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ORIGINAL ARTICLE

In vitro evaluation of probiotic potential of *Pediococcus pentosaceus* L1 isolated from paocai—a Chinese fermented vegetable

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Abstract This study aimed to investigate the probiotic potential of *Pediococcus pentosaceus* L1 isolated from paocai, a Chinese fermented vegetable. In vitro analysis revealed that *P. pentosaceus* L1 had the capability to tolerate simulated gastrointestinal juices. Adhesion of *P. pentosaceus* L1 to HT-29 intestinal epithelial cells (IEC) was also observed. L1 was sensitive to ampicillin, gentamycin, kanamycin, strepomycin, clindamycin, tetracycline and chloramphenicol. L1 showed effective inhibition against *Escherichia coli*, *Salmonella typhimurium*, and *Shigella flexneri*. *P. pentosaceus* L1 also exhibited the abilities of auto-aggregation and co-aggregation with *Shigella flexneri*. Pretreatment of HT-29 IEC with *P. pentosaceus* L1 prior to tumor necrosis factor-alpha (TNFα) challenge down-regulated the expression of pro-inflammatory genes, such as *IL8*, *CCL20*, *CXCL10*, *and CXCL1*. The level of IL-8 released in culture supernatant of TNFα-challenged HT-29 IEC was reduced by strain L1, confirming the observed decrease in TNFα-induced IL-8 mRNA expression. These results indicate the probiotic potential of *P. pentosaceu*s L1, and that this strain could be used to produce functional foods.

Keywords *Pediococcus pentosaceus* · Gastrointestinal tolerance · Antimicrobial activity · Immunomodulatory activity · Chemokines · Probiotics

Zhenhui Cao and Hongbin Pan contributed equally to this work.

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Introduction

Probiotics are defined by the Food and Agriculture Organization/World Health Organization (FAO/WHO 2006) as "live microorganisms, which when administered in adequate amounts confer a health benefit on the host". Mechanisms by which probiotics influence host health have been suggested to act at three levels: interacting with other microorganisms present on the site of action, strengthening mucosal barriers, and affecting the immune system of the host (Leroy et al. 2008). The gastrointestinal tract (GIT) is the site where probiotics are believed to exert most health-modulating activities. Thus, probiotics should have the ability to survive the harsh conditions of GIT such as low pH in the stomach (Maragkoudakis et al. 2006), digestive enzymes, and the bile of the small intestine (Begley et al. 2005). Tolerance to GIT conditions is an important selection criterion for probiotic candidates. In addition to surviving transit through the GIT, adherence to intestinal epithelial cells (IECs) should be assessed as probiotics may provide health benefits by

competitive exclusion of enteric pathogens and interaction with intestinal mucosa (Collado et al. 2007; Lebeer et al. 2008).

Lactic acid bacteria (LAB), particularly certain strains of the genera *Lactobacillus* and *Bifidobacterium*, are the most commonly used probiotics (Saxelin et al. 2005). *Pediococcus pentosaceus* are categorized as LAB because they utilize sugar and produce lactic acid as a major end product. They are found commonly in naturally fermented foods and beverages including fermented sausage (Cocolin et al. 2011), cheese (Carafa et al. 2015), pickles (Halami et al. 2005) and wine (García-Ruiz et al. 2014), for their roles in rapid acidification (Abrams et al. 2011) and control of spoilage as bacteriocin producers (Carafa et al. 2015). Most of these organisms are also known to have the ability to survive in the gastrointestinal conditions in vitro (Maragkoudakis et al. 2006; Monteagudo-Mera et al. 2012; Garsa et al. 2014) and in vivo (Fernandez et al. 2003; Hwanhlem et al. 2010). More than those properties, *P. pentosaceus* have received increasing attention with recently reported healthy effects on the immune system (Masuda et al. 2010), enteric pathogens invasion (Vidhyasagar and Jeevaratnam 2013), obesity and lipid liver (Zhao et al. 2012), cholesterol metabolism (Ilavenil et al. 2015), and cancer (Shukla and Goyal 2014).

Paocai (artisan pickled radish) is a Chinese fermented vegetable rich in LAB (Feng et al. 2012). *P. pentosaceus* is found commonly in paocai (Huang et al. 2009; Gong et al. 2014). However, little is known about the probiotic potential of *P. pentosaceus* derived from paocai. The objective of this study was to evaluate in vitro the probiotic potential of *P. pentosaceus* strain L1 derived from paocai. This strain showed good survival in simulated gastrointestinal juices and effective adhesion to HT-29 IEC. *P. pentosaceus* L1 also had antimicrobial activity on indicator organisms as well as immunomodulatory capability at the intestinal mucosal level. These observations suggest that *P. pentosaceus* L1 exhibits probiotic properties and suggests its potential for incorporation into functional foods.

Materials and methods

Isolation and identification of *P. pentosaceus* L1 from paocai

Artisan paocai (pickled radish; 10 g) from Kunming, China, was homogenized in phosphate buffer saline (PBS) for 3 min using JC-T homogenizer (Luoheiintian Test Equipment Institute). The homogenized suspension was serially diluted in PBS and poured into sterile Petri dishes onto de Man-Rogosa-Sharpe (MRS) agar (Oxoid; http://www.oxoid.com) containing 3 mg ml^{-1} $CaCO_3$ and subjected to incubation at 37 °C for 24 h. Bacterial colonies that exhibited a clear zone on the plates were individually picked and streaked on MRS agar containing 3 mg mL^{-1} $CaCO_3$. Based on morphological differences in colony color, shape and gloss, LAB strains were selected for further tests. Each isolates was first tested for catalase by placing a drop of 3 % (w/v) hydrogen peroxide solution on the cells. Immediate formation of bubbles indicated the presence of catalase in the cells. Only those isolates that were catalase-negative were stained using the Gram staining method, and then Gram-positive isolates were stored in MRS broth containing 25 % (v/v) glycerol at −20 °C.

Genomic DNA extracted using a genomic DNA extraction reagent kit of bacteria (SK8225, Sangon Biotech, Shanghai, China) following lysozyme treatment served as template for PCR; 16S rDNA was amplified by PCR using the universal 16S rDNA primers 27f (AGAGTTTGATCCTGGCTCAG) and 1492r (TACGGCTACCTT GTTACGACTT). PCR products electrophoresed through 1 % agarose gels and DNA bands corresponding to 1450 bp of the16S rDNA amplicon were extracted and sent to Sangon Biotech for DNA sequencing. The partial 16S rDNA sequence was submitted to the GenBank database and compared to similar sequences by BLAST analysis. The 16S rDNA sequences were aligned with the most similar sequences and the sequences of other representative bacteria using Clustal X (Thompson et al. 1997). A phylogenetic tree of the 16S rDNA sequences was constructed with MEGA 4.0 software using the neighbor-joining method (Tamura et al. 2007). Based on morphology, physiological and chemical assays, and 16S rDNA sequences, 35 LAB strains were isolated. In pilot studies, six LAB strains showed greater resistance to simulated gastrointestinal juices. Of these six LAB isolates, *P. pentosaceus* strain L1 was chosen to assess probiotic potential, and its 16S rDNA sequence was deposited with GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under accession number KP792278.

In vitro resistance to simulated gastrointestinal juices

Cells of *P. pentosaceus* L1 from a 24-h incubation were harvested by centrifuging at 10,000 *g* for 10 min at 4 °C. Cells were washed twice with PBS and resuspened in the same buffer. Then, 40 μL 10^8 CFU mL^{-1} bacterial suspension was mixed with 3960 μL simulated gastric juice [125 mmol L^{-1} NaCl, 7 mmol L^{-1} KCl, 45 mmol L^{-1} $NaHCO_3$, and 0.3 % (w/v) pepsin pH 2.0 adjusted with HCl]. Tolerant LAB were assessed in terms of viable colony counts and enumerated after incubation at 37 °C in water bath for 1, 2 or 3 h, which mimicked the gastric transit time for humans.

After 3 h exposure to stimulated gastric juice, the strain was harvested by centrifugation at 10,000 *g* for 10 min at 4 °C and washed twice with PBS before being resuspended in 4 mL simulated small intestinal juice [0.1 % (w/v) pancreatin and 0.3 % (w/v) bovine bile salts]. Tolerance was assessed in terms

of viable colony counts, and enumerated after incubation at 37 °C in water bath for 1, 2, 3 or 4 h.

In vitro adhesion assay

The human cell line HT-29 IEC (ATCC HTB-38) was cultured in RPMI 1640 supplemented with 10 % (v/v) fetal bovine serum, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Applied Biosystems, Foster City, CA). HT-29 IEC were propagated routinely in 25-cm^2 tissue culture flasks at 37 °C in a humidified, 5 % CO_2 incubator (Thermo Scientific, Waltham, MA) until they approached 80–90 % confluence, and were used between passages 10 and 25. To prepare for treatment with *P. pentosaceus* L1 strain, 2×10^4 HT-29 IEC were inoculated into a six-well culture plate (Corning, Sigam, St. Louis, MO) and incubated for 48 h at 37 °C in a humidified, 5 % CO_2 incubator. The broth culture of *P. pentosaceus* L1 was centrifuged at 10,000 *g* for 10 min at 4 °C and the pellets were resuspended in RPMI 1640 medium (HyClone, http://www.gelifesciences.com) without serum and antibiotics. Then, 2 ml of the bacterial suspension (2×10^7 CFU mL^{-1}) was added to each well of a six-well cell culture plate and allowed to incubate with HT-29 IEC for 1 h at 37 °C, 5 % CO_2. HT-29 human IEC were then washed with DPBS to remove non-adherent bacteria and lysed by incubation for 15 min with 0.1 % (v/v) Triton X-100. The lysates were then diluted and plated onto MRS agar to determine the number of adherent bacteria. The adhesion capacity was recorded as the percentage of adherent bacteria of the bacteria applied.

Antibiotic sensitivity testing

The antibiotic susceptibility of *P. pentosaceus* L1 was evaluated according to the technical guidelines of the European Food Safety Authority (EFSA 2012). The minimal inhibitory concentrations (MIC) of nine antibiotics, including ampicillin, vancomycin, gentamycin, kanamycin, streptomycin, clindamycin, tetracycline and chloramphenicol (Sigma-Aldrich), were determined as described by Ryu and Chang (2013). Overnight culture of *P. pentosaceus* L1 in MRS broth were centrifuged at 10,000 *g* for 10 min at 4 °C and resuspended in Mueller-Hinton (MH) broth (Oxoid) containing 0.5 % (w/v) dextrose. The resultant cell suspensions were then further diluted in the same medium to a final concentration of 5.0 log CFU/mL. Each antibiotic was added to aliquots of the diluted cell suspension, which were incubated at 30 °C for 24–48 h without shaking. Cell growth was observed visually and measured based on the turbidity of the suspensions at 600 nm (UNICO 2100, UNICO Instruments, Shanghai, China). MIC values were determined using the serial antibiotic dilution procedure in MH broth containing 0.5 % (w/v) dextrose.

Antimicrobial activity

Effects on the growth of indicator enteropathogen were determined using the agar well diffusion method according to Tejero-Sariñena et al. (2012) with simple modifications. *P. pentosaceus* L1 was incubated in MRS broth at 37 °C for 24 h. The broth culture was centrifuged at 10,000 *g* for 10 min at 4 °C, and cell-free culture supernatant (CFCS) was harvested. CFCS was then filtered through a Millex® 0.22 μm filter (Millipore; https://www.emdmillipore.com) and divided into four aliquots. One aliquot was not treated. One aliquot was adjusted to pH 6.5. One aliquot was adjusted to pH 6.5 followed by being heated at 100 °C for 15 min. One aliquot was adjusted to pH 6.5 followed by being incubated with 1 mg mL^{-1} proteinase K at 37 °C for 3 h. Then, 20 mL 1.2 % (w/v) of LB agar (Oxoid) or TSA (Oxoid) was mixed vigorously with 20 μL of an overnight culture of the indicator pathogen including *Escherichia coli* CMCC44825, *Shigella flexneri* CMCC(B)51592 and *Salmonella typhimurim* CMCC(B)50115 (Guangdong Huankai Microbial SCI. & TECH., Guangdong, China), poured into a Petri dish. Wells 8 mm in diameter were made in the agar using sterile steel cylinders. Then, 90 μL non-treated CFCS or treated CFCS was placed into each well and incubated at 37 °C for 24 h. The inhibition zone (in mm) was measured around each well, and the antimicrobial activity was expressed as the mean inhibition zone diameter.

Auto-aggregation and co-aggregation

Auto-aggregation and co-aggregation were tested as described by Zhang et al. (2011) with simple modifications. An overnight culture of *P. pentosaceus* L1 in MRS broth at 37 °C was centrifuged at 12,000 *g* for 10 min at 4 °C. The pellet was washed twice with PBS and then resuspened in PBS to approximately 10^8 CFU mL^{-1}. The mixture was vortexed for 10 s followed by incubation for 4 h at 37 °C and the absorbance was read at 600 nm. The auto-aggregation percentage was calculated as follows (Kos et al. 2003):

$$\%\,\text{autoaggregation} = [1-(At-Ao)] \times 100$$

where At is the absorbance at time t=4 h ,and Ao is the absorbance at time t=0 h.

For the co-aggregation assay, 2 mL aliquots of pairs of bacterial suspensions (*P. pentosaceus* L1 and *Shigella flexneri*) were vortexed for 10 s. Samples from the auto-

aggregation were also used as control tubes (4 mL aliquots of a single bacterial suspension). Co-aggregation was also determined at 4 h, as described above. Co-aggregation percentage was calculated as follows (Kos et al. 2003):

$$\%\,\text{coaggregation} = \frac{\left(\left(Ax + Ay\big/2\right) - A(x+y)\right)}{(Ax + Ay)\big/2} \times 100$$

where *Ax* and *Ay* are the individual aggregation properties of *P. pentosaceus* L1 and *Shigella flexneri*, and *A*(*x*+*y*), is the combined aggregation of *P. pentosaceus* L1 and *Shigella flexneri*.

IEC pre-treated with *P. pentosaceus* L1

A broth culture of *P. pentosaceus* L1 was centrifuged at 10, 000 *g* for 10 min at 4 °C and the pellets were resuspended in RPMI 1640 medium without serum and antibiotics. To prepare for treatment with *P. pentosaceus* L1 strain, 2×10^6 HT-29 IEC was inoculated into 25-cm^2 flasks and incubated for 48 h to establish confluent monolayers. Four million HT-29 IECs were treated for 16 h with 2×10^5 CFU *P. pentosaceus* L1. TNFα (Pepro Tech, Rocky Hill, CT) was added to a final concentration of 50 ng mL^{-1}, and incubation continued for 3 h, at which point HT-29 IEC were collected for RNA extraction. Six hours later, cell supernatants were collected and frozen at −80 °C until analysis of IL-8 concentrations.

Relative RT-qPCR

Transcript abundance of differentially expressed and reference genes was measured by RT-qPCR on cDNA. Prior to reverse transcription, 4 μg RNA was treated with RQ1 DNase (Promega, Madison, WI) as per the manufacturer's instructions. DNase-treated RNA (1 μg) was reverse transcribed using Superscript III (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The resulting cDNA was diluted 1:9 before amplification, and 2 μL diluted cDNA was used as template in RT-qPCR using 300 nmol gene-specific primers (Table S1) in a 20 μL reaction volume with SYBR® Premix Ex *Taq*™ (TaKaRa Biotechnology, Dalian, China). An initial incubation of 30 s at 95 °C was followed with 40 cycles consisting of template denaturation (5 s at 95 °C) and one-step annealing and elongation (30 s at 60 °C) with an ABI7500 Real-Time PCR System (Applied Biosystems). Melting curve analysis was used to determine amplification specificity. Reaction efficiency was determined with LinRegPCR (Ramakers et al. 2003), normalizing genes were evaluated with BestKeeper (Pfaffl et al. 2004), and relative expression and statistical analysis were conducted with REST2009 (Pfaffl et al. 2002).

Enzyme-linked immuno-sorbant assay

Levels of IL-8 were determined in tissue culture supernatants from HT-29 IEC (4×10^6) incubated for 16 h with 2×10^5 CFU *P. pentosaceus* L1 and then challenged with TNFα for 6 h. IL-8 concentrations were measured by enzyme-linked immuno-sorbant assay (ELISA) using a Human IL-8 ELISA Kit [Multisciences (LIANKE) Biotech, Hangzhou, China]. The optical density was read with a microplate reader (Sunrise, http://www.tecan.com) at 450 nm.

HT-29 IEC viability assay

HT-29 IEC viability was measured using trypan blue staining. The cells were washed with DPBS and detached from the surface using 0.25 % trypsin with EDTA. The cells were then resuspended in RPMI medium supplemented with 10 % fetal bovine serum. Trypan blue solution (0.4 %, w/v) was added in cell suspensions and incubated for 30 s at room temperature. Trypan blue/cell mixture (10 μl) was applied to the hemocytometer and unstained cells (live cells) were counted under the inverted microscope (XDS-37; Shanghai Optic Instrument Co., Shanghai, China).

Statistical analysis

Experiments were conducted in triplicate. Results are presented as the means±standard deviations (error bars) of replicate experiments. Microsoft Office Excel 2013 and GraphPad Prism were used to create graphs. The results from adhesion ability, auto-aggregation and co-aggregation and RT-qPCR assays were analyzed using Student's *t*-test. One-way ANOVA with Duncan's post-test was performed to determine statistical significance of the differences of data from ELISA and cell viability assay. $P<0.05$ was considered statistically significant.

Results

In vitro resistance to simulated gastrointestinal juices of *P. pentosacerus* L1

The ability to survive through gastrointestinal transit is one of the main desirable characteristics required for a probiotic. *P. pentosaceus* L1 isolated from artisan pickled radish survived exposure to simulated gastrointestinal juices for 7 h with a survival rate of 92.1 % (Fig. 1). *P. pentosaceus* L1 retained its viability with a negligible reduction (< 0.3-log) for up to 3 h exposed to simulated gastric juice. *P. pentosaceus* L1 showed a minor reduction (< 0.3-log) 4 h after exposure to simulated intestinal juice. Compared to before exposure to simulated gastrointestinal juice, viability reduced slightly with a minor

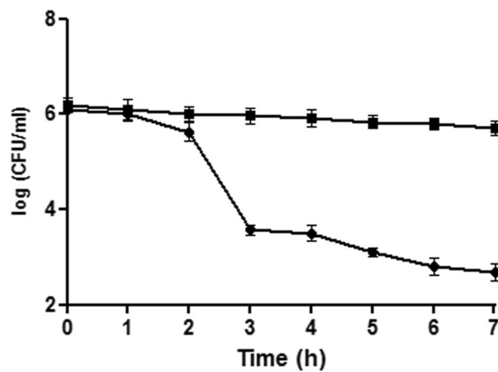

Fig. 1 In vitro resistance to simulated gastrointestinal juices. *Pediococcus pentosaceus* L1 (■) or LGG (●) was exposed to simulated gastric juices for 3 h, followed by simulated intestinal juices for 4 h. Data are presented as mean±SD (n=3)

0.5-log decrease. The reference strain *Lactobacillus rhamnosus* GG showed less tolerance (survival rate of 43.9 %) in simulated gastrointestinal juice during all the tested period than did *P. pentosaceus* L1.

Adhesion of *P. pentosaceus* L1 to HT-29 IEC

The adhesion of LAB to intestinal cells is an essential prerequisite for probiotic strains. In this study, *P. pentosaceus* L1 could adhere to HT-29 IEC. Compared to reference strain LGG (12.53±0.58), L1 showed significantly less capability to adhere to HT-29 IEC (6.24±0.46).

Antibiotic susceptibility

Although resistance of potential probiotics to antibiotics allows them to survive antibiotic treatment, the presence of potentially transferable antibiotic resistances to pathogenic bacteria in potential probiotics is of concern (Ammor et al. 2007). According to the microbiological breakpoints for antimicrobials defined by the *Panel on Additives and Products or Substances in Animal Feed* (FEEDAP) of the European Food Safety Authority (EFSA 2012), *P. pentosaceus* L1 were susceptible to ampicillin, gentamycin, kanamycin, streptomycin, clindamycin, tetracycline and chloramphenicol, except vancomycin (Table 1).

Antimicrobial activity of *P. pentosaceus* L1

The non-treated CFCS of *P. pentosaceus* L1 in MRS showed antibacterial activity against all the three indicator pathogens (*E. coli*, *Shigella flexneri*, *Salmonella typhimurium*) in the agar well diffusion assay (Table 2). The highest inhibitory activity was observed against the indicator strain *Shigella flexneri* with inhibition halo of 9.9 mm (data not shown). The CFCS of *P. pentosaceus* L1 adjusted to pH 6.5 showed antimicrobial activity against all three indicators comparable to non-treated CFCS. Proteinase K treatment diminished inhibitory impact of CFCS on growth of these pathogen strains. Moreover, the heated CFCS of *P. pentosaceus* L1 remained antimicrobial activity.

Auto-aggregation and co-aggregation

Auto-aggregation of probiotic strains is known to be a prerequisite for adhesion to intestinal epithelium. Our results showed that the auto-aggregating ability of *P. pentosaceus* L1 strain (18.3±3.15) was significantly less than that of LGG (25.2±2.03) after 4 h. Co-aggregation of *P. pentosaceus* L1 with *Shigella flexneri* was examined. *P. pentosaceus* L1 aggregated *Shigella flexneri* at 12.8±2.25, significantly greater than that of LGG with *Shigella flexneri* at 3.9±1.09.

Immunomodulatory effect of *P. pentosaceus* L1

In this study, *P. pentosaceus* L1 down-regulated gene expression of *IL8*, *CCL20*, *CXCL10* and *CXCL1* encoding pro-inflammatory chemokines stimulated by TNFα in HT-29 IEC (Fig. 2). Furthermore, we examined the level of IL-8 released in cell-free supernatant of HT-29 IEC pre-incubated with *P. pentosaceus* L1 prior to TNFα treatment. As shown in Fig. 3, strain L1 resulted in a significant decrease in IL-8 production relative to TNFα challenged control. *P. pentosaceus* strain L1 did not affect IL-8 production by HT-29 IEC co-cultured with *P. pentosaceus* alone (Fig. 3), suggesting that strain L1 does not induce an inflammatory response in IECs. To detect any toxic effect of tested *P. pentosaceus* L1 on HT-29 IEC, cell viability after incubation with the *P. pentosaceus* L1 was determined. *P. pentosaceus* L1 had no observed effect on viability relative to TNFα-challenged IECs (Fig. 4).

Discussion

Fermented vegetables have proved a good source of potential LAB probiotics since LAB naturally occupy these plant matrices as well as showing good gastrointestinal tolerance (Peres et al. 2012). Several studies have reported that *P. pentosaceus* originating from fermented vegetables showed probiotic potential such as higher resistance to acid and bile as well stronger adhesive ability to IEC (Ryu and Chang 2013), as well as antimicrobial activity against *Salmonella* species in mice (Chiu et al. 2008), and immunomodulation through affecting cytokine production (Jonganurakkun et al. 2008). In this study, we reported the probiotic potential of *P. pentosaceus* strain L1 isolated from paocai, suggesting that paocai could be a resource of potential LAB probiotics.

P. pentosaceus L1 showed the ability to survive when exposed to simulated gastrointestinal juices, in keeping with

Table 1 Susceptibility of *Pediococcus pentosaceus* L1 to antibiotics, presented as minimal inhibitory concentration (MIC) values

Antibiotic	Ampicillin	Kanamycin	Streptomycin	Gentamicin	Tetracyline	Erythromycin	Clindamycin	Chloramphenicol	Vancomycin
μg mL^{-1}	2	4	16	1	2	0.03	1	4	>512

recent studies demonstrating that certain *P. pentosaceus* strains originated from fermented vegetables have the ability to tolerate the gastrointestinal environment in vitro (Jonganurakkun et al. 2008; Ryu and Chang 2013; Shukla and Goyal 2014) and in vivo (Chiu et al. 2008). Acid adaption of LAB during vegetable fermentation may account for the reason why *P. pentosaceus* derived from these matrices show gastrointestinal tolerance (McDonald et al. 1990). A recent study conducted using genomic analysis has shown that the transit tolerance capability of *P. pentosaceus* strains may be attributed to genes associated with acid and bile tolerance present in the genome as found in *P. pentosaceus* strain LI05 isolated from the human GI tract (Lv et al. 2014), suggesting that molecular and genomic-based studies will likely provide useful insight into the mechanisms underlying adaptation to the GI environment in *P. pentosaceus* derived from fermented vegetables.

In addition to surviving passage through the GIT, adhesion of bacteria to epithelial cells is one of the key features for the selection of probiotics. *P. pentosaceus* strain L1 was able to adhere to HT-29 IEC with less potential than the reference strain LGG. Previous studies have reported that the adhesion abilities of *P. pentosaceus* vary with IEC cell line, source and strain (Ryu and Chang 2013; Vidhyasagar and Jeevaratnam 2013; Varsha et al. 2014). It will be important to evaluate effects of *P. pentosaceus* L1 in the context of different IEC cell lines in future study.

P. pentosaceus L1 was sensitive to all antibiotics tested except vancomycin. Bacteria from the genus *Pediococcus* are known to be intrinsically resistant to vancomycin due to a modified peptidoglycan precursor ending in D-Ala-D-lactate (Billot-Klein et al. 1994). Intrinsic resistance is not horizontally transferable and poses no risk in nonpathogenic bacteria (Ammor et al. 2007). Susceptibility of *P. pentosaceus* L1 to other antibiotics should be tested by further study. The non-treated CFCS of *P. pentosaceus* L1 exerted antimicrobial activities against three tested pathogenic strains. The non-treated CFCS of *P. pentosaceus* L1 showed greater antimicrobial activity than did the reference strain LGG. It is well documented that *P. pentosaceus* strains produce bacteriocin against *Escherichia* and *Salmonella* (Uymaz et al. 2009; Ryu and Chang 2013; Vidhyasagar and Jeevaratnam 2013). Neutralized CFCS of L1 strain treated with proteinase K lost inhibitory activity against indicator strains, suggesting that bacteriocin may contribute to the antimicrobial activity of *P. pentosaceus* L1. Heat treatment for 15 min at 100 °C had no significant effect on antimicrobial activity. To our knowledge, this is the first report showing the antimicrobial activity of *P. pentosaceus* against *Shigella flexneri*.

Auto-aggregation of probiotic strains is known to be a prerequisite for adhesion to intestinal epithelium. Our results showed that the auto-aggregating ability of *P. pentosaceus* L1 strain was less than that of LGG after 4 h, which is lower than that of earlier reports of *P. pentosaceus* (Ruas-Madiedo et al. 2005; Osmanagaoglu et al. 2010; Vidhyasagar and Jeevaratnam 2013; Ilavenil et al. 2015). For example, Osmanagaoglu et al. (2010) reported that *P. pentosaceus* OZF originated from human breast milk auto-aggregated at 85.71 % after 5 h. Six strains of *P. pentosaceus* isolated from *Idly* batter also exhibited significant auto-aggregation properties of 42 % or more after 5 h (Vidhyasagar and Jeevaratnam 2013). Notably, auto-aggregation of *P. pentosaceus* strains in these latter studies were shown to be independent of incubation time, suggesting that further study to examine the auto-aggregation of *P. pentosaceus* L1 at different time points may be of interest. Co-aggregation of *P. pentosaceus* L1 with *Shigella flexneri* was examined. *P. pentosaceus* L1 aggregated *Shigella flexneri* at 12.8±2.25. Co-aggregation of probiotics with pathogens enables them to eliminate pathogens from colonizing the intestinal epithelium (Aslim et al. 2007).

Table 2 Antimicrobial activity of the cell-free culture supernatant (CFCS) of *P. pentosaceus* L1 as measured by agar well diffusion method compared to reference strain LGG

Indicator strains	Inhibition zone (mm)[a]							
	Non-treated CFCS		Neutralized at pH 6.5		Proteinase K		Heat treatment	
	L1	LGG	L1	LGG	L1	LGG	L1	LGG
Escherichia coli	++	++	++	++	−	++	++	−
Shigella flexneri	+++	++	+++	++	−	++	+++	−
Salmonella typhimurium	++	++	++	++	−	++	++	−

[a] − No inhibition zone, + < 3 mm, ++ 3–6 mm, +++ radius inhibit zone>6 mm

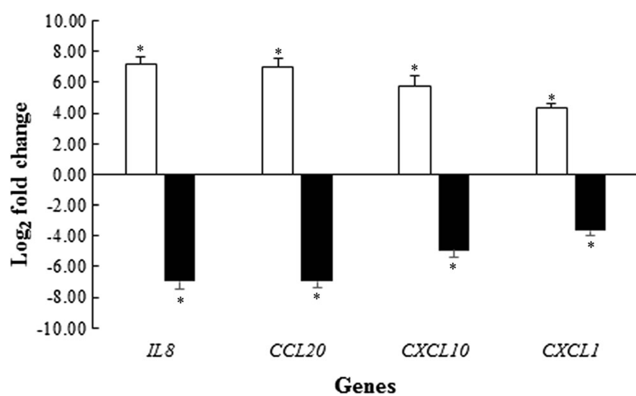

Fig. 2 Effects of pre-incubation *P. pentosaceus* L1 on TNFα-induced gene expression in HT-29 intestinal epithelial cells (IEC). Expression of genes such as *IL8*, *CCL20*, *CXCL10*, *CXCL1* in TNFα-challenged HT-29 IEC following incubation with *P. pentosaceus* L1 were analyzed by RT-qPCR. HT-29 IEC cultured in RPMI1640 served as controls. *Dark-gray filled bars* Ratio of change in gene expression between HT-29 IEC treated with *P. pentosaceus* L1 prior to TNFα challenge versus those exposed to TNFα only. *Open bars* Ratio of change in gene expression between TNFα-treated HT-29 IEC versus untreated control HT-29 IEC. Data are presented as mean±SD (n=3, *P<0.05)

Therefore, our results suggest that *P. pentosaceus* L1 could prevent *Shigella flexneri* infection through co-aggregation with those cells as well as production of antimicrobial substances.

IECs are the initial point of contact with ingested probiotics at the intestinal mucosal interface. Probiotics have been shown to enhance epithelial cell functions (Thomas and Versalovic 2010). Although several reports have shown immunomodulating activities of *P. pentosaceus* originated from varied sources in vitro and in vivo, little is known of the immunomodulatory activities of *P. pentosaceus* at the IEC level. In our study, *P. pentosaceus* strain L1 down-regulated expression of pro-inflammatory genes in TNFα-challenged HT-29 IEC. Similar to our results, feeding *P. pentosaceus* strain LAB4012 (isolated from cobia intestine) in *Vibrion anguillarum* challenged groups increased gene expression of the anti-inflammatory cytokine TGF-β1 and decreased expression of pro-inflammatory genes such as *TNF1*, *TNF2*, and *IL1B* in the head-kidney phagocytes of groupers (Huang et al. 2014). A few studies have also reported immunomodulatory activities of *P. pentosaceus* through affecting the Th1/Th2 immune response. For example, *P. pentosaceus* NB-17 originated from Hokkaido pickle induced secretion of interferon-γ and IL-12 p70 and decreased IL-4 production by ovalbumin-sensitized mouse spleen cells (Jonganurakkun et al. 2008). Similar results that feeding heat-killed *P. pentosaceus* Sn26 derived from Japanese Sunki pickle increased interferon-γ and IL-12 p70 levels in Peyer's patch of BALB/c mice and down-regulated IgE production by splenocytes of ovalbumin-induced allergic diarrheic mice, were reported in a recent study (Masuda et al. 2010). A recent study using an animal model reported that feeding *P. pentosaceus* OZF—a strain derived from human breast milk—in BALB/c mice up-regulated IL-6 production by spleens cells and peritoneal exudates cells (Osmanagaoglu et al. 2013). Our study showed that pre-incubation of HT-29 IEC with *P. pentosaceus* L1 prior to TNFα challenge reduced the expression of TNFα-induced pro-inflammatory genes, suggesting that *P. pentosaceus* L1 has immunomodulatory activity at the IEC level. To our knowledge, this is the first study reporting effects of *P. pentosaceus* derived from fermented vegetables on pro-inflammatory chemokine expression at the intestinal mucosal level.

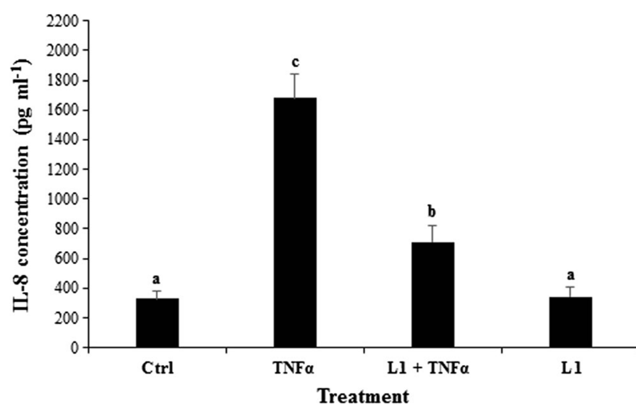

Fig. 3 IL-8 production by TNFα-challenged HT-29 IEC following pre-incubation with *P. pentosaceus* L1. Data are presented as mean±SD (n=3). Bars with different lower case letters are significantly different (P<0.05)

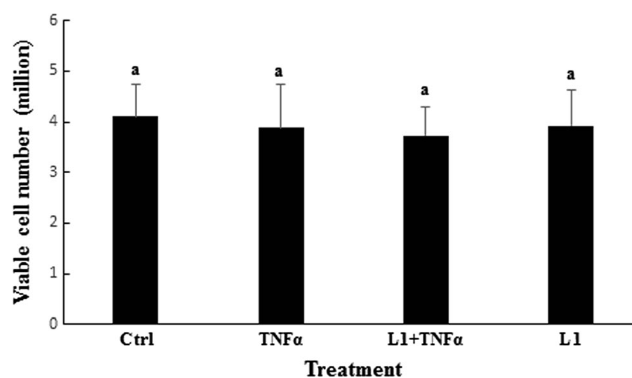

Fig. 4 Viability of HT-29 IEC challenged with TNFα for 3 h following incubation with *P. pentosaceus* L1. Data shown as mean viable cell numbers±SD (n=3). Values with different lower case letters are significantly different (P<0.05)

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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