected LABs under these conditions may be related to the bile salts' capacity to alter the structure of cell membranes, which is toxic to bacterial cells[25].

Autoaggregation and hydrophobicity

In this study, the cell surface properties of autoaggregation and hydrophobicity were tested. The two selected LAB strains exhibited high autoaggregation after incubation for 2 h at 37 °C (Table 3). High autoaggregation contributes toward LAB strains reaching a high density in gastrointestinal tract, enabling their plentiful biological functions such as anti-cholesterol and anti-oxidant activity[2–4].

Cell surface hydrophobicity methods were mainly measured the bacterial adhesion to a certain hydrophobic substratum rather than intrinsic microbial cell surface hydrophobicity[26]. In this work, xylene, chloroform and ethyl acetate were used as hydrophobic substratum. The bacterial adhesion to chloroform and ethyl acetate were regarded as a measure of electron donor/basic and electron acceptor/acidic characteristics of bacterial surface, respectively[27]. As shown in Table 4, both strains

Table 1 Antimicrobial activity and antibiotic resistance of the LAB.

LAB	MIC (μg/ml)								Inhibitory zone (cm)	
	A	CL	S	T	E	C	N	V	*E. coli*	*S. aureus*
L. mesenteroides	1	1	128	32	4[R]	16[R]	16[R]	>1024	1.74	2.60
L. plantarum	1	2[R]	64	32[R]	2[R]	4	16[R]	>1024	1.65	2.65

[R] Antibiotic resistance according to EFSA's breakpoints (EFSA, 2008). A, ampicillin; C, chloramphenicol; S, streptomycin; T, tetracycline; E, erythromycin; CL, clindamycin; N, norfloxacin; V, vancomycin.

Table 2 Effect of simulated gastric juice and simulated intestinal juice on the viable counts of the LAB at different incubation times.

LAB	Gastric		SC[†]	Intestinal
	60 min	120 min	(%)	120 min
L. mesenteroides	13.5 ± 2.4	6.3 ± 0.8	0.15	1.0 ± 0.9
			0.30	0.6 ± 0.7
L. plantarum	18.3 ± 6.2	12.5 ± 5.1	0.15	11.4 ± 0.7
			0.30	7.6 ± 0.7

[†] SC, % survival concentration of bile salts.
Results are shown as average percentages of survived bacteria (%) ± SD.

Table 3 Autoaggregation and coaggregation activities of the LAB.

LAB	Autoaggregation	Coaggregation	
	2 h	2 h	24 h
L. mesenteroides	78.78 ± 2.53	24.24 ± 0.30	44.67 ± 1.74
L. plantarum	76.65 ± 0.23	19.19 ± 0.51	45.72 ± 5.19

Results are shown as average percentages (%) ± SD.

showed high affinity toward all three of the solvents used in this study, ranging from 76.94 ± 2.10% to 94.89 ± 0.76%.

Coaggregation and hemolytic activity

The coaggregation ratios between the LABs and *E. coli* are also shown in Table 3. As expected, the percentage of coaggregation increased with time over the 24 h tested. The coaggregation of the LM and LP with *E. coli* was 44.67 ± 1.74% and 45.72 ± 5.19%, respectively, after 24 h.

Hemolytic activity is commonly assayed to assess the safety of probiotic bacteria[5]. As displayed in Table 4, the two strains showed α- and γ-hemolysis when grown in Columbia blood agar. Neither of the examined strains exhibited β-hemolytic activity when grown in Columbia blood agar.

Table 4 Hydrophobicity and hemolytic activity of the LAB.

LAB	Xylene	Chloroform	Ethyl acetate	HA[†]
L. mesenteroides	85.03 ± 1.44	76.94 ± 2.10	87.94 ± 0.21	α
L. plantarum	94.89 ± 0.76	91.33 ± 0.07	93.35 ± 2.18	γ

[†] HA, hemolytic activity.
Results are shown as average percentages (%) ± SD.

Tolerance toward nitrite

The tolerance of the two strains toward nitrite is displayed in Fig. 1. As shown in Fig. 1a, the nitrite depletion rate of LM was maintained above 70% after 48 h when the nitrite concentration was 0.2 mg/ml. Notably, the LP strain exhibited good levels of nitrite degradation of above 70% after 24 h (Fig. 1b).

DISCUSSION

When probiotics are applied to the gastrointestinal tract, they produce miscellaneous components that can kill pathogens, enhance epithelial barrier function, and modulate immune responses[28]. In addition, LAB are used to improve the safety and shelf-life of minimally processed foods such as sliced apples and lamb's lettuce[29]. Therefore, the antagonistic activity of the probiotic candidate toward specific microbes is extremely important. The two selected LAB exhibited obvious antimicrobial activity, and this activity may be related to the production of hydrogen peroxide or organic acids such as lactic acid. Several researchers[30] have confirmed the effect of the antimicrobial activity of organic acids on Gram-positive and Gram-negative pathogenic bacteria. This antimicrobial activity is probably also related to bacteriocins produced by LAB. Several studies[31] have reported that bacteriocin combined with physicochemical treatments with reagents, such as organic acids or phenolic compounds, could strengthen antimicrobial activity toward Gram-negative bacteria.

Microbial cell autoaggregation ability ensures that the probiotic reaches a high cell density in the gut. Coaggregation with a potential pathogen allows the probiotic strain to inhibit effectively the

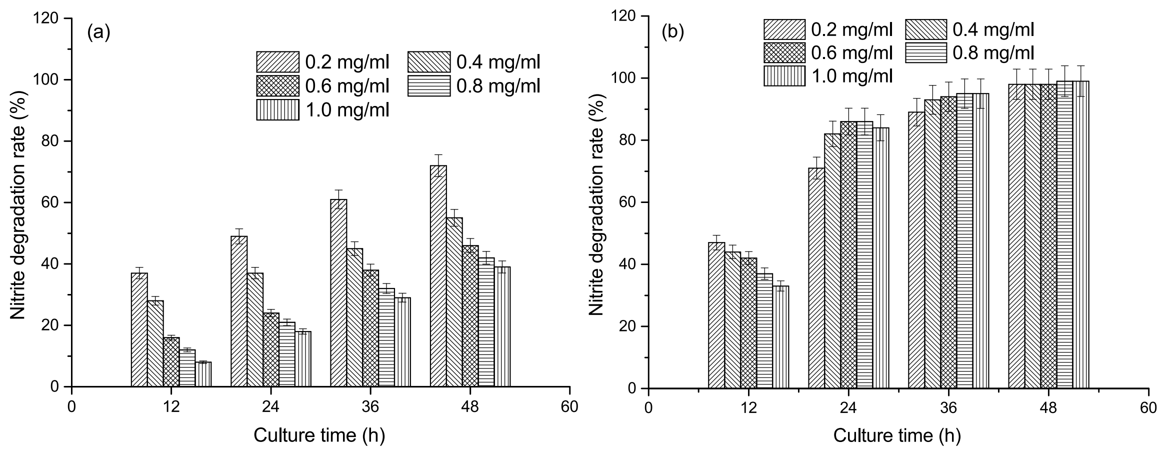

Fig. 1 Tolerance capability of (a) *L. mesenteroides* and (b) *L. plantarum* on nitrite in MRS broth.

growth of pathogenic strains and facilitate its elimination through feces in the gastrointestinal tract. Recently, it was demonstrated that aggregation of probiotic strain effectively produced antimicrobial substances[32], suggesting that autoaggregation and coaggregation are closely associated with the antagonistic effect of LAB.

The absence of hemolytic activity is considered a safety prerequisite for the selection of probiotic strains. It is known that some bacterial species produce hemolysin, which can damage human red blood cells. Hemolysin activity produces a halo around bacterial colonies when cultured on media supplemented with blood. Based on this, hemolytic activity can be divided into α-hemolytic (green halo) as a result of the production of hemoglobin, β-hemolytic (transparent to opaque halos of 2–4 mm) caused by complete hemolysis of red blood cells, or γ-hemolytic (no halo) due to the absence of any hemolytic activity. In this study, both strains showed α-hemolysis and γ-hemolysis, indicating safety for food application.

The fact that a high percentage of LM and LP cells adhered to xylene, chloroform and ethyl acetate, demonstrated hydrophobic cell surface of this strain. Many previous studies on the physicochemistry of microbial cell surfaces have shown that the presence of (glyco-) proteinaceous material at the cell surface results in higher hydrophobicity, whereas hydrophilic surfaces are associated with the presence of polysaccharides[33]. It is known that only pronase- and pepsin-sensitive surface molecules are responsible for cell surface hydrophobicity in bacteria. Hydrophobic cell surface structure of two strains is also to be further studied[33].

The antibiotic resistance mechanisms of bacterial strains include reduced antibiotic intake, increased antibiotic elimination, modifications of the target site of antibiotics, and hydrolysis or modification of the antibiotics[34]. Furthermore, Antibiotic resistance in lactic acid bacteria is mainly distinct from intrinsic and acquired resistance. Intrinsic resistance is an inherent characteristic; for example, *L. mesenteroides* and *L. plantarum* are characterized as having intrinsic resistance to vancomycin, which is not transferable to other species and strains[35]. The reason for the LAB in the present work being resistant to vancomycin may be the result of the replacement with D-Ala-D-lactate instead of the normal D-Ala-D-Ala cell wall precursor, which is the target of the antibiotic[36]. However, the intrinsic resistance of LAB toward antibiotics is not considered a risk to animal and human health[37]. LAB use in this work, antibiotic resistance is probably caused by antibiotic resistance genes or drug efflux mechanisms or even gene mutations, with the two strains showing resistance to erythromycin, chloramphenicol, norfloxacin/erythromycin, norfloxacin, tetracycline, and clindamycin. Furthermore, for some genera of lactic acid bacteria, there are no generally accepted standard procedures for MIC determination and information on MIC ranges is rather limited. It is matter of debate whether LAB considering generally regarded as safe should be resistant or sensitive against antibiotics. What's more, resistance of the probiotic to some antibiotics could be used for both preventive and therapeutic purposes in controlling intestinal infections[21]. Therefore, further reason is also to be studied.

Nitrite intake is a serious human health problem that should not be ignored. Nitrite intake in the human diet mainly comes from vegetables but nitrite can be depleted through the fermentation of pickled vegetable products. Notably, nitrite content tends to vary over a wide range. For example, it was reported that 218 samples of pickled vegetables in northeast China had nitrite content[38] ranging from 0.01–42.03 mg/kg. Researchers[39] have used *L. pentosus* as a starter culture to ferment oyster mushrooms, and their results suggested a nitrite content ranging from 1.85 ± 0.71 to 5.69 ± 0.58 mg/kg. Therefore, it is necessary to investigate the tolerance of probiotic toward nitrite. In the present study, the LP strain exhibited higher nitrite degradation than the LM strain. This may be related to differences in pH and enzymes. Nitrite degradation can occur via nitrite reductase degradation or acid degradation, with the latter being more efficient[40]. *L. mesenteroides* mainly produces CO_2, organic acid, and flavor substances as metabolic products, is predominantly active during the early stages of fermentation, and is responsible for the high sensory quality of fermented products. *L. plantarum*, in contrast, produces mostly acids; therefore, *L. plantarum* exhibits higher nitrite degradation levels than *L. mesenteroides*.

CONCLUSION

In the present study, probiotic potential of two different LAB, namely *L. mesenteroides* and *L. plantarum* was demonstrated. Both strains exhibited high properties based on antimicrobial activity, antibiotic resistance, aggregation, hydrophobicity and hemolytic activity. Meanwhile, we also investigated the tolerance toward nitrite for both strains, indicating that LAB could be used to control nitrite content.

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