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RESEARCH PAPER



In vitro and in vivo defensive effect of probiotic LAB against *Pseudomonas aeruginosa* using *Caenorhabditis elegans* model

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ABSTRACT

This study aimed to investigate in vitro and in vivo the probiotic characteristics of lactic acid bacteria (LAB) isolated from Korean traditional fermented foods. *Caenorhabditis elegans* (*C. elegans*) was used for analytical assays of fertility, chemotaxis, life-span, worm-killing and bacterial colonization in the intestinal lumen of the worm. All 35 strains of LAB reduced fertility and slowed development in the worms. The worm-killing assay showed that LAB significantly increased the lifespan ($P < 0.05$) and reduced the susceptibility to virulent PA14; however, the heat-killed LAB did not. The bacterial colonization assay revealed that LAB proliferated and protected the gut of the worm against infection by *Pseudomonas aeruginosa* PA14. In addition, specific LAB *Pediococcus acidilactici* (*P. acidilactici* DM-9), *Pediococcus brevis* (*L. brevis* SDL1411), and *Pediococcus pentosaceus* (*P. pentosaceus* SDL1409) strains showed acid resistance (66–91%), resistance to pepsin (64–67%) and viability in simulated intestinal fluid (67–73%) based on in vitro probiotic analyses. Taken together, these results suggest that *C. elegans* may be a tractable model for screening efficient probiotics.

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Probiotics; Korean soybean paste (KSP); Gut colonization; *Pseudomonas aeruginosa* (PA 14); *Caenorhabditis elegans* (*C. elegans*); chemotaxis

Introduction

Caenorhabditis elegans are free-living nematodes that reside in the soil and rotten fruits, where they feed on bacteria [1,2]. When used for in vitro studies, they are normally cultured on a lawn of *Escherichia coli* (*E. coli* OP50) on which they feed, leading to the presence of bacterial cells in pharynx and the intestine, which frequently appears distended; it has been widely applied as an experimental system for biological studies due to its transparency, short reproductive cycle, ease of cultivation, and suitability for genetic analysis [2].

C. elegans has been used as a model animal in a variety of experiments, including susceptibility testing for antibiotics and human pathogen experiments, because of numerous advantages over other experimental animal models. In probiotic-based research, there are few studies using non-mammalian hosts. Studies on the nematode *C. elegans* have previously indicated that isolates of both *Bifidobacterium* and *Lactobacillus* spp. are beneficial to the health and lifespan of the worm [3,4]; feeding *C. elegans* with *Lactobacillus* has been proven to protect against the foodborne pathogens *Enterococcus faecalis* and *Staphylococcus aureus* [5].

Furthermore, symbiotic lactobacilli appear to be involved in intestinal development.

Lactic acid bacteria (LAB) are rod shaped non-spore-forming gram-positive, microaerophiles and facultative anaerobes bacteria [6]. The enhancement of life span based on probiotics were first proclaimed by Metchnikoff who conveyed that Bulgarian farmers practiced in consuming fermented milk with *Lactobacilli* experienced improved health and longevity, which leads to antiaging effect [7]. Recently, several research groups were focused on finding novel *Lactobacillus* based probiotics and the functional properties as a potential probiotic candidates [8,9]. Unfortunately, studies on the enhancing life span and defense against specific pathogens based on the influence of probiotics are limited because of a lack of appropriate experimental models to validate host longevity [10]. The Mechanisms underlying the probiotic effects of Lactic acid bacteria are also unclear. In this respect, studies on *C. elegans* have delivered valuable perceptions into the functional aspects of anti-aging and defense against specific pathogen (*P. aeruginosa*) and have led to the exploration of the multiple genetic pathways that determine longevity [11,12]. In addition, lactic acid

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 Supplementary data for this article can be accessed [here](#).

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bacteria are widely used and produced commercially, and they are mainly consumed in the form of fermented foods or dairy products [13]. These products have a positive impact on the immune system and overall health. LAB can elicit various immune responses by affecting specific receptors in the intestinal tract or immune cells of the host [6,7].

Pseudomonas aeruginosa is an opportunistic Gram-negative pathogen that infects immunocompromised individuals, for instance patients with cancer or AIDS, or patients with cystic fibrosis [14,15]. The pathogenic factors of *P. aeruginosa* mainly based on lipopolysaccharide (LPS), flagella endotoxin and extracellular toxin [16]. Most of the Gram-positive pathogens depends on, lipoteichoic acid, which induce pro-inflammatory cytokines mediated by Toll-like receptor 4 is a protein (TLR-2) [17,18]. But Non-pathogenic Gram-positive bacteria, such *Pediococcus*, *Enterococcus*, *Lactobacilli*, persuade a dissimilar pattern of cytokines in peripheral blood cells [19–21]. The mechanism of lactobacillus mainly based on inhibition of peritoneal macrophage apoptosis by infection with *Salmonella typhimurium* [22], studies on invitro and invio have reported that *Lactobacilli* possess antagonistic effects against urinary tract infecting (UTI) pathogens [23,24].

Doenjang (Korean soy bean paste) is a traditional Korean food made from fermented soybeans. Doenjang has many health-friendly ingredients, including anti-carcinogenic flavonoids and other beneficial vitamins, minerals, and phytoestrogens [25]. Moreover, the soybeans used for making soybean paste contain linoleic acid and linolenic acid, which have positive effects on cardiovascular diseases and contain a high level of lysine, which is one of the essential amino acids. Thus, this food is known as nutritional source [26].

Lactic Acid Bacteria was isolated and identified from Korean soy bean paste further screened for in-vitro probiotics characteristics. Further the *C. elegans* model was applied to study the effects of lactic acid bacteria against *Pseudomonas aeruginosa* PA14. Specifically, we aimed to study the effects of LAB on the reproductive ability, colonization and life span of *C. elegans*.

Materials and methods

Bacterial strains and growth media

A total of 35 indigenous LAB (Lactic acid bacteria) strains (Table 1) isolated from the Korean fermented soybean paste, were provided by Soonchang Jang Ryu Saupso Company-Korea. Based on different colony morphology using MRS (de Man, Rogosa and Sharpe) selective media and further identified on the bases of

16S rRNA sequencing using universal primers (27F-5' AGA GTT TGA TCM TGG CTC AG 3' and 1492R-5' GGT TAC CTT GTT ACG ACT 3'). The LAB, *Pseudomonas aeruginosa* PA14 and *Escherichia coli* OP50 bacterial strains were stored at – 80°C in De Man, Rogosa & Sharpe (MRS, BD Biosciences, San Jose, CA, USA) medium. Trypticase soy broth (TSB, BD Biosciences, San Jose, CA, USA) was used to cultivate *Pseudomonas aeruginosa* PA14 and *Escherichia coli* OP50, which were obtained from the US Food Fermentation Laboratory Culture Collection (Wyndmoor, PA., USA) supplemented with 30% glycerol. Each culture was streaked from frozen stocks onto TSA and MRS (BD Biosciences) and incubated at 37°C for 24 h.

Screening for probiotic properties in LAB

Tolerance to low pH

The tolerance to low pH of selected lactic acid bacterial strains was determined using the method described by Mokoena [27], with some modifications. Briefly, the active strains grown in MRS broth were inoculated (1.5%) in 10 ml of fresh MRS broth, incubated at 37°C for 24 h and then centrifuged at 6000 × g for 15 min. The pellets were suspended in sterile phosphate-buffered saline (PBS; GIBCO, USA) containing 9 g/L NaCl (Sigma-Aldrich, South Korea), 9 g/L Na₂HPO₄·2H₂O and 1.5 g/L KH₂PO₄ adjusted to a pH of 2.5. The mixture were incubated at 37°C for 4 h, and samples were taken at time 0 and after 4 h. These samples were serially diluted in sterile saline (0.85% NaCl) solution and plated on MRS agar; the viable cells were determined after incubation at 37°C for 24 h, and the percentage of survival of the bacteria was calculated as follows: % survival = [CFU of viable cells survived/CFU of initial viable cells inoculated] × 100.

Tolerance to pepsin

To test the bacterial tolerance to pepsin, a simulated gastric juice was prepared by adding 3 mg/mL pepsin (Sigma-Aldrich, South Korea) to sterile saline solution (0.85% NaCl, w/v) and adjusting the pH to 2.5. The fluid was inoculated with active cultures at an inoculum size of 1% (v/v) and incubated at 37°C for 4 h. The viable cells were determined at an initial (T1) and final incubation time (T2) by the spread plate method by Oh et al [28]. The percentage survival of the bacteria was calculated as follows: % survival = [CFU of viable cells survived/CFU of initial viable cells inoculated × 100].

Table 1. Bacterial strains used in this study All 35 LAB (lactic acid bacterial) strains were isolated from Korean fermented soybean paste.

SN.o	Accession No	Identification Number	Bacterial Strains
01	637,912	ATCC25922	<i>E.coli</i> OP50
02	208,963	UCBPP-PA14	<i>Pseudomonas aeruginosa</i> P14
L01	SRCM100425	MAD-13	<i>Enterococcus faecalis</i>
L02	SRCM100479	SC54	<i>Enterococcus faecium</i>
L03	SRCM100426	CK-5	<i>Enterococcus faecium</i>
L04	SRCM100328	SCL1421	<i>Enterococcus lactis</i>
L05	SRCM100318	SDL1411	<i>Lactobacillus brevis</i>
L06	SRCM100315	SDL1408	<i>Lactobacillus brevis</i>
L07	SRCM100991	JBNU38	<i>Lactobacillus curvatus</i>
L08	SRCM100476	SC48	<i>Lactobacillus pentosus</i>
L09	SRCM100436	JDFM44	<i>Lactobacillus plantarum</i>
L10	SRCM100435	JDFM33	<i>Lactobacillus rhamnosus</i>
L11	SRCM100434	JDFM6	<i>Lactobacillus rhamnosus</i>
L12	SRCM100478	SC53	<i>Leuconostoc citreum</i>
L13	SRCM100744	JBNU10	<i>Leuconostoc mesenteroides</i>
L14	SRCM100474	SC46	<i>Leuconostoc paramesenteroides</i>
L15	SRCM100327	SCL1420	<i>Pediococcus acidilatic</i>
L16	SRCM100325	SKL1418	<i>Pediococcus acidilatic</i>
L17	SRCM100321	SDL1414	<i>Pediococcus acidilatic</i>
L18	SRCM100313	SDL1406	<i>Pediococcus acidilatic</i>
L19	SRCM100312	SDL1405	<i>Pediococcus acidilatic</i>
L20	SRCM100309	SDL1402	<i>Pediococcus acidilatic</i>
L21	SRCM100427	DM9	<i>Pediococcus acidilatic</i>
L22	SRCM100424	MAC11	<i>Pediococcus pentosaceus</i>
L23	SRCM100323	SDL1416	<i>Pediococcus pentosaceus</i>
L24	SRCM100322	SDL1415	<i>Pediococcus pentosaceus</i>
L25	SRCM100308	SDL1401	<i>Pediococcus pentosaceus</i>
L26	SRCM100879	SCML337	<i>Streptococcus thermophilus</i>
L27	SRCM100872	SCML300	<i>Streptococcus thermophilus</i>
L28	SRCM100182	SCCB2306	<i>Weissella cibaria</i>
L29	SRCM100196	SCSB2320	<i>Weissella confusa</i>
L30	SRCM100194	SCKB2318	<i>Weissella confusa</i>
L31	SRCM100966	JBNU2	<i>Weissella koreensis</i>
L32	SRCM100183	SCCB2307	<i>Weissella cibaria</i>
L33	SRCM100316	SDL1409	<i>Pediococcus pentosaceus</i>
L34	SRCM100320	SDL1413	<i>Lactobacillus plantarum</i>
L35	SRCM100467	SC25	<i>Lactobacillus arizonensis</i>

Resistance to bile salts and pancreatin

The resistance of the lactobacilli to bile salts and pancreatin was tested as described by Oh et al [28], with some modifications. Briefly, 0.3% (w/v) bile salts (Sigma-Aldrich, South Korea) and 1 mg/mL pancreatin (Sigma-Aldrich, South Korea) were dissolved in sterile saline solution (0.85% NaCl, w/v) adjusted to pH 8.0. The fluid was inoculated with 1% (v/v) LAB cultures and incubated at 37°C for 6 h. The viable cells were determined before and after incubation by the spread plate method. The percentage survival of the bacteria was calculated as follows: % survival = [CFU of survivors/CFU of inoculum x 100].

Determination of antibiotic susceptibility

The susceptibility of the lactic acid bacteria towards antibiotics was tested as reported earlier by Oh et al [28]. All the antibiotics were purchased from Sigma Inc.-USA. The lactic acid bacteria were tested against the cell wall-targeting antibiotics 10 µg ampicillin

(Am), 10 µg penicillin (Pe) and 30 µg vancomycin (Va); the protein synthesis-inhibiting antibiotics 15 µg erythromycin (Er), 2 µg clindamycin (Cl), 30 µg tetracycline (Te), 30 µg kanamycin (Ka), 30 µg chloramphenicol (Ch) and 10 µg gentamicin (Ge); and the nucleic acid-targeting antibiotic 25 µg streptomycin (St) using the disc diffusion method. In addition 0.005mg/mL. The MRS agar plates were examined for the zones of inhibition after incubation at 37°C for 24 h.

Antibacterial effects of LAB isolates in vitro

The antibacterial effects of eight selected LAB isolates on six common food borne pathogens or spoilage organism's at 37°C were investigated using the agar diffusion assay described above. Targeted indicator organisms and their respective media used were as follows: *P. aeruginosa* (PA14) (Nutrient Broth (NB), DifcoTM), *L. brevis* SDL1411 (L05), *P. acidilatici* DM9 (L21) and *P. pentosaceus* SDL1409 (L33), *Enterococcus faecium* CK-5 (L03) *Enterococcus faecium* SC54 (L02), *Lactobacillus plantarum* JDFM44 (L09), *Leuconostoc citreum* SC53 (L12), *Pediococcus acidilatic* SDL1414 (L17), *Pediococcus acidilatic* DM9 (L21), *Pediococcus pentosaceus* MAC11 (L22), *Weissella koreensis* JBNU2 (L31) (MRS, DifcoTM). All strains were cultured aerobically for 24 h at 37°C, from which Cell free supernatant (CFS) was prepared and tested against *P. aeruginosa* (PA14). Following inoculation, plates were incubated at 37°C for 24 h, respectively. Inhibition zones were measured.

Thermal stability of antimicrobial peptides produced by LAB isolates

Based on identified LAB species, isolation source and the size of inhibition zones, nine LAB isolates were chosen for thermal stability tests. The pH adjusted CFS described above were treated at 90°C for both 10 and 30 min. pH adjusted CFS without any heat treatments served as a controls. Residual antimicrobial activity of heat-treated CFS was determined by the agar diffusion bioassay compared to the control using *P. aeruginosa* (PA14) as the indicator bacteria.

Digestive enzyme stability of antimicrobial peptides produced by LAB isolates

Based on identified LAB species, isolation source and the size of inhibition zones, nine LAB isolates were chosen for enzyme stability tests. The pH adjusted CFS with < 10Kda (Abcam ab-93,349) peptide separated using spin column, which were treated at 3 mg/mL pepsin + (pH 2.5) and

incubated at 37°C for 4 h, 1 mg/mL bile + pancreatin+ (pH 8.0) and incubated at 37°C for 6 h. The pH adjusted proteins were freeze dried, without any treatments served as a controls. Residual antimicrobial activity of enzyme – treated CFS was determined by the agar diffusion bioassay compared to the control using *P. aeruginosa* (PA14) as the indicator bacteria.

Nitric oxide production in *Lactobacillus*

Qualitative analysis

In addition *Lactobacillus* (L5, L21 and L33) biosynthesis of Nitric Oxide were evaluated by staining with 1, 2-diaminoanthraquinone sulfate (DAAD11582-5G), 50 µg/ml; at 37°C for 30 min) subsequently the bacteria washed from stained observed in confocal microscope [30]. DAA is a colored compound with a maximum of absorption at about 540 nm (fluorescence images obtained using 488 nm and emission measured between 505 and 750 nm – green channel) (Sup.1).

Quantitative analysis

(d) Modified MRS broth (Sharpe.” to test for the production of Nitric oxide from arginine by lactobacilli): MRS broth+ 0–3% (w/v) L-arginine monohydrochloride. After 12 and 24 h incubation at 37 ° C likewise during 3rd, 6th and 9th day of *C. elegans* treated with *Lactobacillus* (L5, L21 and L33). The concentration of NO in sodium nitroprusside solutions was estimated by calibration curve plotted with the use of fresh solutions of NaNO₂ (Sigma Aldrich 237,213) in concentrations of 0–100 µM. Nitrites, NO oxidation products under aerobic conditions, were measured spectrophotometrically (540 nm) using Griess reagent (Sigma-Aldrich G4410) [31].

Nematode and stage of development

The experiment were executed mainly based on [27]. The eggs from strain N2 *C. elegans* were isolated and treated with hypochlorite, and further incubated 20°C for 20 h in S-buffer [28]. The nematodes were maintained on 35 mm diameter NGM (Nematode Growing Medium) agar plates seeded with 50 µL *E. coli* OP50 and stored at 20 ° C. *C. elegans* (N2) strains were obtained from the Caenorhabditis Genetic Center (CGC), Minnesota. A harmonized population of L1 arrested worms were then placed on NGM agar plates seeded with LAB. Five stage [L1 – after egg 5 stage of egg development and immediately after hatching Nervous, Reproductive

and coelomocyte system developmental stage; L2- few cell division after L1 stage leads to possess developed reproductive and nervous system (L2 stage also known as dauer larva; L3- posse matured reproductive system; L4- young adult posse fully developed reproductive system and Adult- hence completing its 3-day reproductive life cycle] [29]. In that L4 nematode were picked manually and moved to a new plate. The worms were then moved twice a day to prevent overcrowding until egg laying ceased. The progeny were counted 3 days after removal of the parents. The experiment was performed in triplicate.

Nematode synchronizing

The young adult worms hatched from eggs were treated with sterile water containing 0.5 M NaOH and 0.5% bleaching solution (sodium hypochlorite and sodium hydroxide) for synchronization. The assay was performed as described by Mokoena [30]. After vortex mixing at 2 min intervals, worms were washed three times in M9 buffer (3.0 g KH₂PO₄, 6.0 g Na₂HPO₄, 0.5 g NaCl, 1.0 g NH₄Cl, H₂O/1 L, sterilized at 121°C) via centrifugation (1200 × g for 200 sec). To prepare worms for all assays, approximately 3000 synchronized eggs were hatched in M9 buffer at 20°C overnight. The L1 larvae were subsequently transferred to nematode growth medium (NGM) agar with a lawn of *E. coli* OP50 and incubated at 25°C for 48 h to reach the L4 stage.

Chemotaxis assay

The chemotaxis assay was adapted from Martin et al., 2017. Briefly, the bacteria (10 µl of OP50) were seeded separately with worms at the initial growth (L4) stage after incubation. Fifty young adult stage worms were washed twice with S-basal buffer and placed in the centre of a (5 cm) plate wherein 20 µl of 0.5 M sodium azide solution had been added to two sites on the plate with a circle marked around the origin to ensure that non-motile worms would be ignored. A two-quadrant system was used to monitor the movement of the worms, which were equidistant from the test and control samples, regardless of which side of the origin they began. In this system, the worms respond to an odorant, which can delay or force them to take a more circuitous route. The number of worms at both sites was checked after 90 min at 25°C and then incubated further at 4°C. Chemotaxis assays were performed in triplicate for each condition. For each experiment, a chemotaxis index (CI) was calculated using the following formula:

Chemotaxis index (CI) = [number of worms in the LAB bacteria strain – number of worms in OP50 (control)]/total number of worms applied]. A + 1.0 score indicates maximal attraction towards the target and represents 100% of the worms arriving in the quadrants containing the chemical target. An index of –1.0 is evidence of maximal repulsion.

C. elegans fertility assay

L4 young adult (N2) worms were transferred to new NGM plates containing 50 µL of the target bacterial strain cultured in TS and MRS broth. Worms were transferred daily until they no longer produced progeny. The NGM plates containing eggs were incubated at 20°C for another 48 h, and the number of progeny was counted for each adult worm tested at the end of 3 days. The fertility of worms was monitored using wild-type L2 [31].

Lifespan assays

The longevity of *C. elegans* was measured using a previously described method by Smolentsevae et al [32]. In the experiment, 50 µL of the target bacterial strain cultured in TS and MRS broth at 25°C for 24 h was spread on a 35 mm diameter NGM agar plate and incubated overnight at 37°C. Each plate was seeded with 30 L4 stage young adults of *C. elegans* grown in *E. coli* OP50. All plates were incubated at 25°C and enumerated for dead worms every 24 h. Live worms were transferred onto fresh NGM agar plates seeded with the target bacterial strain every 2 days. The experiment was performed in triplicate for each strain. Each plate was monitored until all the worms had died. To compare the effects of *Pseudomonas aeruginosa* PA14 alone to the effects of LAB (Table 1) against *Pseudomonas aeruginosa* PA14, worms were first grown with OP50, and the mean life span (MLS) was calculated by applying a previously described formula [33].

Bacterial colonization assay

The influence of selected *Lactobacillus* strains on colonization in *C. elegans* was determined using the bacterial colonization assay described by Antunes et al [35], with some modifications. On the first day, lactic acid bacteria were seeded on NGM plates containing worms. On the 3rd, 5th, 7th, and 9th day of incubation, the total number of colonies present in the gut of the young adult (L4) stage worm was determined using the following procedure: Five worms were washed in 5 µL

drops of M9 buffer to inhibit pharyngeal pumping and expulsion. The washed nematodes were placed in a 1.5 ml centrifuge tube containing 50 µL of PBS buffer with 1% Triton X-100 and mechanically disrupted using a mortar and pestle. Worm lysates were then diluted in PBS buffer and plated on MRS agar at 37°C. LAB colonies were quantified and used to calculate the number of bacteria per nematode. The procedure was repeated using *P. aeruginosa* PA14 as a comparison with lactic acid bacteria [35].

Life span assay of C. elegans with heat killed bacteria

Initially *E. coli* OP50 fed *C. elegans*, were subsequently infected with *P. aeruginosa* PA14, which results in a combination of OP50-PA14: worms were transferred onto PA14-seeded NGM plate after 1st day of incubation in OP50-seeded NGM plate and enumerate dead worms each day to 10th day. OP50-PA14; secondly worms were transferred onto PA14-seeded NGM plate after 1st day of incubation, further seeded with heat-killed (80°C for 10 min) *E. coli* OP50 in NGM plate and dead worms were enumerate each day till 10th day. Likewise the Lactic acid bacteria (The LAB was selected for the experiment based on the colonization assay) seeded NGM plates were then seeded with PA14, from 1st day – 10th day of incubation the life span of the worm were enumerated, in addition the heat killed LAB were tested similarly against PA14. Each experiment for each strain administrated 30 worms and repeated twice and plotted the results using OASIS II program

Statistical analysis

Results are reported as the mean of at least 2 independent experiments ± standard error (s.e.m). In the life span assay and the killing assay, the OASIS2 (Online Application for Survival Analysis 2) program was used for statistical analysis [38]. Differences in the survival of *C. elegans* in the infection assays were determined using Graph Pad Prism version 5.00 [39] (www.graphpad.com).

Results

In vitro probiotic properties of LAB

Among the 35 total lactic acid bacterial strains, based on the lifespan assay, a drastic reduction in the survival ability of LAB in the *C. elegans* model with respect to the pathogen *P. aeruginosa* PA14 could indicate less

chances of surviving further harsh conditions [40]. However, only 3 selective isolates (*L. brevis*, *P. latici* and *P. pentosaceus*) showed promising results; hence, these three strains were further tested for resistance against acid (pH 2.5), pepsin (pH 2.5), Bile + Pancreatin (pH 8.0), antibiotics resistance with different mode of action and antimicrobial activity (Table 2, 3(a-e)).

Acid tolerance

All the LAB isolates were subjected to a 2.5 pH. Among the 35 LAB were presented in (Table 2), *Lactobacillus brevis* SDL1411 (L05) showed > 90% survival abilities in low pH, *Enterococcus faecium* SC54 (L12), *Lactobacillus plantarum* JDFM44 (L09), *Leuconostoc citreum* SC53 (L12), *Pediococcus acidilatic*

Table 2. Acid resistance of lactic acid bacteria in phosphate-saline buffer (pH 2.5). Values are expressed in mean $\pm$ standard deviation (n = 3) Different superscripts (a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r, s, t, u) represent significantly different values (P < 0.05).

SN.o	Bacterial Strains	Identification Number	Acid resistance (pH 2.5)		
			Initial count (cfu/mL)	Survival after 4 h (cfu/mL)	Percentage survival (%)
L1	<i>Enterococcus faecalis</i>	MAD-13	0.81 $\pm$ 0.11	0.32 $\pm$ 0.06	39.02 ^k
L2	<i>Enterococcus faecium</i>	SC54	3.47 $\pm$ 0.36	2.28 $\pm$ 0.24	65.60^e
L3	<i>Enterococcus faecium</i>	CK-5	17.37 $\pm$ 0.44	8.69 $\pm$ 0.50	50.02 ^l
L4	<i>Enterococcus lactis</i>	SCL1421	0.75 $\pm$ 0.06	0.07	9.33 ^r
L5	<i>Lactobacillus brevis</i>	SDL1411	1.17 $\pm$ 0.14	1.17 $\pm$ 0.10	91.45^a
L6	<i>Lactobacillus brevis</i>	SDL1408	1.82 $\pm$ 0.30	0.15 $\pm$ 0.06	8.33 ^r
L7	<i>Lactobacillus curvatus</i>	JBNU38	2.45 $\pm$ 0.30	0.01	0.41 ^u
L8	<i>Lactobacillus pentosus</i>	SC48	11.22 $\pm$ 0.90	0.21 $\pm$ 0.09	1.88 ^t
L9	<i>Lactobacillus plantarum</i>	JDFM44	28.18 $\pm$ 0.20	19.57 $\pm$ 0.30	69.44^d
L10	<i>Lactobacillus rhamnosus</i>	JDFM33	0.17 $\pm$ 0.02	0.05 $\pm$ 0.01	29.41 ⁿ
L11	<i>Lactobacillus rhamnosus</i>	JDFM6	0.52 $\pm$ 0.1	0.16 $\pm$ 0.03	31.13 ⁿ
L12	<i>Leuconostoc citreum</i>	SC53	28.18 $\pm$ 0.20	19.57 $\pm$ 0.30	69.44^d
L13	<i>Leuconostoc mesenteroides</i>	JBNU10	4.57 $\pm$ 0.50	0.35 $\pm$ 0.03	7.75 ^r
L14	<i>Leuconostoc paramesenteroides</i>	SC46	9.77 $\pm$ 0.30	0.53 $\pm$ 0.09	5.44 ^s
L15	<i>Pediococcus acidilatic</i>	SCL1420	6.16 $\pm$ 0.13	0.29 $\pm$ 0.09	0.48 ^u
L16	<i>Pediococcus acidilatic</i>	SKL1418	11.22 $\pm$ 0.90	0.21 $\pm$ 0.09	1.88 ^t
L17	<i>Pediococcus acidilatic</i>	SDL1414	12.30 $\pm$ 0.30	7.94 $\pm$ 0.46	64.55^f
L18	<i>Pediococcus acidilatic</i>	SDL1406	1.51 $\pm$ 0.26	0.49 $\pm$ 0.09	32.45 ^m
L19	<i>Pediococcus acidilatic</i>	SDL1405	20.89 $\pm$ 0.43	2.03 $\pm$ 0.16	9.66 ^q
L20	<i>Pediococcus acidilatic</i>	SDL1402	1.32 $\pm$ 0.21	0.51 $\pm$ 0.10	38.64 ^l
L21	<i>Pediococcus acidilatic</i>	DM9	1.41 $\pm$ 0.17	1.06 $\pm$ 0.11	75.17^c
L22	<i>Pediococcus pentosaceus</i>	MAC11	2.34 $\pm$ 0.22	1.86 $\pm$ 0.23	79.74^b
L23	<i>Pediococcus pentosaceus</i>	SDL1416	20.89 $\pm$ 0.43	2.03 $\pm$ 0.16	9.66 ^q
L24	<i>Pediococcus pentosaceus</i>	SDL1415	2.75 $\pm$ 0.34	0.76 $\pm$ 0.21	27.64 ^a
L25	<i>Pediococcus pentosaceus</i>	SDL1401	4.27 $\pm$ 0.24	1.03 $\pm$ 0.21	24.13 ^p
L26	<i>Streptococcus thermophilus</i>	SCML337	2.75 $\pm$ 0.34	0.76 $\pm$ 0.21	27.64 ^a
L27	<i>Streptococcus thermophilus</i>	SCML300	18.19 $\pm$ 0.26	10.16 $\pm$ 0.11	55.86 ^h
L28	<i>Weissella cibaria</i>	SCCB2306	17.37 $\pm$ 0.44	8.69 $\pm$ 0.50	50.02 ^l
L29	<i>Weissella confusa</i>	SCSB2320	3.39 $\pm$ 0.22	1.45 $\pm$ 0.32	42.63 ^j
L30	<i>Weissella confusa</i>	SCKB2318	3.02 $\pm$ 0.08	0.75 $\pm$ 0.01	24.92 ⁿ
L31	<i>Weissella korensis</i>	JBNU2	0.43 $\pm$ 0.13	0.27 $\pm$ 0.09	62.79^g
L32	<i>Weissella cibaria</i>	SCCB2307	20.89 $\pm$ 0.43	2.03 $\pm$ 0.16	9.66 ^q
L33	<i>Pediococcus pentosaceus</i>	SDL1409	1.91 $\pm$ 0.34	1.17 $\pm$ 0.17	66.00^e
L34	<i>Lactobacillus plantarum</i>	SDL1413	1.82 $\pm$ 0.30	0.15 $\pm$ 0.06	8.33 ^r
L35	<i>Lactobacillus arizonensis</i>	SC25	3.02 $\pm$ 0.08	0.75 $\pm$ 0.01	24.92 ⁿ

Table 3. Probiotic characteristics of the selected Lactic acid bacterial strain.

(a) Stability of Lactic acid bacteria towards pepsin at (pH = 2.5)					
S.No	Bacterial Strains	Identification Number	Pepsin + (pH 2.5)		
			Initial count (cfu/mL) x 10 ⁹	Survival after 4h (cfu/mL) x 10 ⁹	Percentage survival (%)
L03	<i>Enterococcus faecium</i>	CK-5	1.82 ± 0.30	0.15 ± 0.06	8.33 ^g
L05	<i>Lactobacillus brevis</i>	SDL1411	12.30 ± 0.30	7.94 ± 0.10	64.52 ^b
L09	<i>Lactobacillus plantarum</i>	JDFM44	20.89 ± 0.43	2.03 ± 0.16	9.66 ^f
L12	<i>Leuconstoc citreum</i>	SC53	2.45 ± 0.30	0.01	0.41 ^h
L17	<i>Pediococcus acidilatic</i>	SDL1414	2.75 ± 0.34	0.76 ± 0.21	27.64 ^e
L21	<i>Pediococcus acidilatic</i>	DM9	8.71 ± 0.51	5.82 ± 0.47	66.86 ^a
L22	<i>Pediococcus pentosaceus</i>	MAC11	24.55 ± 0.56	10.0 ± 0.47	41.0 ^c
L31	<i>Weissella korensis</i>	JBNU2	1.32 ± 0.21	0.51 ± 0.10	38.64 ^d
L33	<i>Pediococcus pentosaceus</i>	SDL1409	8.70 ± 0.48	5.82 ± 0.37	66.86 ^a

Tolerance of lactic acid bacteria to pepsin (pH = 2.5). Values are expressed as the mean $\pm$ standard deviation (n = 3). Different superscripts (a, b, c, d, e, f, g, h) represent significantly different values (P < 0.05).

(b) Stability of Lactic acid bacteria towards Bile + Pancreatin at (pH = 8.0)

S.No	Bacterial Strains	Identification Number	Bile + Pancreatin+ (pH 8.0)		
			Initial count (cfu/mL) x 10 ⁹	Survival after 4h (cfu/mL) x 10 ⁹	Percentage survival (%)
L03	<i>Enterococcus faecium</i>	CK-5	4.27 ± 0.24	1.03 ± 0.21	24.13 ^d
L05	<i>Lactobacillus brevis</i>	SDL1411	3.02 ± 0.08	2.05 ± 0.01	68.00^b
L09	<i>Lactobacillus plantarum</i>	JDFM44	9.77 ± 0.30	0.53 ± 0.09	5.44 ^g
L12	<i>Leuconostoc citreum</i>	SC53	6.16 ± 0.13	0.29 ± 0.09	0.48 ⁱ
L17	<i>Pediococcus acidilatic</i>	SDL1414	11.22 ± 0.90	0.21 ± 0.09	1.88 ^h
L21	<i>Pediococcus acidilatic</i>	DM9	9.77 ± 0.30	6.53 ± 0.09	67.83^c
L22	<i>Pediococcus pentosaceus</i>	MAC11	3.71 ± 0.23	0.97 ± 0.08	21.35 ^e
L31	<i>Weissella koreensis</i>	JBNU2	3.63 ± 0.40	0.55 ± 0.9	15.21 ^f
L33	<i>Pediococcus pentosaceus</i>	SDL1409	11.22 ± 0.90	8.21 ± 0.09	73.17^a

Viability of lactic acid bacteria in the presence of simulated intestinal fluid (0.3% bile salt and 1 mg/mL pancreatin in 0.85% NaCl, w/v, pH 8.0). Values are expressed as the mean ± standard deviation (n = 3). Different superscripts (a, b, c, d, e, f, g, h, i) represent significantly different values (P < 0.05).

(c) Antibiotic sensitivity of Lactic acid bacteria

S.No	Bacterial Strains	Identification Number	Different Mode of Antibiotics									
			Cell wall Targeting				Protein Synthesis Inhibiting				Nuclic Acid Targeting	
			Am	Va	Pe	Er	Cl	Te	Ka	Ch	Ge	St
			10 µg	30 µg	10 µg	15 µg	2 µg	30 µg	30 µg	30 µg	10 µg	25 µg
L03	<i>Enterococcus faecium</i>	CK-5	NR	R	R	R	NR	NR	R	NR	NR	R
L05	<i>Lactobacillus brevis</i>	SDL1411	R	R	NR	NR	NR	NR	NR	NR	NR	R
L09	<i>Lactobacillus plantarum</i>	JDFM44	R	R	R	R	NR	NR	R	NR	NR	R
L12	<i>Leuconostoc citreum</i>	SC53	R	NR	R	R	R	R	NR	NR	R	R
L17	<i>Pediococcus acidilatic</i>	SDL1414	R	R	R	R	NR	NR	R	NR	NR	R
L21	<i>Pediococcus acidilatic</i>	DM9	R	R	NR	NR	NR	NR	NR	NR	NR	R
L22	<i>Pediococcus pentosaceus</i>	MAC11	R	R	NR	R	NR	R	R	R	NR	NR
L31	<i>Weissella koreensis</i>	JBNU2	R	R	R	NR	NR	R	R	NR	NR	R
L33	<i>Pediococcus pentosaceus</i>	SDL1409	R	R	NR	NR	NR	NR	NR	NR	NR	R
L03	<i>Enterococcus faecium</i>	CK-5	NR	R	R	R	NR	NR	R	NR	NR	R
L05	<i>Lactobacillus brevis</i>	SDL1411	R	R	NR	NR	NR	NR	NR	NR	NR	R
L09	<i>Lactobacillus plantarum</i>	JDFM44	R	R	R	R	NR	NR	R	NR	NR	R
L12	<i>Leuconostoc citreum</i>	SC53	R	NR	R	R	R	R	NR	NR	R	R
L17	<i>Pediococcus acidilatic</i>	SDL1414	R	R	R	R	NR	NR	R	NR	NR	R
L21	<i>Pediococcus acidilatic</i>	DM9	R	R	NR	NR	NR	NR	NR	NR	NR	R
L22	<i>Pediococcus pentosaceus</i>	MAC11	R	R	NR	R	NR	R	R	R	NR	NR
L31	<i>Weissella koreensis</i>	JBNU2	R	R	NR	R	NR	R	R	NR	NR	R
L33	<i>Pediococcus pentosaceus</i>	SDL1409	R	R	NR	NR	NR	NR	NR	NR	NR	R

Viability of Lactic Acid Bacteria (LAB) to antibiotics (R: resistant, NR: not resistant).

(d) Antimicrobial activity of Lactic Acid Bacteria (LAB) against cell free supernatant and < 10Kda peptide purified

S.No	List of Lactic Acid Bacteria	LAB cell free supernatant against <i>Pseudomonas aeruginosa</i> (PA14)	< 10Kda peptide purified from LAB against <i>Pseudomonas aeruginosa</i> (PA14)	Zone of inhibition (mm)
		Zone of inhibition (mm)		Zone of inhibition (mm) (1mg/mL)
L03	<i>Enterococcus faecium</i> CK-5	15	16	^d
L05	<i>Lactobacillus brevis</i> SDL1411	25	28	^g
L09	<i>Lactobacillus plantarum</i> JDFM44	11	12	^a
L12	<i>Leuconostoc citreum</i> SC53	12	14	^b
L17	<i>Pediococcus acidilatic</i> SDL1414	13	10	^c
L21	<i>Pediococcus acidilatic</i> DM9	19	26	^a
L22	<i>Pediococcus pentosaceus</i> MAC11	15	14	^e
L31	<i>Weissella koreensis</i> JBNU2	12	13	^g
L33	<i>Pediococcus pentosaceus</i> SDL1409	23	28	^c

Antimicrobial activity of Lactic Acid Bacteria (LAB) against cell free supernatant and < 10Kda peptide purified from LAB against *Pseudomonas aeruginosa* (PA14) Values are expressed as the mean ± standard deviation (n = 3). Different superscripts (a, b, c, d, e, f, g) represent significantly different values (P < 0.05)

SDL1414 (L17), *Pediococcus acidilatic* DM9 (L21), *Pediococcus pentosaceus* MAC11 (L22), *Weissella koreensis* JBNU2 (L31), *Pediococcus pentosaceus* SDL1409 (L33) exhibited 60–75%, *Enterococcus faecalis* MAD-13 (L01), *Enterococcus faecium* CK-5 (L03), *Enterococcus lactis* SCL1421 (L04),

Lactobacillus brevis SDL1408 (L06), *Lactobacillus curvatus* JBNU38 (L07), *Lactobacillus pentosus* SC48 (L08), *Lactobacillus rhamnosus* JDFM33 (L10), *Lactobacillus rhamnosus* JDFM6 (L11), *Leuconostoc mesenteroides* JBNU10 (L13), *Leuconostoc paramesenteroides* SC46 (L14), *Pediococcus acidilatic* SCL1420

(e) Stability of Antimicrobial peptides towards heat (90°C for 10 and 30 Min) and digestive enzymes (Pepsin + (pH 2.5) Bile + Pancreatin+ (pH 8.0)

S.No	List of Lactic Acid Bacteria	Thermo-stability of the Antimicrobial peptides (< 10Kda peptide) (1mg/mL)		Stability of Antimicrobial (< 10Kda) peptide produced from LAB in digestive enzymes and tested against <i>Pseudomonas aeruginosa</i> (1mg/mL)	
		Heat stability of Peptide produced from LAB and <i>Pseudomonas aeruginosa</i>			
		90°C for 30 Min	90°C for 60 Min	Pepsin + (pH 2.5)	Bile + Pancreatin+ (pH 8.0)
L03	<i>Enterococcus faecium</i> CK-5	15	NZ	NZ	NZ
L05	<i>Lactobacillus brevis</i> SDL1411	25	27	18	13
L09	<i>Lactobacillus plantarum</i> JDFM44	11	NZ	NZ	NZ
L12	<i>Leuconostoc citreum</i> SC53	12	NZ	NZ	NZ
L17	<i>Pediococcus acidilatic</i> SDL1414	13	NZ	NZ	NZ
L21	<i>Pediococcus acidilatic</i> DM9	19	24	17	14
L22	<i>Pediococcus pentosaceus</i> MAC11	15	NZ	NZ	NZ
L31	<i>Weissella koreensis</i> JBNU2	12	NZ	NZ	NZ
L33	<i>Pediococcus pentosaceus</i> SDL1409	23	25	20	14

Stability of Antimicrobial peptides in heat (90°C for 10 and 30 Min) and digestive enzymes (Pepsin + (pH 2.5) Bile + Pancreatin+ (pH 8.0) were tested against *Pseudomonas aeruginosa* (PA14) Values are expressed as the mean $\pm$ standard deviation (n = 3). Different superscripts (a, b, c, d, e, f, g, h, i) represent significantly different values (P < 0.05)

(f) Nitric Oxide production in Lactic Acid Bacteria (LAB) under aerobic condition and anaerobic condition at 37°C and in *C. elegans* (Invivo)

S.N.o	Bacterial Strains	Aerobic condition at 37°C		Anaerobic condition at 37°C		<i>C. elegans</i> (Invivo)		
		NO (ppb) 12 h	NO (ppb) 24 h	NO (ppb) 12 h	NO (ppb) 24 h	NO (ppb) in 3 day	NO (ppb) in 6 day	NO (ppb) in 9 day
1	Control (<i>E. coli</i> OP50)	ND	ND	12	36	36	40	48
2	<i>Pseudomonas aeruginosa</i> PA14	ND	ND	ND	ND	ND	ND	ND
3	<i>P. acidilactici</i> DM-9 (L21)	8	11	16	77	58	167	164
4	<i>L. brevis</i> SDL1411 (L05)	5	12	14	82	79	130	138
5	<i>P. pentosaceus</i> SDL1409 (L33)	12	13	12	102	84	121	123

Nitric Oxide production in *Lactobacillus* under aerobic condition, anaerobic condition at 37°C and *C. elegans* (Invivo), nd = not determined, (p < 0.001).

(L15), *Pediococcus acidilatic* SKL1418 (L16), *Pediococcus acidilatic* SDL1406 (L18), *Pediococcus acidilatic* SDL1405(L19), *Pediococcus acidilatic* SDL1402(L20), *Pediococcus pentosaceus* SDL1416 (L23), *Pediococcus pentosaceus* SDL1415 (L24), *Pediococcus pentosaceus* SDL1401(L25), *Streptococcus thermophiles* SCML337 (L26), *Streptococcus thermophiles* SCML300 (L27), *Weissella cibaria* SCCB2306 (L28), *Weissella confuse* SCSB2320 (L29), *Weissella confuse* SCKB2318 (L30). The strains showing < 50% (Table 2(a)) survival capability were omitted from further analysis. Since the extreme decline in their survival ability could specify lesser chances of surviving further harsh conditions [30]. However, the remaining 9 strains were tested for their resistance against pepsin.

Resistance to pepsin

Nine LAB were tested for their resistance to pepsin. Our results showed that *L. brevis* SDL1411 (L05), *P. acidilactici* DM9 (L21) and *P. pentosaceus* SDL1409 (L33) showed the highest survival ability in pepsin (> 60%) while *Enterococcus faecium* CK-5 (L03) *Enterococcus faecium* SC54 (L12), *Lactobacillus plantarum* JDFM44 (L09), *Leuconostoc*

citreum SC53 (L12), *Pediococcus acidilatic* SDL1414 (L17), *Pediococcus acidilatic* DM9 (L21), *Pediococcus pentosaceus* MAC11 (L22), *Weissella koreensis* JBNU2 (L31), showed the least survival ability (< 41%) these least survival abilities were therefore excluded from subsequent experiments. Our results showed that all three (*L. brevis*, *P. latici* and *P. pentosaceus*) strains showed the highest survival ability in pepsin (> 64%), as shown in Table 3(a).

Resistance to bile salts and pancreatin

In this study, the LAB isolates showed varying levels of resistance to bile salts and 1 mg/mL pancreatin after 4 h of exposure (Table 3(b)). Six LAB strains were found to be less tolerant (< 24%) to the simulated intestinal fluid after 4 h of incubation. *L. brevis* SDL1411 (L05), *P. acidilactici* DM9 (L21) and *P. pentosaceus* SDL1409 (L33) showed strong resistance to bile salts and pancreatin with survival abilities of 68.00%, 67.00% and 73.17% respectively. Among the *Pediococcus* species, only *P. acidilactici* DM9 (L21) and *P. pentosaceus* SDL1409 (L33) showed high resistance against simulated intestinal conditions respectively.

Antibiotic susceptibility

The breakpoints for testing lactobacilli are not reported in the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines, and the procedure proposed by the CLSI (ISO/IDF standard for antimicrobial susceptibility testing) is not adapted to the growth requirements of all *Lactobacillus* species. Hence our interpretation of the results was based on the breakpoint values suggested by FEDAP (EFSA Panel on Additives and Products or Substances used in Animal Feed – European Food Safety Authority, 2012) [41] for ampicillin, tetracycline, erythromycin, streptomycin, gentamicin and chloramphenicol. For kanamycin, vancomycin, clindamycin and penicillin we adopted the breakpoints of tetracycline and erythromycin, respectively. In addition 0.001mg/mL concentration was tested according to against selected nine LAB strains as per the European Food Safety Authority (EFSA), 2012, Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). All three LAB strains tested in this study were found to be sensitive to ampicillin, a cell wall-inhibiting antibiotic. Likewise, they were sensitive to the nucleic acid-targeting antibiotic streptomycin. However, among the cell wall-targeting antibiotics, they were resistant to penicillin. Additionally, they were resistant to the rest of the protein synthesis-inhibiting antibiotics (tetracycline, kanamycin, erythromycin, gentamicin, clindamycin and chloramphenicol) (Table 3(c)). There was no change in the results with higher concentration 0.001mg/mL of the respective antibiotics against LAB.

Antibacterial effects of LAB isolates in vitro

In this study, the CFS and < 10Kda peptide of 9 LAB isolates showed varying levels of antimicrobial activity against *P. aeruginosa* (Table 3(d)). Three LAB [*L. brevis* SDL1411 (L05), *P. acidilatici* DM9 (L21) and *P. pentosaceus* SDL1409 (L33)] strains were found to be strong bactericidal activity (19 – 25 mm) *Enterococcus faecium* CK-5 (L03), *Lactobacillus plantarum* JDFM44 (L09), *Leuconostoc citreum* SC53 (L12), *Pediococcus acidilatic* SDL1414 (L17), *Pediococcus pentosaceus* MAC11 (L22), *Weissella koreensis* JBNU2 (L31), showed the least survival ability (< 15 mm) respectively. Low molecular weight (< 10 Kda) peptide showed enhanced antimicrobial activity due to high in low molecular weight peptides respectively.

Heat stability of antimicrobial peptides (< 10Kda peptide)

The < 10Kda peptide produced by the nine selected LAB isolates were heat-treated at 90°C for 30 and

60 min, respectively. Peptides were thermal stable at these heat conditions above as their inhibitory effects against *P. aeruginosa* were retained (Table 3(e)). However, < 10Kda from *Enterococcus faecium* CK-5 (L03), *Lactobacillus plantarum* JDFM44 (L09), *Leuconostoc citreum* SC53 (L12), *Pediococcus acidilatic* SDL1414 (L17), *Pediococcus pentosaceus* MAC11 (L22), *Weissella koreensis* JBNU2 (L31) were less sensitive to 90°C for 30 and 60 min displaying either smaller or no inhibition zones compared to the control. But LAB Isolates [*L. brevis* SDL1411 (L05), *P. acidilatici* DM9 (L21) and *P. pentosaceus* SDL1409 (L33)] showed strong antimicrobial activity against *P. aerogenasa* and retained their anti – *pseudomonas* activity subsequent to exposure to 90°C for 60 min.

Digestive enzyme stability of antimicrobial peptides

The enzymatic stability of peptide produced from nine LAB isolates revealed that among the isoaltes *L. brevis* SDL1411 (L05), *P. acidilatici* DM9 (L21) and *P. pentosaceus* SDL1409 (L33) showed moderate sensitivity (17–20 mm) against *P. aerogenasa* with enzyme treated peptided, but subsequently other six LAB *Enterococcus faecium* CK-5 (L03), *Lactobacillus plantarum* JDFM44 (L09), *Leuconostoc citreum* SC53 (L12), *Pediococcus acidilatic* SDL1414 (L17), *Pediococcus pentosaceus* MAC11 (L22), *Weissella koreensis* JBNU2 (L31) were found to be sensitive with digestive enzymes hence it lost the anti-*pseudomonas* activity

Nitric oxide production from LAB

The Production of nitric acid (NO) was estimated qualitatively (Sup. 1) and quantitatively determined in L5, L21, and L33 (Table 3(f))

NO generation had be stimulated by dietary supplementation with substrate lactobacilli in vivo. The NO ($p < 0.001$) content were also estimated in modified media, NO contents in *C. elegans* supplemented with probiotics showed increase in NO nitrate, had significantly higher at 9th day nitrite levels (Table 3(f), Sup.1).

Chemotaxis assay

Feeding nematodes with 35 *Lactobacilli* strains resulted in increases in the average life span of the nematodes compared to their life span when fed with *E. coli* OP50 strain (Figure 2(a-j, a1-z2)). Between the cell pellet and broth culture of LAB, the chemotaxis assay indicates that the worms were maximally attracted towards the

broth culture (Figure 1(b)) and showed lesser attraction towards the pellet and solvents.

Fertility assay

To increase the fertility of nematodes, LAB strains were fed to worms in NGM plate, and their effects were compared with those of *E. coli* OP50. The results indicated that worms fed the 35 different LAB strains showed lower fertility compared with worms fed the OP50 strain (Figure 1(c)). To examine if there were any beneficial effects of feeding LAB to worms compared to OP50 and the survival rate of worms fed on LAB were compared with that of nematodes fed on *P. aeruginosa* PA14. OP50 did not prolong the worms' longevity as much as LAB did (Figure 2(a)). We observed only negligible change in the body sizes of the nematodes

fed on *P. aeruginosa* and LAB compared with those of control worms during the first 3 days after the food source was changed (Data not shown). On the other hand, 80% of the worms fed OP50 remained alive after 5 days. The pathogenicity of *P. aeruginosa* against *C. elegans* has been reported elsewhere, slow killing of *C. elegans* by PA14 involves the colonization and proliferation of pathogen in the host gut. The widespread problems associated with PA14 is principally due to the production of a series of virulence factors including protease, elastase, pyocyanin, and alginate [1,16] and was confirmed in this study; the life span of worms fed with newly isolated LAB strains from fermented soy bean paste were significantly different from the life spans of those grown on *E. coli* OP50 (Figure 1(c)).

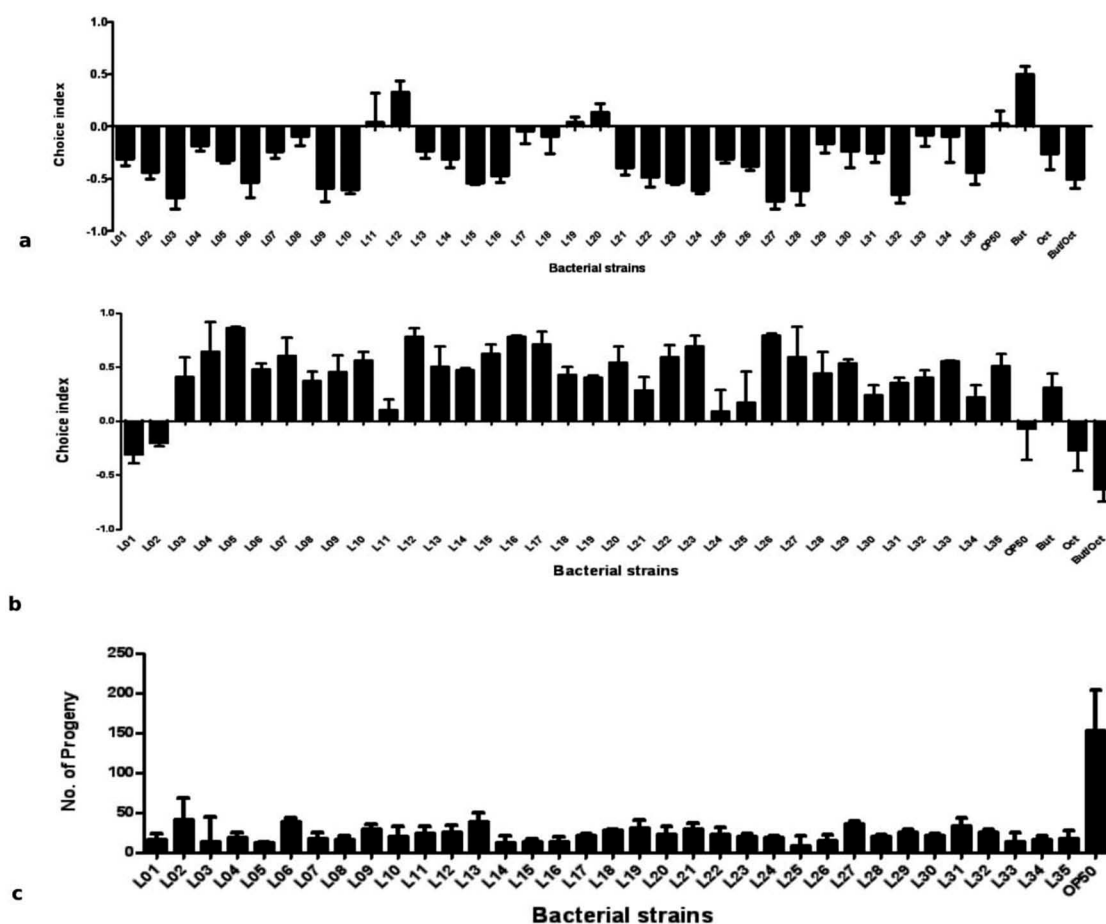


Figure 1. (a) Selection of preferred LAB among 35 strains (either broth or cell pellet) as food to *C. elegans*. (a) Cell pellet of LAB strains collected by micro-centrifuge and used as bacterial food in NGM plates with worms ($n = 150$ per plate). From chemotaxis assay results, worms showed a high preference for 4 LAB strains and low preference for 31 LAB strains compared with solvent (butanol and octanol). (b) Chemotaxis assay Broth of LAB strains used as food in NGM plates with worms ($n = 150$ per plate). A total of Worms showed high preference for 33 LAB strains and low preference for 2 LAB (L1 and L2) strains. Preference was obtained from the choice index as a numerical value (Y axis) and the threshold value from bacterial strains as the horizontal axis (X axis). (c) Fertility in *C. elegans* fed on various lactic acid bacteria. To explore effects of lactic acid bacteria on the fertility of nematodes, we used lactic acid bacteria as food instead of *E. coli* OP50 normally used in experimental conditions. The results indicate that worms fed LAB (L01-L35) had low reproduction and developmental rates compared with worms fed control *E. coli* OP50.

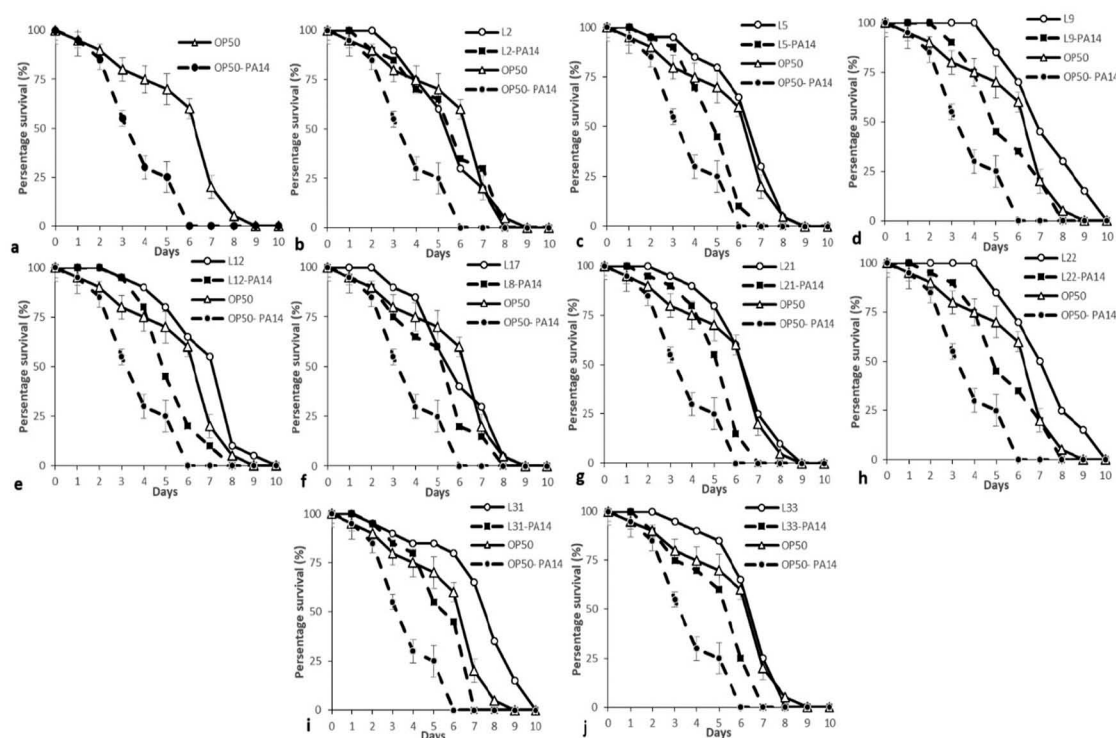


Figure 2. Life span and killing of *C. elegans* fed on various lactic acid bacteria and pathogens. (a) *E. coli* OP50 as their basic food for 24 h in NGM plates and then transferred onto *P. aeruginosa* (PA14)-seeded NGM plates, and the number of dead worms was enumerated each day until the 8th day of incubation. (b-j) Initially, the worms were fed for 24 h with lactic acid bacteria, which were seeded on NGM plates, and later, the worms were transferred onto PA14-seeded NGM plates (b) L2: *Enterococcus faecium* SC54, (c) L5: *Lactobacillus brevis* SDL1411, (d) L9: *Lactobacillus plantarum* JDFM44, (e) L12: *Leuconostoc citreum* SC53, (f) L17: *Pediococcus acidilatic* SDL1414, (g) L21: *Pediococcus acidilatici* DM-9, (h) L22: *Pediococcus pentosaceus* MAC-11, (i) L31: *Weissella koreensis* JBNU2, and (j) L33: *P. pentosaceus* SDL 1409. In each experiment, 30 worms were administered, repeated twice and plotted using OASIS II. (a1-z2) Initially, the worms were fed for 24 h with lactic acid bacteria, which were seeded on NGM plates, and later, the worms were transferred onto PA14-seeded NGM plates (a1) L1: *Enterococcus faecalis* SC54, (b1) L3: *Enterococcus faecium* CK-5, (c1) L4: *Enterococcus lactis* SCL1421, (d1) L6: *Enterococcus lactis* SCL1421, (e1) L7: *Lactobacillus curvatus* JBNU38, (f1) L8: *Lactobacillus pentosus* SC48, (g1) L10: *Lactobacillus rhamnosus* JDFM33, (h1) L11: *Lactobacillus rhamnosus* JDFM6, (i1) L13: *Leuconostoc mesenteroides* JBNU10, (j1) L14: *Leuconostoc paramesenteroides* SC46, (k1) L15: *Pediococcus acidilatic* SCL1420 (l1) L16: *Pediococcus acidilatic* SKL1418, (m1) L18: *Pediococcus acidilatic* SDL1406, (n1) L19: *Pediococcus acidilatic* SDL1405, (o1) L20: *Pediococcus acidilatic* SDL1402, (p1) L23: *Pediococcus pentosaceus* SDL1416, (q1) L24: *Pediococcus pentosaceus* SDL1415, (r1) L25: *Pediococcus pentosaceus* SDL1401, (s1) L26: *Streptococcus thermophilus* SCML337, (t1) L27: *Streptococcus thermophilus* SCML300, (w1) L28: *Weissella cibaria* SCCB2306, (x1) L29: *Weissella cibaria* SCCB2320, (y1) L30: *Weissella confusa* SCKB2318, (z1) L32: *Weissella cibaria* SCCB2307 and (z2) L34: *Lactobacillus plantarum* SDL1413, (z3) L35: *Lactobacillus arizonensis* SC25. In each experiment, 30 worms were administered, repeated twice and plotted using OASIS II.

C. elegans life span assay

The OP50 fed *C. elegans* indicates life span up to 8th days (Figure 2(a)), but their life span was shortened to 6th days when treated with *P. aeruginosa* (Figure 2(a)). The average life span of the 35 different LAB strains individually fed nematodes compared to the life span of nematode fed with *P. aeruginosa* (Figure 2(a-j, a1-z2)). Based on the results obtained, pretreated *P. aeruginosa* with post treated with LAB strains showed increase in life span (Figure 2(b-j)). Among the 35 LAB isolates,

nine strains showed strong acid tolerance at pH 2.5 (Table 2) with range of (62.79 – 91.45%). Based on the acid tolerance and life span assay (Figure 2(b-j, a1-z2)), selectively 9 strains were proceed for probiotic characterization. Among the selected 9 strains, only *L. brevis* SDL1411 (L05), *P. acidilatici* DM9 (L21) and *P. pentosaceus* SDL1409 (L33) showed strong antimicrobial activity against *P. aeruginosa* (Table 3(d)) and the selected three strains (L05, L21 and L33) showed efficient probiotic properties, such as Pepsin (pH 2.5) stability (Table 3(a)), Bile + Pancreatin (pH 8.0) stability

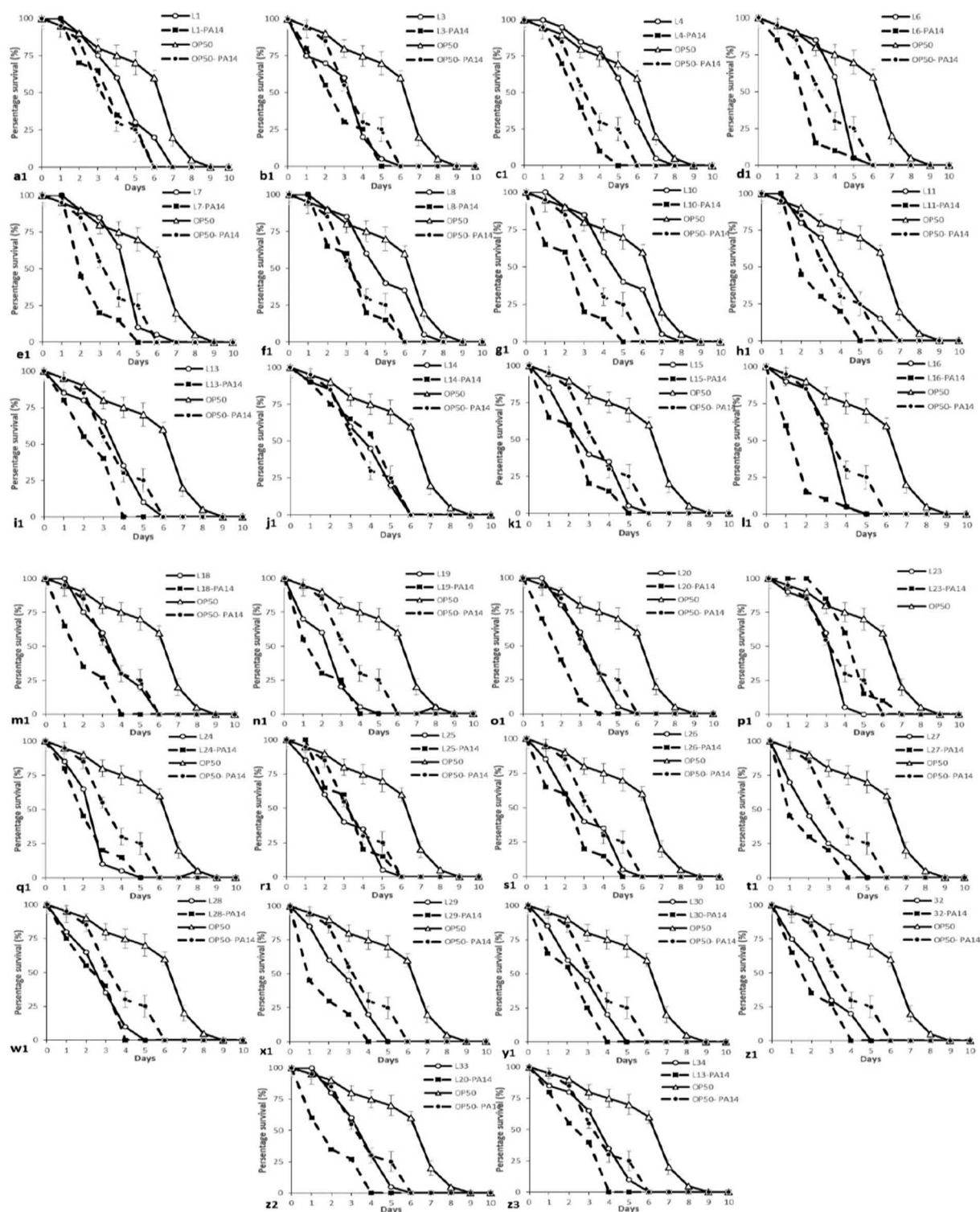


Figure 2. Continued.

(Table 3(b)), and lower antibiotic resistance towards most of the antibiotics (Table 3(c)). Hence the selected efficient LAB (L05, L21 and L33) strains were further analyzed for heat killed bacterial life span assay in comparison with OP50 and *P. aeruginosa* PA14 (Figure 4)

Nematode gut colonization ability of LAB

Although LAB strains in cultured broth may act as a major food source for worms, the L4 to young adult stage of the worms failed to lay eggs, and the L1 to L3 or L4 stages of the worms did not grow well compare to

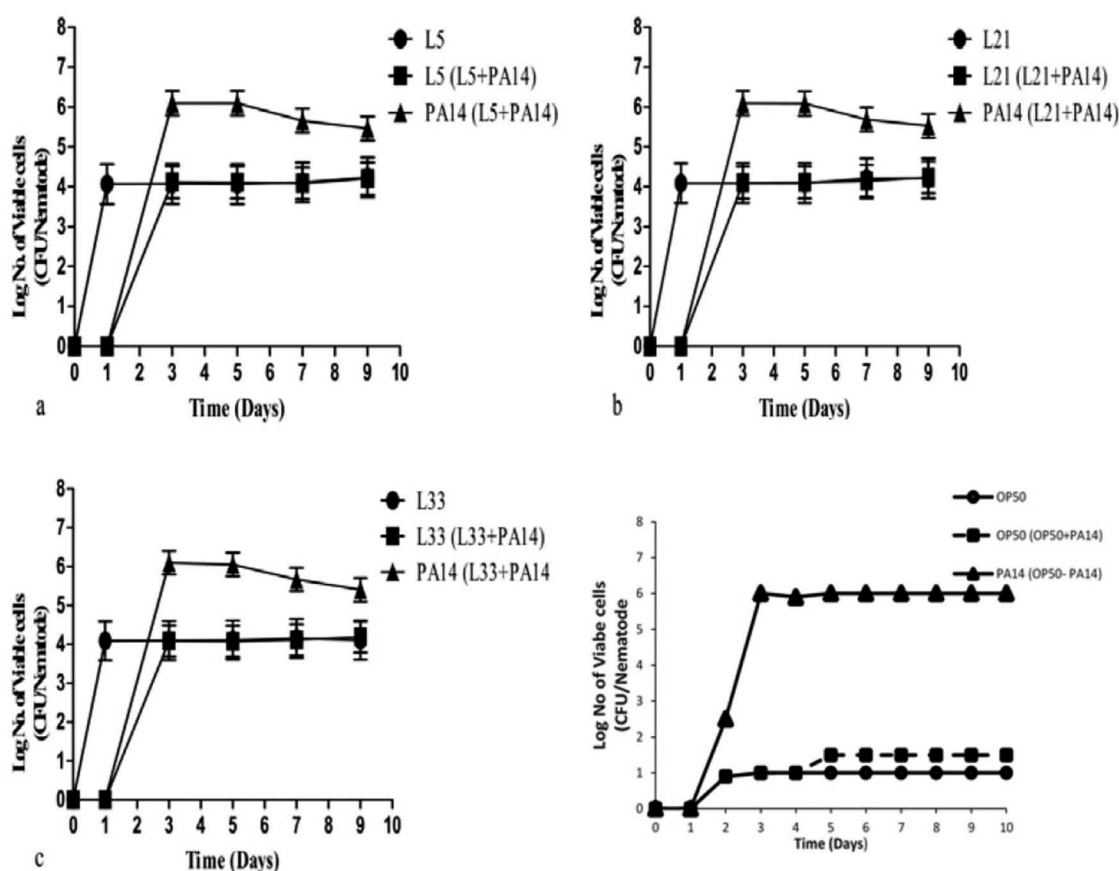


Figure 3. Bacterial colonization in *C. elegans*. Evaluation of LAB colonization (numbers of log CFU/nematode) in the nematode intestine. (a, b, c) Representation of pre-exposure to LAB or *P. aeruginosa* PA14. The habituation of LAB in the worm showed a minor degree of inhibition with the increase in the number of *P. aeruginosa* PA14 in the intestine of nematodes at the seventh and ninth days of incubation. (a) L5: *Lactobacillus brevis* SDL 1411 (b) L21: *Pediococcus acidilatici* DM – 9 (c) L33: *Pediococcus pentosaceus* SDL 1409. All results are presented as the means $\pm$ standard error mean.

worms fed with OP50 (Figure 3(d)). However, the results indicated longevity and protective effects of LAB on nematodes, which show that most of the LAB strains have beneficial effects on the nematode when challenged with *P. aeruginosa* PA14 (Figure 3(a-c)). In these experiments, the nematodes were initially grown with LAB strains (pre-exposed) before being transferred to NGM plates with the PA14 strain comparatively the vice-versa. Post-exposure resulted in increased life span and LAB colonization in the intestine of *C. elegans* (Figure 3(a-d)).

Life span assay of *C. elegans* with heat killed bacteria

The OP50 fed *C. elegans* indicates life span up to 8th days (Figure 4(a)), but their life span was shortened to 6th days when treated with *P. aeruginosa* (Figure 4(a)). Intestinal colonization experiment with heat-killed lactic acid bacteria were conducted to determine the detailed factors for increasing nematode

longevity and protection against pathogens. As a result, the initial number of *P. aeruginosa* recovered from those worms during the initial stage (3-day-old L4 stage to young adult stage worms) was less than the number recovered from worms infected on the 7th day (Figure 4(a-d)). The 7-day-old nematodes were fed separately with three different bacteria (*L. brevis* SDL1411 (L05), *P. acidilatici* DM9 (L21) and *P. pentosaceus* SDL1409 (L33) (Figure 4(a-c) and Table 1). The results show that worms fed with any of the three tested strains, namely, *L. brevis* SDL1411 (L05), *P. acidilatici* DM9 (L21) and *P. pentosaceus* SDL1409 (L33) from 3 days old were clearly resistant to subsequent *P. aeruginosa* infection compared with *C. elegans* fed with OP50 before the *Pseudomonas* infection (Figure 4(a-d)). However, the number of *Pseudomonas* cells recovered from worms fed LAB was the same as that recovered from worms grown on OP50 (Figure 4(a)). Hence, the results reveal that compared with heat-killed bacteria, most of the live bacteria express bioactive compounds that provide

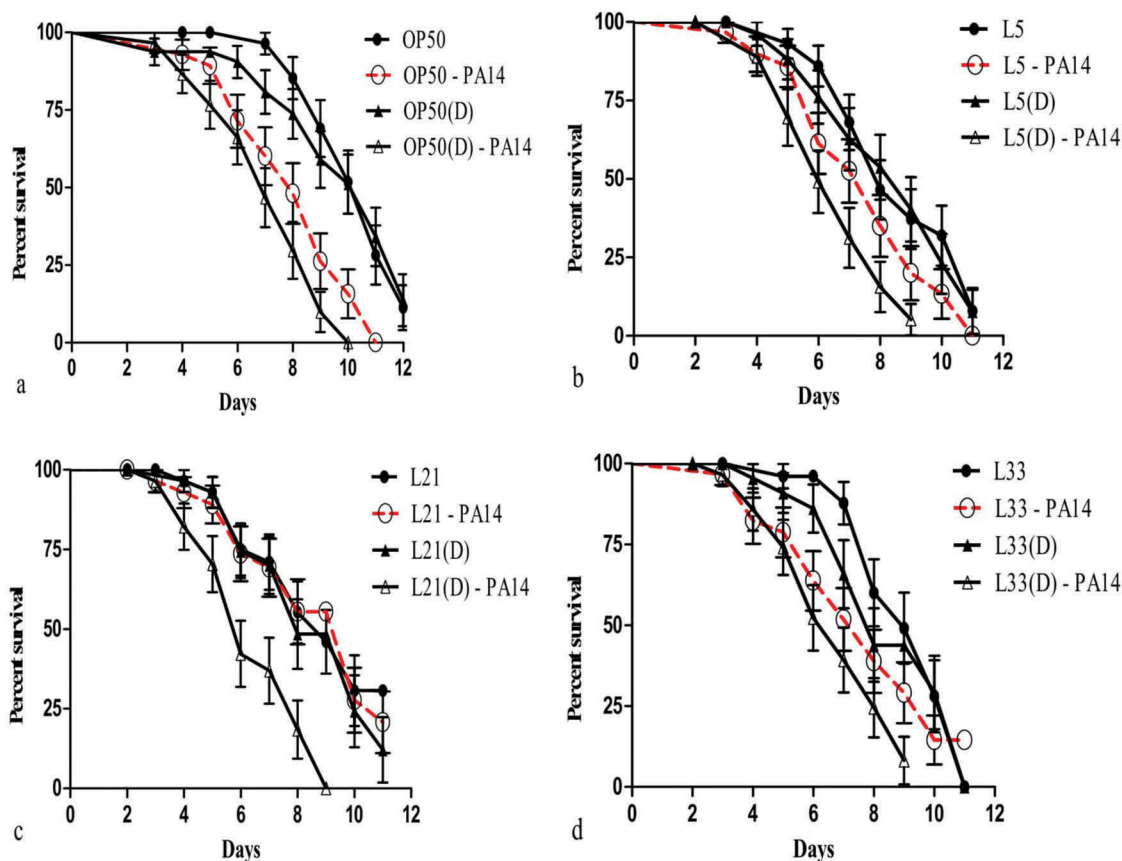


Figure 4. Lifespan and killing of *C. elegans* fed on heat-killed lactic acid bacteria and pathogens. (a) Young adult stage worms were transferred onto PA14-seeded NGM plates after 24 h incubation, and later, the worms were transferred onto *E. coli* OP50-seeded NGM plates; the dead worms were enumerated each day until the 8th day of incubation. (D): Worms were transferred onto PA14-seeded NGM plates, which were pre-exposed with heat-killed *E. coli* OP50 for 24 h on NGM plates; later, the dead worms were enumerated each day until the eighth day. (B-H) Lactic acid bacteria-seeded NGM plates and PA14-seeded NGM plates after 1-day incubation on LAB-seeded NGM plates. (b) L5: *Lactobacillus brevis* SDL 1411, (c) L21: *Pediococcus acidilactici* DM – 9, (d) L33: *Pediococcus pentosaceus* SDL 1409. Each experiment administered 30 worms and was repeated twice, and the results were plotted using OASIS II program. (Each experiment groups labelled “D” – heat-killed bacteria in experiment).

protection and longevity (Figure 4(a-c)). Apart from the selected LAB, other bacterial strains showed different levels of probiotic effects (Figure 4(b,c)). The positive effects of LAB were brought about by their beneficial nature compared to OP50. The survival rate of worms fed on LAB was higher than that of nematodes fed on heat-killed *P. aeruginosa* PA14 (Figure 4(a,b)), but OP50 did not prolong the worms’ longevity as much as LAB (Figure 4(d)).

Discussion

Food and nutrition are known to be essential for survival and life. There is a particularly high interest in highly nutritious foods and functional foods [23,25,26,30,42,43].

It is known that soybean paste, a traditional Korean fermented food, is rich in soybean protein, organic acids, amino acids, and minerals, all of which are

beneficial to health; has antioxidant, anti-obesity, anti-mutagenic and fibrinolytic properties; and harbours many beneficial bacteria as probiotics [43]. The probiotic effect of lactic acid bacteria on *C. elegans*, which is a widely known in vivo model. However, there are no studies that used *C. elegans* or applied various experimental methods to confirm the effect gradually.

In this study, we investigated the probiotic effect of 35 lactic acid bacteria strains isolated from soybean paste using *C. elegans*, a model animal. Among 35 strains isolated from Korean traditional foods, the bioactive compounds secreted from certain *Lactobacillus* spp. inhibit both gram-positive and gram-negative bacteria [14]. In the present study, the life span assay in *C. elegans*, indirectly showed that three selective *Lactobacillus* spp. isolates from Korean traditional foods have strong antimicrobial activity against *P. aeruginosa*. Antimicrobial molecules modulate the composition of the microbiota and influence

the host immune system [44]. Hence, *Lactobacillus* spp. that possess antimicrobial activity can boost the host defence response towards pathogens. In this study, the worm was used as a simple in vivo defence response model. This model has been accepted as an alternative, efficient host model that resembles other animal models and has been used to study evolutionarily conserved aspects of innate immunity [45].

Probiotic lactobacilli present in fermented foods may have positive impacts on health [60]. Before an efficient probiotic bacterium is confirmed, it has to be tested for its biological impact on the host during gut passage [60]. The physiologic activity of probiotics should be able to withstand the stress environment imposed by digestive enzymes (Pepsin, pancreatin), bile salts, and acidity (2.0 pH) [61]. Resistance to these stresses is now considered the basic criteria for screening potential probiotic strains [62]. Acid resistance is also a prerequisite for the use of efficient probiotics as dietary supplements [63]. Most LAB strains survive for a long time in highly acidic environments without large reductions in viable bacterial counts [64]. However, the capacity of lactobacilli to survive in low pH remains debatable [64]. For example, *L. delbrueckii* subsp. *bulgaricus* strain has very poor survival rates when exposed to low pH [64].

Previous studies showed that the capacity of LAB and *Pediococcus* to survive at low pH were variable, even within the same species. In this study, the selected three strains exhibited good tolerance to pH 2.5 with significant 60% cell count (Table 2(a)). Previous studies support the testing of bacteria for resistance to gastric and intestinal conditions. However, few of these studies included testing for digestive enzymes [65]. Other relevant studies on probiotics examined combined effects, for example, of pepsin and pH or pancreatic enzyme and bile salts [66]. Notably, well-studied probiotics were subjected to individual and multiple stress-based assays and were found to be highly resistant to bile salts and digestive enzymes under an acidic pH of 2.0 [67]. The relevant physiological concentrations of human bile ranges from 0.3% [68]. Assessing the bile resistance of LAB strains is imperative during the selection of proficient probiotics [68]. Since most bile- and pancreatic enzyme-tolerant strains are highly beneficial as a remedy for lactose intolerance. A significant variability in pepsin tolerance has been observed among different LAB species. 1, 2-diaminoanthraquinone sulfate (DAA) with nitric oxide is water insoluble, this dye makes it possible to visualize the real NO distribution in native bacterial cells [69]. Although NO molecules have high permeability through the membranes of lactobacillus and intracellular structures due to their small

sizes. This result indicates that bacterial NO is generally beneficial to worms [70] indicates NO Promotes heat – tolerance (stress tolerance) in *C. elegans* [71–73].

Because the evolution of multi-drug-resistant pathogens may have developed from the antibiotic resistance genes (transmissible resistance) from probiotic strains, this has become a serious safety issue [73]. Some studies reported that *Lactobacillus* had high levels of resistance to ampicillin vancomycin and streptomycin [59]. Similar results were confirmed by our study. Most of the *Lactobacillus* strains were kanamycin and vancomycin resistant but were susceptible to protein synthesis-inhibiting antibiotics. Further studies are needed to estimate the antibiotic resistance on the basis of genetic stability of the examined strains. Immunocompromised patients generally are more vulnerable to infection with pathogens and have a higher incidence of opportunistic infections. However, there is no published evidence that consumption of probiotics that contain *Lactobacillus* or *Bifidobacteria* increases the risk of opportunistic infection among such individuals. In addition, 2 clinical studies have been conducted to assess the safety of probiotics in small groups of specific immunocompromised patients (e.g. patients with HIV infection), and the findings of these studies support the safety of probiotics consumed by such groups [74–76] Immunocompromised treated with – (*Pediococcus pentosaceus*, *Leuconostoc mesenteroides*, *Lactobacillus paracasei* subspecies *paracasei* F19, *Lactobacillus plantarum*, LGG, *L. johnsonii* LA 1, VSL 3, *L. plantarum* 299V (liver transplant), *S. boulardii* (HIV) – Absence of toxicity were determined [77–79] previously likewise pregnant women – (LGG, LC705, Bb99, and *Propionibacterium freudenreichii* spp *shermanii*) Absence of toxicity [80], Inflammatory bowel disease – (*Lactobacillus acidophilus* LA-5, *Lactobacillus delbrueckii* subsp. *bulgaricus* LBY-27, *Bifidobacterium animalis* subsp. *lactis* BB-12, *Streptococcus thermophilus* STY-31, LGG) – Absence of toxicity [81] Hospitalized adults – LGG, BioK+ -Absence of toxicity [82].

In the current study, *C. elegans* killing assay were performed by transferring 30 young adult to L4 stage worms onto a lawn of lactobacillus species for 24 h at 25°C and then transferring them to *P. aeruginosa* PA14. The results revealed that all the tested *Lactobacillus* spp. elicited negligible changes in the physiological characteristics of *C. elegans*, including size, compared with those of worms given *E. coli* OP50 as a control (data not shown) for 24 h. Remarkably, the viability of *C. elegans* was significantly enhanced. When they were pre-exposed to the non-pathogenic *E. coli* OP50 strain [46]. The chemotaxis assay has been used to reveal the behavioural characteristics of nematodes in

the field of neurobiology [47]. In this study, the nematodes showed low preference for the cell pellet of LAB and high preference for the broth (liquid) culture. This finding may be due to the attraction of nematodes to the diacetyl group, which is one of the secondary metabolites produced by lactic acid bacteria [48]. In addition, the behavioural characteristics of nematodes during the chemotaxis assay clearly showed that the LAB strains are non-toxic to the nematodes [49]. Based on these results, it is evident that LAB act as growth enhancers when they are used as food supplements for nematodes.

Results obtained from the fertility assays show that LAB reduced the reproductive ability but enhanced the life span development of the nematode, and showed a protective effect against the tested pathogen, *P. aeruginosa* PA14. Coenzyme Q (CoQ) is an important coenzyme in aerobic respiration, and mainly due to calorie restriction, it plays a role in increasing the lifespan of *C. elegans*. Similar life span enhancement based on LAB in *C. elegans* was reported by Martin [50]. Coenzyme Q (CoQ) or ubiquinone (2, 3-dimethoxy-5-methyl-6-multiprenyl-1, 4- benzoquinone) molecules are located in the hydrophobic domain of the phospholipid bilayer of cellular membranes [51]. Ubiquinone (or coenzyme Q [CoQ]), an isoprenylated benzoquinone lipid important for electron transport in aerobic respiration, is another possible factor to explain our findings. Since nematodes fed an *E. coli* mutant that was defective in the ability to synthesize CoQ were reported have an decreased life span [52], it is possible that the LAB do have a full set of enzymes to synthesize CoQ and that this resulted in the extension of life span. In addition, Ishi et al. observed that the life span of worms could be prolonged by adding CoQ10 into the medium for the worms and discussed the possible role of ubiquinone as an antiaging substance (Ishii et al., 2004). Likewise the Coenzyme Q production leads to extending the lifespan of *Drosophila*. CoQ has been linked to the lifespan extension. Ishii and co-workers reported that dietary supplement of CoQ10 extended the lifespan of *C. elegans* by releasing oxidative stress [53]. In contrast, another study showed that wild type *C. elegans* fed with CoQ-depleted *E. coli* extended their lifespan [54]. Worms heterozygous for *clk-1*, a homolog of yeast COQ7 that is required for CoQ biosynthesis, showed extended lifespan as well [27]. In mammals, although CoQ has been administrated for therapeutic use in human heart failure and ischemic heart disease [55], its relationship with lifespan has, like in worms, been controversial. Kalen and co-workers detected an age-associated loss of CoQ in homogenized human tissues, while a constant level of CoQ with age was reported

from homogenates of rat brains and lungs [55, 56, 57]. The biosynthesis of CoQ in *S. cerevisiae* is a complex process. Nine genes which are designated as COQ1–COQ9 have been identified for this series of enzymatic process in *S. cerevisiae* [58]. If LAB is producing CoQ enzyme, then it leads to increased life span of *C. elegans*. Similar to *S. cerevisiae* the CoQ gene has not been identified. Previously documented study's also provided sufficient proof, about the reduction in fertility due to LAB feeding is based on balanced diet and as an added advantage, when compared with OP50 fed *C. elegans* the LAB fed *C. elegans* showed increased life span.

The results from bacterial colonization and killing assays that applied heat-killed LAB pellet revealed that the life span and fertility were increased in the nematodes fed with heat-killed LAB cell pellets, which indirectly prove the protective nature of the LAB and the heat stability of the bioactive compounds. In addition, when the cell pellet-treated nematodes were fed with *P. aeruginosa* PA14, a dramatic reduction in pathogen colonization when compared with the control (not fed with heat-treated cell pellets). Likewise, there was a steep increase in selected LAB strains (L5- *L. brevis* SDL 1411; L21- *P. acidilatici* DM – 9; L33- *P. pentosaceus* SDL 1409) that were initially fed to *C. elegans* 24 h before being fed with *P. aeruginosa* PA14. This finding proves the efficacy of LAB strains against the pathogen. This defensive mechanism may be due to the probiotic efficiency of the LAB²⁶. In addition, some of the previously reported secondary metabolites that enhance the immune modulating activity may also act as defensive factors [59].

In summary, the work presented here demonstrates, *P. aeruginosa* PA14 significantly reduced the life span of *C. elegans* through pathogenicity. However, *L. brevis* (L5), *P. acidilatici* (L21) and *P. pentosaceus* (L33) increased the life span of the *C. elegans* pathogenicity model. The antibacterial activity exhibited by these isolates may be useful for controlling undesirable microbiota either in the food system or in the GI tract. Our results demonstrate promising probiotic characteristics for LAB by their survival in gut-simulated fluids and gut colonization abilities. However, the multiple-antibiotic resistance exhibited by most of the LAB requires further investigation. Among the strains, those with intrinsic antibiotic resistance may be used as probiotics as they cannot transfer resistant genes to pathogenic bacteria. Nevertheless, in the future, it is essential to match the potential application with the functional properties of these probiotic candidates. Moreover, the *L. brevis* (L5), *P. acidilatici* (L21) and *P. pentosaceus* (L33) strains demonstrated potential probiotic features.

The combination of in vitro platforms coupled with in vivo screening in the simple model organism, *C. elegans*, is a powerful tool to characterize industrially relevant bacterial isolates.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Author Contributions

RC., B.J.P., D.H.O., J.G.C and E.B.M., designed the experiments. R.C., B.J.P., D.H.O., J.G.C., E.B.M., S.H., carried out the experiments. D.H.O., and B.J.P. provided expertise and guidance. R.C., J.G.C., B.J.P. wrote the manuscript.

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