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Antilisterial potential of bacteriocinogenic *Lactobacillus plantarum* L001 and *Enterococcus faecium* L103 isolated from fermented foods

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Abstract

Listeriosis is a severe gastrointestinal disease that affects human health significantly. It is caused by the invasion of *Listeria monocytogenes*, which secretes virulence factors such as invasion-associated protein (IAP) and listeriolysin O (LLO). Although antibiotics are commonly prescribed for the treatment of listeriosis, overuse can lead to antimicrobial resistance. Lactic acid bacteria (LAB), which produce bioactive metabolites, and exhibit probiotic and antibacterial effects, have been explored as an alternative to antibiotics. In the present work, 83 LAB isolates were screened for 51 bacteriocinogenic-structural genes, and 4.8% of the isolates harboured these genes. Bacteriocinogenic LAB strains were evaluated for their antilisterial activity and mechanism of action against haemolytic *L. monocytogenes* ATCC19111. The results showed that only *Enterococcus faecium* L103 and L104, which produce Enterocin L50 (EntL50), showed antilisterial activity in the disc diffusion assay. The mechanism of action of these two strains was determined using HCT-8 cells, in which the exclusion assay was the main inhibition factor contributing to a 50% reduction in the infection assay, and was visualised using field-emission scanning electron microscopy (FESEM). The inhibition of infection was also supported by the absence of p60 protein expression. Heat-inactivated (HI) bacteriocinogenic LAB also conferred immunomodulatory effects by controlling the secretion of TNF-α and CXCL8 from *L. monocytogenes* infected cells. In conclusion, heat-inactivated LAB can potentially reduce the pathogenicity of *L. monocytogenes in vitro*, and should be further evaluated in pre-clinical studies.

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Introduction

Listeria monocytogenes is a foodborne pathogen that causes listeriosis, a life-threatening disease. Individuals with weakened immune systems are at higher risk of listeriosis (Jibo *et al.,* 2022). The bacterium employs several virulence factors, including listeriolysin O (LLO) and p60 protein, to aid in its invasion and intracellular survival. LLO is crucial for the escape of bacteria from phagocytic vacuoles, and their subsequent growth within the host cells. The p60 protein, with a molecular weight of 60 kDa, is one of the primary extracellular proteins secreted by *L. monocytogenes*, and plays a significant role in the invasion of non-phagocytic cells (Silva *et al.,* 2020).

Intestinal epithelial cells (IECs) are the main target sites of *L. monocytogenes* pathogenesis, and associated with attachment, invasion, and resistance to host immunity (Regan *et al*., 2013). IECs are also said to be the first-line defence mechanism against *L. monocytogenes* invasion, consisting of mucin, which strengthens the epithelial physical barrier function and integrity against *Listeria* invasion (Galdeano *et al*., 2019; Vieco-Saiz *et al*., 2019).

The treatment of listeriosis includes the administration of ampicillin or penicillin combined with aminoglycosides, and a combination of trimethoprim and sulfamethoxazole (Arslan and Baytur, 2019). The use of antibiotics in sensitive individuals can result in allergic reactions. Overuse or misuse of antibiotics has led to the emergence and

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spread of antimicrobial resistance, which has become an increasingly significant global public health issue. In recent years, lactic acid bacteria (LAB) have been the subject of extensive research, owing to their potential use as antibacterial agents. Studies have demonstrated the potential of LAB to be used in fermented foods and dairy products to produce added value, in terms of their antimicrobial properties through the production of bacteriocins. These antimicrobial properties have been shown to be effective against both pathogenic and spoilage microorganisms, including extremely drug-resistant (XDR) strains of *Acinetobacter baumannii* (Yap *et al.*, 2021), *Gallibacterium anatis* (Zhang *et al.*, 2021), and *Salmonella* spp. (Hossain *et al.*, 2022), *etc*. The antiviral potential of LAB and their metabolites against the influenza virus, gastroenteritis, and enterovirus has been widely reviewed by Ögel and Özturk (2020) and Tiwari *et al.* (2020). LAB can reinforce the antibacterial mechanisms possessed by the microflora in IECs to maintain the intestinal microbial balance. LAB enhance IECs' functions, regulate intestinal immune cell responses, eliminate gastrointestinal pathogens, and prevent post-infectious inflammation or overreactions in adaptive immunity (Galdeano *et al.* 2019). Protection of the gastrointestinal tract by LAB has been consistently reported to be effective in the early control of *L. monocytogenes* infection (Becattini and Pamer, 2018). The safety recognition of LAB from a natural source, such as Generally Recognized as Safe (GRAS) by the U.S. FDA (Parlindungan *et al.,* 2019), makes it an attractive candidate for combating bacterial infections and a biopreservative in the food industry for sustainable development. Therefore, the present work aimed to screen and evaluate bacteriocinogenic LAB isolated from fermented foods, and determine their antilisterial mechanisms of action in HCT-8 cells.

Materials and methods

Culture of L. monocytogenes and lactic acid bacteria (LAB) strains

L. monocytogenes ATCC19111 (haemolytic), *L. monocytogenes* ATCC15313 (non-haemolytic, control), *Pediococcus acidilactici* ATCC8042 (reference LAB, control), and *Escherichia coli* ATCC8739 (non-*Listeria*, negative control) were obtained from the American Type Culture Collection (ATCC, USA). *Lactobacillus plantarum* L001, previously reported to exert strong antibacterial activity against XDR *A. baumannii*, was used as the positive bacteriocinogenic LAB control (Yap *et al.*, 2021). *L. monocytogenes* ATCC19111 and ATCC15313 were grown in Brain Heart Infusion (BHI) broth (BD Biosciences, USA), and incubated at 37°C for 24 h. Purified single colonies were transferred to the BHI broth, and incubated under the appropriate conditions. *P. acidilactici* ATCC8042 was used as a reference strain. *L. plantarum* L001 and 83 LAB isolates from a previous study were retrieved from glycerol stock, and grown on de Man, Rogosa, and Sharpe (MRS) lactobacilli agar (HiMedia, India), whereas *E. coli* ATCC8739 was cultured on nutrient agar (HiMedia, India) using the streak-plate method. Purified single colonies of *E. coli* ATCC8739 and the LAB isolates were transferred to their respective broths, and incubated at 37°C for 24 - 36 h, under aerobic and anaerobic conditions (5%, v/v, CO_2), respectively.

Detection of bacteriocin-structural genes in LAB isolates

DNA of LAB isolates were extracted using the crude cell lysis method, and LAB bacteriocin structural genes were detected using different primers, as described previously (Macwana and Muriana, 2012; Fontana *et al.*, 2015; Ribeiro *et al.*, 2017) by polymerase chain reaction (PCR). The PCR mix, using the Gotaq® Taq Flexi DNA Polymerase kit (Promega, USA), consisted of 1× colourless buffer, 3 mM $MgCl_2$, 2.5 mM dNTP mix, 100 pmol primers, 1.5 U Taq Polymerase, and 3 µL DNA template. The following PCR cycle conditions were applied: an initial denaturation step at 95°C for 2 min, followed by 35 cycles of 30 s denaturation at 95°C, 30 s annealing in the range of 45 - 55°C, 1 min extension at 72°C, a final extension at 72°C for 10 min, and a final hold at 4°C. The amplified PCR products were visualised on 2% (w/v) agarose gel stained with GelView (BioTeke, China). All PCR runs included a reference ladder, a 100 bp D2100 ladder (Smobio, Taiwan), and a blank control consisting of sterile distilled water. The PCR products that harboured the bacteriocinogenic-structural gene were subjected to DNA sequencing at the FIRSTBASE Sequencing Service and bacterial ID using 16s rRNA. The resulting sequences were analysed using the National Center for Biotechnology Information (NCBI) BLAST program.

Production of bacteriocin crude extracts from LAB isolates by disc diffusion antibacterial assay

Bacteriocinogenic LAB isolates, L101, L102, L013, and L104, were cultured under suboptimal conditions (25°C, aerobic conditions) to stimulate bacteriocin production (Sreedharan *et al.,* 2024). LAB cell cultures were centrifuged at 9,391 *g* for 10 min at 4°C to separate the bacterial cells from the cell-free supernatant (CFS). LAB CFS was adjusted to pH 6.5 with 2 M NaOH solution to eliminate the antimicrobial effect resulting from organic acids. Catalase from bovine liver (Sigma-Aldrich C9322) was added to CFS at a final concentration of 1 mg/mL, and incubated at 25°C for 30 min to eliminate the inhibitory effect of hydrogen peroxide (Voidarou *et al.,* 2020). CFS was sterilised using a 0.22 µm syringe filter, and concentrated for methanol extraction using a rotary evaporator. The concentration of crude bacteriocin was adjusted to 1 g/mL as a stock solution for further use.

Overnight broth cultures of *L. monocytogenes* ATCC19111 and ATCC15313 were adjusted to approximately 10^6 CFU/mL, and inoculated into 0.7% (w/v) soft Tryptic Soy Agar (HiMedia, India) using the pour-plate method. Blank discs were applied, and 10 µL of 1 g/mL of bacteriocin crude extract was transferred onto each disc, and incubated at 37°C overnight. The antilisterial activity of LAB bacteriocins was determined using Eq. 1, and expressed as AU/ml (Heidari *et al*., 2021).

$$Antilisterial\ activity = \frac{Area\ of\ clear\ zone\ (mm^2)}{Volume\ of\ bacteriocin\ crude\ extract(ml)}\ (AU/ml)$$

(Eq. 1)

In vitro listeria invasion assay

HCT-8 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 (ATCC 30-2001) media supplemented with 10% (v/v) foetal bovine serum (FBS), and incubated at 37°C under anaerobic conditions. The culture was maintained regularly, and sub-passaged when the cells reached sub-confluence (80 - 90%). HCT-8 cells were seeded in 24-well tissue culture plates at a density of 0.5 × 10^6 cells/well approximately, in 10% (v/v) FBS RPMI medium, and grown overnight at 37°C under 5% CO_2 anaerobic conditions. Subsequently, the HCT-8 monolayer was washed with 1× PBS (pH 7.2) prior to the invasion assay.

The protective effect of LAB against *L. monocytogenes* invasion of HCT-8 cells was determined using previously described procedures, with minor modifications (Popovic *et al*., 2019). Briefly, overnight cultures of *L. monocytogenes* ATCC19111 and ATCC15313 in BHI broth, and the selected viable and heat-inactivated (HI) LAB strains in MRS broth, were harvested by centrifugation (9,391 *g*, 5 min), and washed thrice with 1× PBS. The concentrations of *L. monocytogenes* and LAB were adjusted to approximately 10^6 and 10^7 CFU/mL, respectively, in RPMI media before the HCT-8 cells were infected.

Mechanism of action by LAB to reduce L. monocytogenes invasion

The use of *L. plantarum* L001 as a positive control, and *L. monocytogenes* ATCC19111, was evaluated using competitive, exclusion, and displacement assays, as previously described (Popovic *et al*., 2019), to determine the antilisterial activity of the LAB. The mechanism of action was selected based on the evaluation of invasion and inhibition abilities. The selected strains, *Enterococcus faecium* L103 and *P. acidilactici* ATCC8042, were tested. The invasion ability of *L. monocytogenes* and inhibition ability of LAB were calculated using Eqs. 2 and 3, respectively:

$$\text{Invasion efficiency (\%)} = 1 - \left(\frac{Number\ of\ invaded\ L.monocytogenes_{T0}\ \left({}^{cfu}/_{ml}\right)}{Number\ of\ invaded\ L.monocytogenes_{T1}\ \left({}^{cfu}/_{ml}\right)}\right) x\ 100\%$$

(Eq. 2)

where, the number of invaded *L. monocytogenes*$_{T0}$ represents the number of invaded *L. monocytogenes* at 0 h, and the number of invaded *L. monocytogenes*$_{T1}$ represents the number of invaded *L. monocytogenes* at 1 h.

$$\text{Inhibition efficiency (\%)} = 1 - \left(\frac{Number\ of\ invaded\ L.monocytogenes_{C}\ \left({}^{cfu}/_{ml}\right)}{Number\ of\ invaded\ L.monocytogenes_{LAB}\ \left({}^{cfu}/_{ml}\right)}\right) x\ 100\%$$

(Eq. 3)

where, the number of invaded *L. monocytogenes*$_{C}$ represents the number of invaded *L. monocytogenes* in the absence of LAB, and the number of invaded *L. monocytogenes*$_{LAB}$ represents the number of invaded *L. monocytogenes* in the presence of LAB.

Competitive, exclusion, and displacement assays

The three mechanisms of action for evaluating LAB inhibition of *Listeria* infection were based on a previously described method (Popovic *et al*., 2019). HCT-8 monolayer was co-cultured with *L. plantarum* L001 and *L. monocytogenes* at multiplicities of infection (MOI) of 10 and 1, respectively. The addition of bacterial cultures in 12-well plates (Dutra *et al*., 2016) was performed based on different order to simulate the mechanism of action. Antibiotic treatment was performed using 100 µg/mL penicillin-streptomycin in RPMI medium for 30 min of incubation to eliminate extracellular or weakly attached bacteria outside the cells. After incubation, the killed extracellular bacteria were removed, and the cells were gently washed with sterile 1× PBS. Enumeration of *L. monocytogenes* was performed with an appropriate dilution, using sterile 1× PBS as the diluent on *Listeria* selective agar (HiMedia, India), and then using the pour-plate method, and incubated at 37°C for 24 - 48 h. The assay with the highest inhibitory effect was selected to test the inhibitory activity of *E. faecium* L103 and *P. acidilactici* ATCC8042, by replacing *L. plantarum* L001.

Detection of invasion-associated protein production by L. monocytogenes

The mechanism of action of *L. monocytogenes* inhibition was validated based on the presence of IAP produced by *L. monocytogenes* using western blotting. Cell lysates infected with *L. monocytogenes*, with or without treatment, with *L. plantarum* L001, *E. faecium* L103, or *P. acidilactici* ATCC8042, were collected from the exclusion assay to obtain cellular protein extracts. A negative control, *E. coli* ATCC8739, was used to replace LAB. The cellular protein extract was then subjected to protease inhibitor treatment, and the concentration was measured using a Pierce™ bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, USA), with bovine serum albumin as the standard. Cellular protein extracts were mixed with 10× loading dye, denatured at 95°C for 10 min, and incubated on ice. Denatured cellular protein extracts and a dual-colour reference ladder (Bio-Rad, USA) were subjected to SDS-PAGE. Proteins on the gel were transferred to a polyvinylidene difluoride (PVDF) membrane, and blocked with 3% skim milk at room temperature for an hour. The membrane was then incubated with fresh 1% skim milk, and incubated with primary antibody p60 (Novus Bio, USA) at a ratio of 1:5000 at room temperature overnight. On the second day, the membrane was washed thrice with 1× Tris-buffered saline-Tween 20 (TBST), and a secondary antibody (HRP-conjugated anti-mouse IgG) was added to fresh 1% skim milk at 1:5000 and incubated for an hour. The membrane was then washed with 1× TBST, incubated with an enhanced chemiluminescence (ECL) substrate (Bio-Rad, USA), and viewed.

Immunomodulation potential of LAB in L. monocytogenes infected HCT-8 cells based on mechanism of action

The immunomodulatory characteristics of LAB were profiled based on chemokine and cytokine expression in *Listeria*-infected HCT-8 cells. The HCT-8 monolayer was infected with HI LAB and *L. monocytogenes* at MOI of 10 and 1, respectively, at different time points (0 - 8 h). To determine the relationship between HI L001 and HI L103, a 1:1 mixture was used. The controls included HCT-8 cell baseline (with no bacteria), HI L001, HI L103, or the mixture, and haemolytic *L. monocytogenes* ATCC19111. Briefly, the treatments, including each or a mixture of HI LAB, were added to HCT-8 cells, and incubated for an hour before *L. monocytogenes* infection. After an hour, the HI LAB were removed from the wells, and washed twice with sterile 1× PBS. *L. monocytogenes* ATCC19111 was added to each well. At each 2 h interval (up to 8 h), the CFS of the controls and treatments was collected from the wells by centrifugation (9,391 *g*, 5 min), and stored at -80°C for determination of chemokine and cytokine production. The attached cells were then collected for visualisation using field-emission scanning electron microscopy (FESEM).

Chemokine production by HCT-8 cells was measured using a Cytometric Bead Array (CBA) Human Chemokine Kit (BD Biosciences, USA). Five chemokines, CXCL8, CCL5, CXCL9, CCL2, and CXCL10, were measured and detected using flow cytometry (FACS Canto II), following the manufacturer's instructions (BD Biosciences, USA). The samples were sent for the detection of eight immune analytes (Human Serum/Plasma, 32 × 8; ST01E-PS-003426), including IL-1β/IL-1F2, IL-2, IL-4, IL-6, IL-10, IL-12P70, IFN-γ, and TNF-α, using ELLA analysis (Biotechne ®, Protein Simple).

Visualisation of interaction between heat-inactivated (HI) L001 and L. monocytogenes ATCC19111 by field emission scanning electron microscopy (FESEM)

The attached cells were collected using a cell scraper, and fixed in 4% glutaraldehyde at 4°C. The samples were further washed with distilled water, and dehydrated using different concentrations of ethanol (0 - 100%) and acetone before critical point drying. The samples were then subjected to FESEM.

Statistical analysis

All experiments were performed at least in duplicate, and each set of experiments was performed in duplicate. All data were expressed as the mean ± standard error of the different experiments. One-way analysis of variance with the Kruskal-Wallis *post hoc* test was used to evaluate significant differences among the groups. All analyses were conducted using the GraphPad Prism 9.4.0 software (GraphPad Software, USA).

Results

Bacteriocin-producing LAB isolated from food

The prevalence of bacteriocinogenic LAB isolated from food and soil was 4.8% (4/83). The four LAB isolated from food were found to contain the bacteriocin structural gene (Figure 1), and none of the LAB were isolated from soil. In the disc diffusion assay, not all LAB strains with amplified bacteriocin structural genes showed an inhibitory effect against *L. monocytogenes*. Only *L. plantarum* L001 from a previous study and *E. faecium* isolated from fermented vegetables (L103) and fermented probiotic drinks (L104) were proven to exert an antilisterial activity of at least 550 AU/mL (Table 1).

Mechanism of action of LAB against L. monocytogenes in HCT-8 cells

L. plantarum L001, as a positive control, was further tested for antilisterial invasion *in vitro* using competitive, displacement, and exclusion assays. Among the three assays, the exclusion assay showed the most efficient inhibition effect, up to approximately 50% ($p < 0.05$), compared with the control (Figure 2A). Thus, an exclusion assay was performed to determine the antilisterial invasion activity of *E. faecium* L103 and *P. acidilactici* ATCC8042. As shown in Figure 2B, all selected

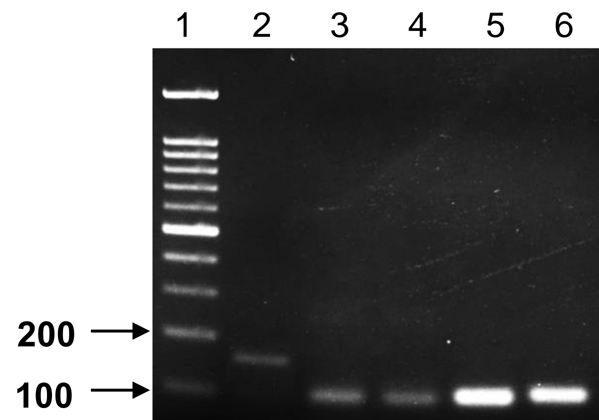

Figure 1. Bacteriocin-structural genes detected in LAB food isolates in 2% (w/v) agarose gel. Lane 1: 100 bp DNA ladder; lane 2: lcnB (L101); lane 3: entL50 (L103); lane 4: entL50 (L104); lane 5: plnc8A (L102); and lane 6: plnc8B (L102).

Table 1. Bacteriocin-structural genes detected in LAB food isolates and their antilisterial activity (AU/mL).

Sample ID	LAB strain	Source	Amplified gene	Antilisterial activity (AU/mL)	
				L. monocytogenes ATCC19111	*L. monocytogenes* ATCC15313
-	*P. acidilactici*	ATCC8042	ND	-	-
L101	*Lactococcus lactis* subsp. *lactis*	Fermented vegetable	*lcnB*	-	-
L102	*L. plantarum* NC8	Fermented vegetable	*plnc8A* *plnc8B*	-	-
L001	*L. plantarum*	Fermented vegetable	ND	566.7 ± 19.24	584.4 ± 32.05
L103	*E. faecium*	Fermented fish products	EntL50	577.8 ± 11.11	577.8 ± 11.11
L104	*E. faecium*	Probiotic drinks	EntL50	577.8 ± 11.11	577.8 ± 11.11

ND: not detected.

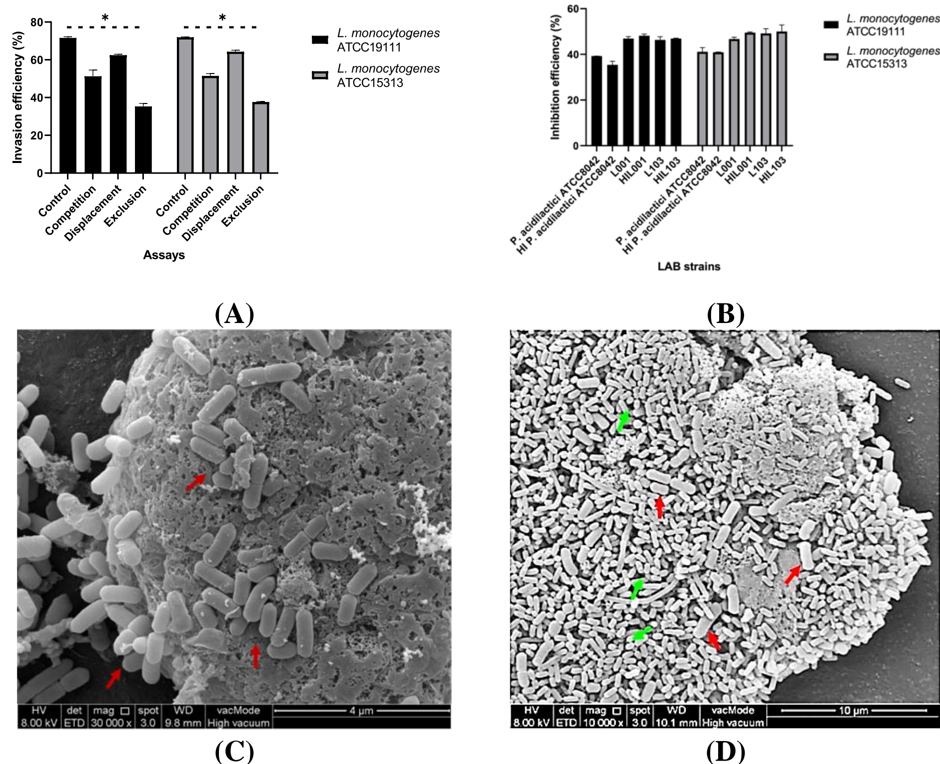

Figure 2. **(A)** Invasion efficiency (%) of *L. monocytogenes* ATCC19111 and ATCC15313 to HCT-8 cells in competition, displacement, and exclusion assay with viable *L. plantarum* L001 treatment. **(B)** Inhibition efficiency (%) of viable and HI LAB against *L. monocytogenes* ATCC19111 and ATCC15313. **(C)** *L. monocytogenes* ATCC19111 invasion to HCT-8 cells. **(D)** Interaction of HI L001 (green arrow) and *L. monocytogenes* ATCC19111 (red arrow) on the HCT-8 cells. Data are representative of duplicates, and values are expressed as mean ± standard error. (*) One-way ANOVA and Kruskal-Wallis post-comparison tests ($p < 0.05$).

viable and HI LAB strains showed at least a 30% inhibitory effect against *L. monocytogenes* invasion in HCT-8 cells. The bacteriocinogenic LAB *L. plantarum* L001 and *E. faecium* L103 showed a greater antilisterial effect (≥ 40%) on HCT-8 cells than the non-bacteriocinogenic *P. acidilactici* ATCC8042. Interestingly, HI LAB exerted antilisterial invasion activity to a similar extent as viable LAB ($p > 0.05$). Figure 2C shows the invasion of *L. monocytogenes* ATCC19111 into HCT-8 cells, whereas Figure 2D shows the aggregation of HI L001 and *L. monocytogenes* ATCC19111 around the HCT-8 cells.

Virulence factor (p60 protein) detection by western blot

The antilisterial activity of the selected LAB was further evaluated by the presence of the invasive protein p60 produced by *L. monocytogenes* upon the invasion of HCT-8 cells, using western blotting (Figure 3). *L. monocytogenes* ATCC19111 produced p60 protein upon HCT-8 cell infection, for as short as 10 min and up to 1 h. No detectable bands for p60 protein were observed with any of the LAB treatments (lanes D to J), including both viable and HI LAB, where *P. acidilactici* ATCC8042 acted as a reference control.

Immunomodulation effect of LAB against L. monocytogenes ATCC19111 invasion

HI L001 (*L. plantarum*) and HI L103 (*E. faecium*) were further studied for their immunomodulatory effects against haemolytic *L. monocytogenes* ATCC19111 infection in HCT-8 cells. Among the five chemokines tested using CBA, untreated HCT-8 cells, *L. monocytogenes* ATCC19111, and HI LAB showed no effect on CXCL9 and CXCL10 production in HCT-8 cells. The untreated HCT-8 cells constitutively secreted CCL2 and CCL5 at very low levels (< 40 pg/mL).

Among the eight immune analytes tested in the ELLA assay, only TNF-α showed a positive result.

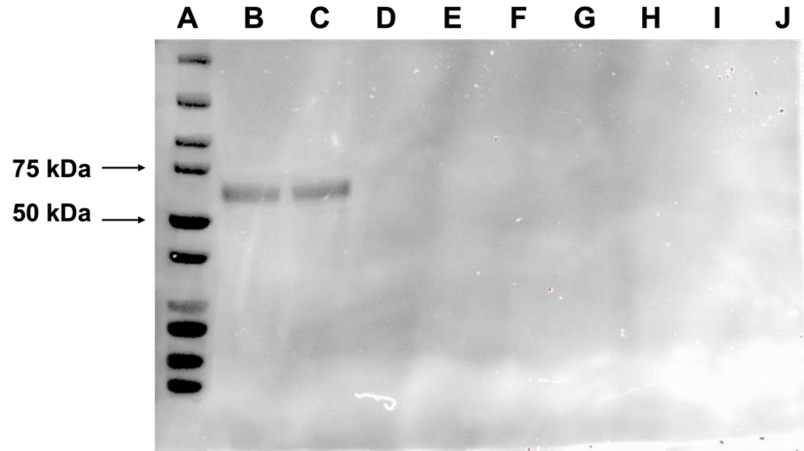

Figure 3. Virulence factor p60 protein detected in *L. monocytogenes* ATCC19111 *in vitro*, with or without LAB treatment. A: ladder; B: *L. monocytogenes* (control); C: pre-treatment with *E. coli* ATCC8739; D: pre-treatment with *L. plantarum* L001; E: pre-treatment with HI L001; F: pre-treatment with *E. faecium* L103; G: pre-treatment with HI L103; H: pre-treatment with *P. acidilactici* ATCC 8042; I: pre-treatment with HI *P. acidilactici* ATCC8042; and J: negative control (Baseline-RPMI media only).

Throughout the 8 h post-infection period, untreated HCT-8 cells and HI LAB produced low levels of TNF-α (< 5 pg/mL), whereas *L. monocytogenes* ATCC19111 increased the secretion of TNF-α up to 16 pg/mL. Pre-treatment with HI LAB reduced TNF-α secretion from *L. monocytogenes* ATCC19111 infected HCT-8 cells, with the greatest effect observed in the HI mixed culture (HIM) (Figure 4A). In contrast, as shown in Figure 4B, CXCL8 was constitutively secreted at low levels (< 500 pg/mL) for up to 8 h in untreated HCT-8 cells. After 8 h, *L. monocytogenes* ATCC19111 and HI LAB alleviated the secretion of CXCL8 from HCT-8 cells compared with the baseline. Pre-treatment with HI LAB, especially HIM, effectively reduced CXCL8 production when challenged with *L. monocytogenes* ATCC19111, from the 1st to 8th h of infection.

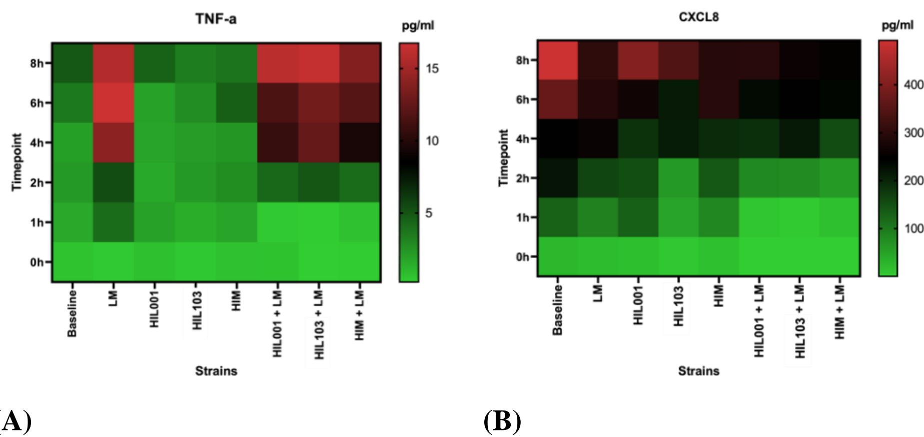

Figure 4. Production of CXCL chemokine from HCT-8 cells with bacterial infection and treatment for 8 h (pg/mL). **(A)** TNF-α, and **(B)** CXCL8. LM: *L. monocytogenes* ATCC19111.

Discussion

Antilisterial activity of bacteriocinogenic LAB

The antilisterial potential of bacteriocinogenic *L. plantarum* L001 and *E. faecium* isolated from fermented food was demonstrated in the present work. *E. faecium* L103 and L104 produced Enterocin L50 (EntL50), which exhibited antilisterial activity. It has been reported that *P. acidilactici* ATCC8042 exerted antibacterial activity against *L. monocytogenes via* membrane-bound proteins (Garcia-Cano *et al.*, 2015). However, metabolites from *P. acidilactici* ATCC8042 did not show an inhibitory effect on *L. monocytogenes* in the present

work. The secondary metabolites secreted by the positive control *L. plantarum* L001 consisted of cyclodipeptides (CDPs) and cyclic organic compounds belonging to diketopiperazine with proline, which have been identified as potential bioactive compounds. The CDPs not only possessed good binding affinity to the bacterial 16S rRNA, but also the targeted crystal structure complementary to geneticin, namely 1 MWL and ILC4, as the targeted crystal structure. Geneticin, an antibiotic structurally related to gentamicin and tobramycin, is an aminoglycoside that exerts antilisterial activities (Olaimat *et al*., 2018).

The production of bacteriocins from certain LAB, such as EntL50-producing *E. faecium* L103 in the present work, is closely associated with their growth environment, especially temperature, which is supported by Perez *et al.,* 2018), who reported that the bacteriocin activity of EntL50 at an optimal growth temperature of 37°C or above, diminished or vanished, and maximised at 25°C. The metabolic adaptation of LAB to this environment through a multitude of extracellular stimuli and stress-responsive gene regulation is crucial for the production of metabolites, such as bacteriocins (Parlindungan *et al.,* 2019). EntL50 is a unique class II bacteriocin with both class IIb (consisting of two peptides) and IIc (synthesised and secreted without an N-terminal extension) characteristics (Basanta *et al*., 2010). The antilisterial potential of EntL50 has been reported in previous studies (Tymoszewska *et al.,* 2021; Garcia-Vela *et al.,* 2024). Because bacteriocins are heat-stable, loss of EntL50 bacteriocin activity by heat-inactivation is unlikely (Garcia-Vela *et al.,* 2024).

The bacteriocin crude extract of L102 that amplified plnc8A and plnc8B showed no antibacterial activity, which was consistent with the findings of Jiang *et al*. (2016), who reported no inhibitory activity against six *L. monocytogenes* strains. Other plantaricins, such as plnA, plnEF, and plnJ, which contain GxxxG-motifs in both peptides, have been reported to exert antilisterial activity against *L. monocytogenes* ATCC19111 (Bengtsson *et al*., 2020; Butorac *et al*., 2020). However, instead of the GxxxG motif in both peptides, the plnc8B peptide contained the SxxxG-motif. Thus, the loss of the antilisterial activity of Plnc8 may be due to the polarity difference between glycine (G) and serine (S) (Bengtsson *et al*., 2020). LcnB is a one-peptide bacteriocin from the subclass IIc thiolbiotic AMP consisting of a cysteine residue, which is well known for its limited antibacterial activity against lactococci through dissipation of the proton motive force of sensitive cells (Tumbarski *et al*., 2018).

Viable and heat-inactivated LAB can function in exclusion mechanism in L. monocytogenes invasion to HCT-8 cells

The mode of action of the three different assays in inhibiting *L. monocytogenes* invasion is complicated and involves many factors, including variations in surface characteristics and environmental alterations owing to metabolite production among bacteria (Singh *et al*., 2017). In the present work, the exclusion assay was the strongest inhibition assay for preventing *L. monocytogenes* invasion of HCT-8 cells, which agreed with Ciandrini *et al*. (2017). LAB strains have been proposed to colonise host cells, creating a barrier known as colonisation resistance *via* saturation of the host cell receptor, and simultaneous production of postbiotics, such as bacteriocin, to defend *L. monocytogenes* (Ciandrini *et al*., 2017; Zielinska *et al*., 2021).

Interestingly, a reduction in *Listeria* invasion in HCT-8 cells was also observed when HI LAB were introduced in advance, highlighting the importance of cell wall components contributing to this antilisterial effect. Heat treatment stresses bacteria, and stimulates the formation of extracellular polymeric substances (EPS) as a protective barrier from the bacterial cell wall (Pique *et al*., 2019; Zielinska *et al*., 2021). Abundant LAB, including *Lactobacillus*, *Pediococcus,* and *Leuconostoc*, have been reported to synthesise exopolysaccharides, either loosely attached to the bacterial surface or secreted into the surrounding cell environment, and are suggested to play a role in the aggregation of bacteria (Pique *et al*., 2019), forming a biosurfactant that exhibits strong anti-biofilm activity against the growth of pathogens (Teame *et al*., 2020; Zielinska *et al*., 2021). The released cell wall molecules have been shown to interact with and saturate host cell receptors, preventing the attachment of the pathogens (Dutra *et al*., 2016). Singh *et al*. (2017) and Karbowiak *et al*. (2022) reported that HI LAB can reduce the number of pathogenic bacteria, including *L. monocytogenes*, *E. coli*, *Staphylococcus aureus*, and *Salmonella* Typhi. The ability of HI LAB to retain their antilisterial properties and stability during the thermal process could be a selective advantage as a natural strategy in the food control and pharmaceutical

industries (Ciandrini *et al*., 2017; Pique *et al.,* 2019). The identification of HI LAB that retain their antilisterial effect is a promising alternative, particularly for reducing the use of antibiotics.

LAB can also inhibit the growth of *L. monocytogenes via* metabolic, biological, and morphological alterations, and simultaneously inhibit or degrade the toxins produced by *L. monocytogenes* (Dutra *et al*., 2016; Teame *et al*., 2020), as shown in the present work. In the present work, the mechanism of action of HI LAB in counteracting toxins, such as the p60 protein, and preventing the diffusion of the toxin, was proposed to be controlled by bacterial cell wall components *in vitro* (Pique *et al*., 2019). *P. acidilactici* ATCC8042, which produces membrane-associated lytic enzymes as a reference control, was reported by Garcia-Cano *et al*. (2015) to exert antibacterial effects against various Gram-positive pathogens, including *L. monocytogenes*, suggesting that bacterial cell wall hydrolysis causes cell death.

Immunomodulation potential of HI LAB in L. monocytogenes infection

The integrity of human IECs is crucial for health preservation, and plays a bifunctional role that must be permeable for nutrient intake and ion transmission, while being impermeable to pathogens and antigens (Andrews *et al*., 2018). Communication between IECs and immune cells is important for maintaining intestinal homeostasis, which can be regulated by chemokines and cytokines to initiate the mucosal inflammatory response (Andrews *et al*., 2018; Rocha-Ramirez *et al*., 2020). In response to pathogen infection, proinflammatory chemokines and cytokines are secreted to activate and recruit inflammatory cells (*e.g*., neutrophils) to the infection site, and promote pathogen clearance (Rocha-Ramirez *et al*., 2020).

TNF-α, along with other chemokines and cytokines, is a proinflammatory cytokine released during infection with *L. monocytogenes*. It plays a crucial role in eliminating pathogens from the host, including *L. monocytogenes* (D'Orazio, 2019). CXCL8, also known as interleukin-8 (IL-8), is a proinflammatory chemokine/cytokine induced upon pathogen infection, including *L. monocytogenes*, which triggers an acute inflammatory response (Popovic *et al*., 2019). In the present work, upon *L. monocytogenes* ATCC19111 infection of HCT-8 cells, TNF-α levels were significantly increased, clearly showing the occurrence of inflammation, whereas CXCL8 levels were slightly decreased. Marion *et al.* (2005) reported that under inflammatory conditions, such as those stimulated by TNF-α, L-arginine significantly reduced CXCL8 secretion from HCT-8 cells by increasing nitric oxide levels, thus improving the integrity of the mucosal barrier and host immune response. A phenotypic microarray analysis revealed that *L. monocytogenes* ATCC19111 can produce arginine from pyruvate, which is an intermediate metabolite in its metabolic pathway (Yap *et al.,* 2024). *L. monocytogenes* also synthesises *de novo* arginine during its intracellular growth in macrophages. This may indicate inflammatory conditions, and a limit of arginine in the macrophage cytosol (Sauer *et al*., 2019). The synthesis of arginine could be speculated as one of the possibilities that *L. monocytogenes* infection reduced CXCL8 secretion from HCT-8 cells in the present work, and further studies are necessary. The decreasing trend in CXCL8 production from *L. monocytogenes* ATCC19111 infected HCT-8 cells compared to the baseline could have been due to HCT-8 cell injury or cell death caused by *L. monocytogenes* ATCC19111, and thus the inability to secrete CXCL8.

LAB can modulate the host immune response either through the inhibition or induction of proinflammatory cytokines *e.g.* such as TNF-α (Vincenzi *et al*., 2021). The inappropriate release of chemokines and cytokines may cause intestinal inflammation and cell damage (Andrews *et al*., 2018; D'Orazio, 2019), which can be alleviated by LAB through downregulation of proinflammatory chemokines or cytokine secretion (Galdeano *et al.,* 2019). In contrast, LAB can stimulate TNF-α secretion to strengthen the intestinal epithelial barrier through IECs proliferation (Vincenzi *et al*., 2021). It has been reported that pre-incubation of Caco-2, HT-29, and T84 cell lines with LAB, namely the exclusion assay, elicited better immunomodulatory activity against pathogen infection than the other two assays (competitive and displacement assay) against pathogen infection (Popovic *et al*., 2019). CXCL8 secretion in the absence of *L. monocytogenes* and the presence of HI LAB could be explained by their protective mechanism to prepare for defence against pathogen infection with the pre-recruitment of neutrophils. Genis *et al.* (2017) reported that CXCL8 secretion in the pre-treatment of LAB was reduced in the presence of *E. coli*, but increased in the absence of *E. coli*. *L. acidophilus* has been reported to be capable of downregulating IL-8 secretion through the

inhibition of TNF-α, thus improving epithelial barrier function and protecting against *L. monocytogenes* infection (Zhao *et al.,* 2020). LAB have been suggested to control TNF-α secretion *via* the NF-κB signalling pathway (Vincenzi *et al*., 2021). A study conducted by Popovic *et al.* (2019) revealed that pre-treatment or co-treatment with heat-killed *E. faecium* resulted in a significant reduction in the expression level of IL-8 mRNA in *L. monocytogenes*-infected Caco-2 cells. The most pronounced reduction in proinflammatory chemokines and cytokines was observed with the combination of *L. plantarum* L001 and *E. faecium* L103, which may be attributed to different mechanisms of action involving distinct intracellular signalling pathways. These findings suggested that a combination of these strains may be effective for controlling multifactorial diseases. Furthermore, it has been reported that different strains of heat-killed *L. acidophilus* can inhibit *Salmonella* invasion through immunoregulation (Lin *et al*., 2007), indicating the importance of strain-specific factors in affecting immunomodulatory activity (Rocha-Ramirez *et al*., 2020).

The immunomodulatory functions of HI LAB have been widely studied because they stimulate more complex immunomodulation than viable LAB (Pique *et al*., 2019). The immunomodulatory role of LAB cell wall components and their interaction with IECs have also been reported (Pique *et al*., 2019; Teame *et al*., 2020). LAB cell wall components, such as exopolysaccharides, peptidoglycans, and lipoteichoic acid, are highly likely to be dissociated by heat treatment, and act as ligands for immune cells, stimulating cellular signal transduction and the host immune system (Lin *et al*., 2007; Rocha-Ramirez *et al*., 2020). The exopolysaccharides of *L. delbrueckii* have been reported to act as ligands for toll-like receptors (TLR), exerting anti-inflammatory effects by modulating signalling pathways (Teame *et al*., 2020). Popovic *et al*. (2019) also reported that heat-killed *E. faecium* modulated TLR mRNA expression in *L. monocytogenes*-infected Caco-2 cells, suggesting that LAB cell wall components play a role in the temperature-induced dissociation of active molecules from the LAB cell cytoplasm or surface. HI LAB are unlikely to induce immunopathological responses; however, proinflammatory chemokines/cytokines could be beneficial to the host by enhancing resistance against pathogen infection (Rocha-Ramirez *et al*., 2020).

Conclusion

Our results demonstrated the antilisterial potential of *L. plantarum* L001 and *E. faecium* L103 as alternative strategies using a bacteriocin-based approach. The heat-inactivated (HI) LAB sourced from fermented foods maintained their antilisterial and immunomodulatory properties after exposure to heat. This makes them an attractive option for use in the food industry and in drug manufacturing, where heating is a common process, and the loss of antilisterial properties can be minimised. However, it should be noted that the present work was limited to *in vitro* findings, and further research is needed to validate these results using *in vivo* models.

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