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Probiotic properties of *Lactobacillus pentosus* GP6 isolated from fermented ground pork

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Abstract

Lactobacillus spp. is one of the lactic acid bacteria with probiotic properties. This strain plays important roles for many fermented foods. *Lactobacillus pentosus* GP6 isolated from fermented ground pork showed antimicrobial potential against bacterial gastrointestinal pathogens, including Enteropathogenic *Escherichia coli* (EPEC) DMST 30546, *Pseudomonas aeruginosa*, *Salmonella* Typhimurium and *Shigella sonnei*. It was susceptible to all tested antibiotics except vancomycin. *L. pentosus* GP6 survived in low pH and was tolerant to bile salt. It exhibited good growth in simulated gastric and intestinal tracts. This strain had a good autoaggregation and a high surface hydrophobicity. Moreover, this lactobacillus showed coaggregation with EPEC DMST 30546 to affect its adhesion and colonization. *L. pentosus* GP6 showed high adhesion ability to Caco-2 cell line and the adhesion of EPEC DMST 30546 to this cell line was decreased in the presence of this lactobacillus. Overall, the results indicated *L. pentosus* GP6 is a potential probiotic for dietary supplement formulations.

Keywords: *fermented ground pork, gastrointestinal pathogens, GP6, Lactobacillus pentosus, potential probiotic*

1. Introduction

Lactic acid bacteria (LAB) are typically involved in a large number of spontaneous food fermentations. There has been much recent interest in the use of various strains of LAB as probiotics. Probiotics are defined as live microorganisms that when administered in adequate amounts, confer beneficial effects on the health of the host (FAO/WHO, 2001). These health benefits include prevention of pathogens infection (Juárez Tomás, Ocaña, Wiese, & Nader-Macías, 2003; Saunders, Bocking, Challis, & Reid, 2007; Lin, Tsai, Lin, Tsen, & Tsai, 2008), alleviation of lactose intolerance (Marteau, Seksiik, & Jian, 2002), reduction of the risk associated with colon cancer (Iannitti & Palmieri, 2010), reduction of serum cholesterol level (Nguyen, Kang, & Lee, 2007) and modulation of the immune system (Oelschlaeger, 2010).

Lactobacillus pentosus GP6 used in this research was isolated from fermented ground pork that was purchased from Muang Ake Plaza market, Patumthani, Thailand. This strain was identified by Gram staining, catalase test, oxidase test and confirmed by 16S rDNA analysis by the National Center for Genetic Engineering and Biotechnology, Thailand. The 16S rDNA gene analysis showed a 100% probability of identity to *L. pentosus* strains available in the GenBank database system. Furthermore, *L. pentosus* GP6 was registered in the GenBank database system under accession number KT004663.

Before probiotic strains exert their beneficial effects on the host, they have to show probiotic potential characteristics. The microorganisms selected as probiotic strains must be able to survive during passage through the gastrointestinal tract of the host. They should have high acid and bile tolerance properties (Erkkila & Petaja, 2000; Zarate, Chaia, Gonzalez, & Oliver, 2000), inhibit infectious pathogens (Røssland, Langsrud, Granum, & Sørhaug, 2005), exhibit intrinsic and non-transmissible antibiotic resistance genes (Curragh & Collins, 1992; Salminen et al., 1998), exhibit self-aggregation (Del Re, Sgorbati, Miglioli, & Palenzona, 2000; Kos et al., 2003), and coaagregation with pathogens (Bao et al., 2010), adhere to host epithelial cells and interference with the adherence of pathogens (Merk, Borelli, & Korting, 2005; Ren et al., 2012). *In vitro* models involving human intestinal epithelial cell lines, mostly Caco-2 cells have been used to assess the adhesion properties of potential probiotic strains in several publications (Messaoudi et al., 2012; Tulini, Winkelströter, & De Martinis, 2013).

2. Objectives

This study aimed to assess the probiotic properties of *Lactobacillus pentosus* GP6 isolated from fermented ground pork. Antibacterial activity, antibiotics resistance, survival ability in gastrointestinal simulation model, aggregation characteristics and adhesion activities were included.

3. Materials and methods

3.1 Bacterial strain

L. pentosus GP6 was kept at -80°C in Mann Rogosa and Shape (MRS) broth (Difco, USA) containing 20% (v/v) glycerol as a cryoprotectant. Bacterial gastrointestinal pathogens, including Enteropathogenic *Escherichia coli* (EPEC) DMST 30546, *Pseudomonas aeruginosa*, *Salmonella* Typhimurium and *Shigella sonnei* were stored as stock solutions in brain heart infusion (BHI) broth (Difco, USA) containing 20% (v/v) glycerol at -80°C.

3.2 Inhibition of bacterial gastrointestinal pathogens

To examine the antimicrobial activity of *L. pentosus* GP6 against the selected bacterial gastrointestinal pathogens, a spot-on-lawn method was used as previously described by Santos et al. (2016). Briefly, *L. pentosus* GP6 was spotted (5 µl) on the surface of MRS agar in a 10 cm plate and incubated anaerobically at 37°C for 48 h. One ml of a 24 h tested pathogen culture (10^8 cfu/ml) was mixed with 100 ml of BHI soft agar (1% (w/v) agar). Four ml of the mixture was poured into the plate to cover the lactobacilli spots and incubated in aerobic conditions at 37°C for 24 h. After incubation, the inhibition zones were measured. Gentamicin (10 µg/ml) was used as positive control. Each test was performed in triplicate.

3.3 Antibiotic susceptibility assay

The antibiotics used for the antibiotic susceptibility assay were ampicillin, ceftazidime, chloramphenicol, clindamycin, doxycycline, erythromycin, gentamicin, kanamycin, neomycin, streptomycin, tetracycline and vancomycin. The antibiotic susceptibility of *L. pentosus* GP6 was determined by the disc diffusion method (Boyle, Fancher, Ross, & Jr, 1973). *L. pentosus* GP6 was grown with MRS broth at 37°C for 48 h in anaerobic conditions, then this culture (10^8 cfu/ml) was inoculated onto MRS agar plate. The discs were placed on the inoculated MRS agar plate. Inhibition zones after incubation were measured using an antibiotic zone reader (Fisher-Lilly, USA), and interpreted to be either susceptible (S), moderately susceptible (MS), or resistant (R).

3.4 Survival under conditions simulating the human gastrointestinal tract

The ability of *L. pentosus* GP6 to grow under low pH conditions, the presence of bile and the simulation of the human GI tract were tested as previously described method (Maragkoudakis et al., 2006). Briefly, overnight culture of this strain (18 h) culture was harvested by centrifugation (2,000 × g, 5 min, 4°C) and washed once with phosphate buffered saline (PBS, Sigma-Aldrich, USA), pH 7.4. The cell pellet was then resuspended (10^8 cfu/ml) in the following tested solutions including PBS pH 2, 3 and 4, PBS, pH 7.4 supplemented with 0.1, 0.2, 0.3 and 0.4% (w/v) oxgall (Sigma-Aldrich, USA), simulated gastric fluid pH 2, 3 and 4 (SGF, 3 g/l pepsin) (Sigma-Aldrich, USA) and simulated intestinal fluid pH 6.8 and 8 (SIF, 1 g/l pancreatin) (Sigma-Aldrich, USA). The resistance to low pH solutions and SGF were tested under incubation of 37°C for 0, 1, 2 and 3 h, and the bacterial cell growth in bile solution and SIF were incubated for 0, 1, 2, 3 and 4 h. The survival ability of *L. pentosus* GP6 in these conditions was exhibited in terms of viable colony count on MRS agar after the treatment. Survival rates were calculated according to the following equation:

$$\text{Survival rate (\%)} = (N_1/N_0) \times 100\%$$

N_1: The total viable count of bacterial cell after treatment

N_0: The total viable count of bacterial cell before treatment

3.5 Aggregation test

3.5.1 Autoaggregation

The autoaggregation of *L. pentosus* GP6 was determined (Pascual et al., 2008). Bacterial cells (18 h culture) were resuspended in PBS, pH 6.2 (10^8 cfu/ml). The cell suspension was dropped onto a glass slide and observed with a light microscope. Autoaggregation was considered positive if the cells aggregated within 2 min.

3.5.2 Surface hydrophobicity

The surface hydrophobicity of the bacterial cells was assayed by the salt aggregation test (SAT) (Andreu, Stapleton, Fennell, Hillier, & Stamm, 1995). *L. pentosus* GP6 cells were resuspended in

0.02 mol/l of sodium phosphate (Merck, Germany), pH 6.8 (10^8 cfu/ml). Cell suspension were mixed with an equal volume of solutions of ammonium sulfate (Merck, Germany) at 4.0, 2.0, 1.5 and 0.5 mol/l on glass slides. The lowest final concentration of ammonium sulfate that caused the bacteria to aggregate was defined as the SAT value. The value of < 0.9 mol/l, 0.9 - 1.5 mol/l and > 1.5 mol/l was defined as a high surface hydrophobic, intermediate hydrophobic and hydrophilic, respectively.

3.5.3 Coaggregation

L. pentosus GP6 was tested for its ability to coaggregate with pathogens (Reid et al., 1990). The suspension of this strain and EPEC DMST 30546 (10^8 cfu/ml) was mixed and then incubated in a 24-well tissue culture plates (Corning Inc, USA) for 4 h at 37°C with gentle agitation. The treated cells were Gram-stained. Coaggregation was observed under a light microscope.

3.6 Adhesion to Caco-2 cells

Caco-2 cells (ATCC HTB 37™) were purchased from the American Type Culture Collection (USA) and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) with L-glutamine, 20% (v/v) fetal bovine serum, 100 U/ml of penicillin G and 100 µg/ml streptomycin sulfate under 5% (v/v) CO_2 at 37 °C. Cells (4.5 x 10^5 cells/ml) were seeded into 24-well tissue culture plates and maintained for 21 d to allow for good differentiation (Tuomola & Salminen, 1998). The cells were washed twice with PBS, pH 7.4. One milliliter of *L. pentosus* GP6 suspension (10^8 cfu/ml in antibiotic-free DMEM) was added into each well and incubated under 5% (v/v) CO_2 at 37 °C for 1 h. The treated cells were washed twice with PBS, pH 7.4 to remove the unbound bacterial cells. Cell monolayers were detached with 0.05% (v/v) Triton X-100 (Sigma-Aldrich, USA) at 37 °C for 5 min. Adherent bacterial cells were enumerated by plate counting on MRS agar. The experiment was carried out in triplicate. The adhesion percentage was calculated according to the following equation:

Adhesion (%) = (N_1/N_0) x 100%

N_1: Amount of adherent bacterial cells

N_0: Amount of added bacterial cells

3.7 Inhibition of pathogens adhesion to Caco-2 cells

L. pentosus GP6 and EPEC DMST 30546 (10^8 cfu/ml in antibiotic-free DMEM) were used in the inhibition of the adhesion assay. *L. pentosus* GP6 was added simultaneously with EPEC DMST 30546 and incubated for 1 h to assess the competition (Gagnon, Kheadr, Le Blay, & Fliss, 2004). To examine the exclusion of the pathogens by *L. pentosus* GP6, *L. pentosus* GP6 was added before EPEC DMST 30546. Conversely, EPEC DMST 30546 was added before *L. pentosus* GP6 to determine the displacement of the pathogens by *L. pentosus* GP6. The monolayer cells with the first strain was incubated for 30 min. Furthermore, the second strain was added and incubated for another 30 min (Lee et al., 2000). After incubation, the adherent EPEC DMST 30546 were enumerated by plate counting on McConkey agar (Difco, USA).

3.8 *Statistical analysis*

All data were expressed as a mean ± SD. The Student's *t* test was used for statistical analysis by comparing treatment groups versus the control group. Results were regarded as statistically significant at $p < 0.05$.

4. Results

4.1 Inhibition of bacterial gastrointestinal pathogens

L. pentosus GP6 exhibited antimicrobial activity against tested pathogens, i.e. EPEC DMST 30546, *P. aeruginosa*, *S.* Typhimurium and *S. sonnei* (Table 1). The activity was significantly higher than gentamicin. The highest inhibitory activity was observed against EPEC DMST 30546, followed by *S.* Typhimurium, *S.* Typhimurium and *P. aeruginosa*.

Table 1 Antibacterial activity of *L. pentosus* GP6 on bacterial gastrointestinal pathogens

Pathogens	Inhibition zone (mm) ± SD	
	L. pentosus GP6	Gentamicin (10 µg/ml)
EPEC DMST 30546	70.00±0.10*	23.00±0.10
P. aeruginosa	32.67±0.12*	17.00±0.00
S. Typhimurium	39.33±0.12*	8.67±0.06
S. sonnei	38.00±0.20*	8.67±0.06

*The mean difference is significant at 0.05 when compared with gentamicin.

4.2 Antibiotic susceptibility

L. pentosus GP6 was sensitive to most tested antibiotics, including ampicillin, ceftazidime, chloramphenicol, clindamycin, doxycycline, erythromycin, gentamicin, kanamycin, neomycin, streptomycin and tetracycline. However, this strain showed resistance to vancomycin.

4.3 Survival under conditions simulating the human gastrointestinal tract

The resistance testing of *L. pentosus* GP6 to acid (Figure 1A) showed that after 3 h there was still more than 98% survival at pH 2 and 3, but there was no change in numbers at pH 4.

The tolerance of *L. pentosus* GP6 in the present of bile salts (oxgall) was tested (Figure 1B). The results showed that was able to survive at all bile salt concentrations tested (up to 0.3%) to give an exponential growth from the inoculation (0 h) until 4 h of incubation.

Resistance of *L. pentosus* GP6 to SGF and SIF is shown in Figure 1C and 1D, respectively. When exposed to SGF (pepsin) at every tested pH there were still more than 96% viable cells after 3 h. Exposure to pancreatin (SIF) at either pH 6.8 or 8 had virtually no effect on viable cell numbers but increased the growth of this strain gradually up to 137%.

4.4 Aggregation property

L. pentosus GP6 showed good autoaggregation within 2 min. This strain also aggregated at 0.5 mol/l ammonium sulfate. This result exhibited *L. pentosus* GP6 was also classified as having a high surface hydrophobicity with a SAT value < 0.9 mol/l. To determine coaggregation, *L. pentosus* GP6 was able to coaggregate with EPEC DMST 30546.

4.5 Adhesion of *L. pentosus* GP6 and inhibition of pathogen adhesion to Caco-2 cells

L. pentosus GP6 adhered well (98.06%) to Caco-2 cells. and inhibited the adhesion of EPEC DMST 30546 to Caco-2 cells. On the other hand, the adhesion of EPEC DMST 30546 to Caco-2 cells was only 86.64%. Following the examination of inhibition of pathogen adhesion, *L. pentosus* GP6 was shown to significantly reduced the adhesion of EPEC DMST 30546 to Caco-2 cells by 6.95, 8.79 and 6.25% in the competition, exclusion and displacement assays, respectively (Figure 2).

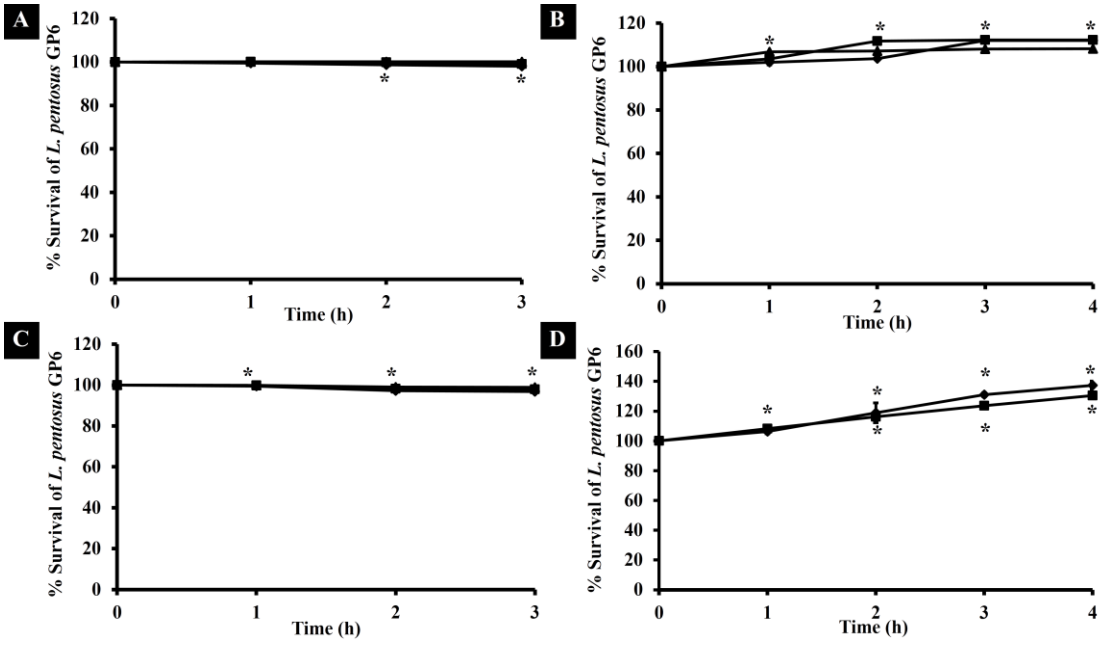

Figure 1 Survival of *L. fermentum* SK5 under condition simulating human GI tract: the presence of acid (A): pH 2 (♦), pH 3 (■), pH 4 (▲), bile-containing buffer (B): 1% oxgall (♦), 2% oxgall (■), 3% oxgall (▲), simulated gastric fluid

(C): SGF pH 2 (♦), SGF pH 3 (■), SGF pH 4 (▲), simulated intestinal fluid (D): SIF pH 6.8 (♦), SIF pH 8 (■). * = $p < 0.05$ (compared with before treatment).

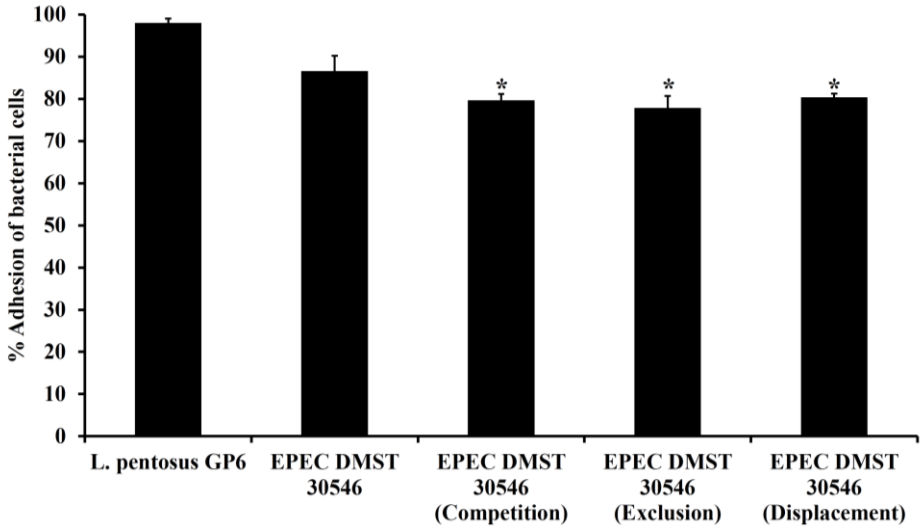

Figure 2 Adhesion of *L. pentosus* GP6 and inhibition of EPEC DMST 30546 adhesion to Caco-2 cells. * = $p < 0.05$ (compared with EPEC DMST 30546 alone).

5. Discussion

L. pentosus GP6 isolated from fermented ground pork showed desirable characteristics for a probiotic strain. This strain exhibited high broad spectrum of antimicrobial activity, inhibiting EPEC DMST 30546, *P. aeruginosa*, *S.* Typhimurium and *S. sonnei*. Similar results have been seen in several related publications. Many LABs are known to produce inhibitory substances to inhibit pathogens (Santos et al., 2016; Fadda, Mossa, Deplano, Pisano, & Cosentino, 2017).

The antibiotic resistance profile is one of the most important properties to select effective a probiotic strain. *L. pentosus* GP6 were exhibited resistances to vancomycin. The genus *Leuconostoc*, *Pediococcus* and *Lactobacillus* are intrinsically resistant to glycopeptides such as vancomycin (Nelson, 1999). According to Tulumoglu et al. (2013) and Lee et al. (2014), lactobacilli species were inherently resistant to vancomycin. These data corroborate the results obtained with respect to *L. pentosus* GP6 antibiotic resistance profile and could be used to recommend its safety as a probiotic candidate.

In order to select isolates with probiotic characteristics, the resistance to gastric acidity, bile salts pepsin and pancreatin is an importance factor in survival and growth of bacteria in the gastrointestinal tract. The pH of the stomach generally ranges from pH 2.5 to 3.5 and the concentration of 0.15-0.3% of bile salt has been recommended as a suitable concentration for selecting probiotics bacteria for human use (Holzapfel, Haberer, Snel, Schillinger, & Huis in't Veld, 1998; Erkkila & Petaja, 2000). The results from this study showed that *L. pentosus* GP6 was tolerant to acid (pH 2-4) and bile (0.1-0.3%). In addition, this strain could survive when tested in SGF and SIF. These results are related to a previous study, where *L. plantarum* DGK-17 isolated from Kimchi was able to survive under low pH and high bile salt conditions and simulated gastric juice environment (Khan & Kang, 2016).

Autoaggregation is necessary for colonization potential of lactobacilli in the environments and prevention of pathogens. The hydrophobic nature of the surface of microorganisms has been also shown to be involved in the attachment of bacteria to host tissues. Adherent lactobacilli show a high surface hydrophobicity, whereas nonadherent lactobacilli are much more hydrophilic (Rosenberg, Gutnick, & Rosenberg, 1980). In addition, coaggregation leads to the formation of a

barrier that prevents colonization by pathogens (Boris, Suárez, Vazquez, & Barbés, 1998). In the present study, *L. pentosus* GP6 exhibited autoaggregation and a high surface hydrophobicity. These characteristics showed that *L. pentosus* GP6 possessed high potential capability to adhere to mucosal surfaces and epithelial cells. A similar finding was reported by Angmo, Kumari, Savitri, and Bhalla (2016), where they demonstrated that LAB isolated from fermented foods and beverage of Ladakh possessed high autoaggregation and hydrophobicity. Furthermore, *L. pentosus* GP6 coaggregated with tested gastrointestinal pathogens c. The coaggregation could be an important factor that interferes with the adhesion ability of the pathogens. Caggia, De Angelis, Pitino, Pino, and Randazzo (2015) also reported that the thirteen selected *Lactobacillus* strains could coaggregate with the tested pathogen *E. coli* 555.

The ability to adhere to intestinal mucosa is considered as an important selection criterion for LAB intended to be used as a probiotic (Rinkinen, Westermarck, Salminen, & Ouwehand, 2003). The adhered probiotics also prevent the pathogenic adhesion to intestinal cells (Lee, Puong, Ouwehand, & Salminen, 2003). Caco-2 cells used in this study are derived from colon carcinomas and represent the typical characteristics of enterocytic differentiation found in the human intestinal mucosa (Gopal, Prasad, Smart, & Gill, 2001). *L. pentosus* GP6 was able to adhere to Caco-2 cells (98.06%). This indicated that *L. pentosus* GP6 has the potential to be used as a probiotic strain. The result was in agreement with previous study that *L. fermentum* strains isolated from Tunisian camel raw milk exhibited an important level of adhesion to human Caco-2 (Mahmoudi et al., 2016). In addition, *L. pentosus* GP6 prevented adhesion of EPEC DMST 30546 to Caco-2 cells by competitive, exclusive, and displacement mechanisms which were reported earlier (Vidhyasagar & Jeevaratnam, 2013; García-Ruiz et al., 2014).

6. Conclusion

In conclusion, *L. pentosus* GP6 exhibited good activity against bacterial gastrointestinal pathogenic EPEC DMST 30546, *P. aeruginosa*, *S.* Typhimurium and *S. sonnei*. It was susceptible to the majority of the antibiotics tested and survived under conditions simulating the human gastrointestinal tract. In addition, this strain had good aggregation properties, adhered to an intestinal cell line and inhibited the adhesion of pathogens to these cells. It is likely that *L. pentosus* GP6 could be a good probiotic candidate for application in dietary supplements.

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8. References

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