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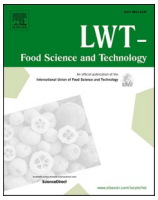

Probiotic *Bacillus* strains inhibit growth, biofilm formation, and virulence gene expression of *Listeria monocytogenes*

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ABSTRACT

This research aimed to evaluate the probiotic potential and anti-biofilm properties of *Bacillus* species isolated from dairy sludge, namely *Bacillus subtilis* GS3 (DS-1), *Bacillus cereus* BF2 (DS-2), *Bacillus velezensis* DM (DS-3), and *Bacillus thuringiensis* JB (DS-4). These strains exhibited tolerance to gastrointestinal fluids, bile salt, phenol, NaCl, and temperature variations. Moreover, they demonstrated notable cell surface hydrophobicity (70.80–89.30%), auto-aggregation (79.30–92.70%), and cholesterol removal (20.12–59.46%). Additionally, the strains displayed antioxidant activity and susceptibility to a wide range of antibiotics. The cell-free supernatants (CFS) of the strains exhibited inhibitory effects against *Listeria monocytogenes* ATCC 19115, with minimum inhibitory concentrations (MIC) observed at 12.5%, 25%, 25%, and 50% v/v for DS-1, DS-2, DS-3, and DS-4, respectively. The CFS also showed degradation and inhibition of *L. monocytogenes* biofilms, affecting adhesion, hydrophobicity, auto-aggregation, and exopolysaccharide production. Notably, DS-1 CFS suppressed the expression of genes associated with biofilm formation in *L. monocytogenes*, including those related to quorum sensing, virulence factors, and flagella. The antibacterial activity of CFS against *L. monocytogenes* was further confirmed by scanning electron microscopy. Particularly, the CFS of *Bacillus* spp., especially *B. subtilis* GS3, exhibited a strong inhibitory effect on the biofilm formation of *L. monocytogenes*.

1. Introduction

The global problem of pathogenic bacteria contaminating food is a serious concern, as it has a substantial impact on public health and can lead to increased death rates. As per the World Health Organization (WHO), contaminated food causes illness to nearly 600 million people and accounts for approximately 4.2 million deaths each year (Park, Kim, Yu, Lee, & Paik, 2023). Several virulence factors are produced by foodborne pathogens such as *Campylobacter*, *Salmonella*, *Escherichia coli*, and *Listeria monocytogenes*, which can lead to pathogenic infections (Liu, Yao, Zhao, & Ge, 2023).

L. monocytogenes is a Gram-positive bacterium known to cause listeriosis, a serious food-borne illness characterized by various symptoms such as perinatal infections, septicemia, spontaneous abortion, meningitis, and gastroenteritis. This illness can be fatal, with death rates ranging from 30% to 75%. The bacterium is primarily spread through faecal-oral transmission and has been found in a variety of food products. It is capable of growing at low temperatures and forming biofilms on biological and non-biological surfaces, making it challenging to eradicate in the food industry and leading to cross-contamination (Xedzro, Tano-Debrah, & Nakano, 2022).

Biofilms refer to groups of microorganisms that adhere to living or non-living surfaces, and are a prevalent aspect of microbial growth in the natural world. These communities of microorganisms are capable of withstanding harsh conditions such as extreme temperatures, high salinity, malnutrition, UV radiation, high pressure, and antibiotics. Essentially, biofilms offer a type of protective barrier or shielding for microorganisms (Liu et al., 2023; Roy, Song, & Park, 2022). Quorum sensing is a mechanism of paramount importance in facilitating bacterial communication that involves the regulation of gene expression through the use of autoinducers. This process is responsible for controlling various cell behaviours, including biofilm formation and the secretion of virulence factors. In the realm of food production, the resilience of biofilms in the face of environmental challenges can result

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in the continuous proliferation of foodborne disease-causing organisms, thereby escalating the likelihood of contaminating the products (Park et al., 2023).

Probiotics are purported to enhance human and animal wellbeing, restore intestinal equilibrium by suppressing pathogenic colonization, and alleviate gastrointestinal inflammation. Probiotics are garnering growing attention in the realm of human health-focused functional food studies and animal nutrition as a substitute for antibiotics (Falah, Vasiee, Yazdi, & Behbahani, 2021; Falah; Vasiee; Alizadeh Behbahani et al., 2021; Falah, Zareie, et al., 2021; Nooshkam & Zareie, 2022; Vasiee, Falah, Sankian, Tabatabaei-Yazdi, & Mortazavi, 2020; Vasiee, Falah, & Mortazavi, 2022; Alizadeh Behbahani et al., 2020).

In the realm of probiotics for monogastric animals, a number of commercially available strains of *Bacillus* spp. are currently employed. These bacteria, which are Gram-positive, capable of forming spores, and exhibit catalase activity, can survive under both aerobic and facultative anaerobic environments. They are typically found in soil and the digestive systems of animals. Among the *Bacillus* species that have been the subject of extensive study as probiotics for monogastric animals are *Bacillus subtilis*, *Bacillus coagulants*, *Bacillus licheniformis*, *Bacillus velezensis*, *Bacillus amyloliquefaeciens*, and *Bacillus cereus*. These *Bacillus* spp. have been shown to possess several advantageous properties, including the ability to produce antimicrobial compounds and enzymes. Moreover, their ability to form spores provides them with a distinct survival advantage in various environments (Luise et al., 2022; Mingmongkolchai & Panbangred, 2018).

In recent times, there have been a number of studies that have identified agents with anti-biofilm properties produced by *Bacillus* species. For instance, *B. subtilis* produces 5-Hydroxymethyl-2-furaldehyde (HMF) which is effective in inhibiting the pathogenicity of *Candida albicans* due to its anti-biofilm effect (Subramenium, Swetha, Iyer, Balamurugan, & Pandian, 2018), while *B. cereus* produces α-amylase which is effective in preventing the formation of biofilms by *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Vaikundamoorthy, Rajendran, Selvaraju, Moorthy, & Perumal, 2018). Nonetheless, there is limited research on the anti-biofilm properties of *Bacillus thuringiensis*, *B. subtilis*, *B. cereus*, and *B. velezensis* on *L. monocytogenes*.

The objective of the study was to examine the efficacy of *Bacillus* strains isolated from dairy sludge on *L. monocytogenes* ATCC 19115 in terms of their antibacterial and anti-biofilm properties. Additionally, the research evaluated the expression of genes responsible for biofilm formation and cell surface structural changes in *L. monocytogenes* ATCC 19115 to identify the mechanisms behind the anti-biofilm properties of the *Bacillus* strains.

2. Materials and methods

2.1. Isolation of Bacillus strains

Pegah Khorasan Co., located in Khorasan-e-Razavi, Iran, provided dairy sludge, which was transported to the laboratory under sterile conditions and stored at 4 °C before analysis. A 10-g sample was mixed with 90 mL of sterile physiological saline solution (0.85% w/v) and shaken for 10 min. Dilutions were made using physiological saline, and then subjected to a temperature of 100 °C for 15 min. From the appropriate dilutions, 100 μL was evenly spread on trypticase soya agar medium. The plates were incubated aerobically at 37 °C for 24–48 h. Randomly selected bacterial colonies showing spore-forming characteristics and morphological properties consistent with *Bacillus* spp. were purified through repeated streaking on trypticase soya agar medium. Stock cultures were maintained at 4 °C in brain heart infusion medium containing 15% glycerol (Dabiré et al., 2021).

2.2. Identification tests

The morphological characteristics, biochemical analysis, and molecular identification were performed according to methods described by Elsadek et al. (2023).

2.2.1. Morphological observation

To perform the bacterial analysis, 100 μL of the bacterial solution was collected and evenly spread on sterile Luria-Bertani (LB) medium plates. The plates were inverted and incubated at a constant temperature of 37 °C for 24 h. Visual characteristics of each colony were examined, and a Gram staining procedure was conducted on the strains. Gram-positive (G^+) bacteria displayed a blue or purple color, while Gram-negative (G-) bacteria exhibited a red color.

2.2.2. Biochemical analysis

For the biochemical analysis, the strains were subjected to treatment using a biochemical micro-reaction tube. The following tests were performed: Voges-Proskauer, propionate utilization, citrate utilization, L-arabinose utilization, D-xylose utilization, D-glucose utilization, gelatin liquefaction, hydrolysis of starch, indole formation, catalase activity, and nitrate reduction. Additionally, the strains' ability to grow at pH 5.7, 5% NaCl, and 7% NaCl was evaluated.

2.2.3. Molecular biological identification

The strains were cultured on a plate at 37 °C for 24 h. A single colony was selected and cultured in LB medium under the same conditions. Genomic DNA extraction was performed using the Mini BEST Bacteria Genomic DNA Extraction Kit (TaKaRa, Dalian, China) following the manufacturer's instructions. The 16S rDNA was amplified using universal primers (27F and 1492R). The PCR product was visualized on a 1% agarose gel and purified using a PCR purification kit (TaKaRa, Dalian, China). The PCR amplification samples were then sequenced. Sequence analysis was performed using Basic Local Alignment Search Tool (BLAST).

2.3. Probiotic properties

2.3.1. Gastrointestinal tract (GIT) tolerance

The *in vitro* digestion model was employed to simulate the conditions of the GIT and assess the viability of strains in the stomach and intestines. In brief, a solution of 3 g/L pepsin (Sigma Aldrich) in phosphate-buffered saline (PBS) at pH 3.0 was used to mimic gastric fluid, while a solution of 1 g/L trypsin (Sigma Aldrich) in PBS at pH 8.0 was used to simulate intestinal fluid. Initially, the bacteria were inoculated at a concentration of 10^7 in the simulated gastric juice. After an incubation period of 1 h at 37 °C, 1 mL of this mixture was combined with 9 mL of simulated intestinal juice and further incubated at 37 °C for 2 h. The viable bacteria were then cultured on deMan, Rogosa, and Sharpe (MRS) media at different time points, including time 0, 1, 2, and 3 h.

2.3.2. Bile tolerance

In summary, LB medium with bovine cholic acid (0.3 mg/mL) was prepared and mixed with a 2 mL bacterial suspension. The mixture was incubated at 37 °C for different time intervals (0, 1, 2, and 3 h), and the viable counts were determined on LB agar plates (Elsadek et al., 2023).

2.3.3. Phenol tolerance

The method described by Dabiré et al. (2021) was used to determine the survival of *Bacillus* strains against toxic metabolites formed during digestion. The overnight culture (1 mL) was inoculated with 10 mL of phenol (0.4%) rich-sterile nutrient broth and incubated at 37 °C for 24 h. The relationship of Burgain (2013) was then used to determine phenol resistance.

2.3.4. Sodium chloride and temperature tolerance

Bacterial strains were cultured overnight in MRS broth with different sodium chloride concentrations (1%, 3%, and 5%). Incubation was

carried out at 15 °C and 45 °C with shaking (200 × rpm) for 24 h. The absorbance at 605 nm was measured at the beginning and end of the incubation to monitor cell growth. The plate-agar method was used to assess the impact of sodium chloride on cell survival, and the percentage of cell survival was calculated. The control sample consisted of non-modified MRS broth (Tenea, Gonzalez, & Moreno, 2022).

2.3.5. Cell surface hydrophobicity

The hydrophobicity of *Bacillus* strains' cell surface was assessed using a method described by Dabiré et al. (2021). After growing a culture for 24 h at 37 °C, the cells were centrifuged and washed with PBS. The cells were then resuspended in 2 mL of PBS, and their OD adjusted to 0.5 at 600 nm (A_{0h}). Next, the cell suspension was mixed with an equal volume of toluene and vortexed for 5 min. The mixture was allowed to separate into two layers at 37 °C for 1 h. The organic layer was extracted, and the absorbance of the remaining aqueous layer was measured at 600 nm. This value, A_{1h}, was used in the formula surface hydrophobicity (%) = [1-(A_{1h}/A_{0h})] × 100 to determine the hydrophobicity of the cell surface. Strains that had a surface hydrophobicity value exceeding 50% were classified as hydrophobic.

2.3.6. Auto-aggregation

The auto-aggregation ability of the strains was evaluated using a method described by Dabiré et al. (2021). Briefly, the bacterial cells in PBS (10^8 colony forming unit (CFU)/mL) were incubated at 37 °C for 2 h. The absorbance of the upper layer was read at 600 nm before incubation (A_{0h}) and after 2 h of incubation (A_{2h}). The auto-aggregation was calculated using the below formula:

$$\text{Auto-aggregation } (\%) = [(1 - (A_{2h}/A_{0h})] \times 100$$

2.4. Safety evaluation

2.4.1. Haemolytic activity

The strains of *Bacillus* were introduced into a medium of sheep blood agar (7%), and then incubated for a period of 48 h at a temperature of 37 °C. Identifying the alpha and beta haemolytic isolates was based on the formation of a green or clear zone around the colony, respectively (Nwagu et al., 2020).

2.4.2. DNase activity

To assess the DNase activity of the isolates, the method of Shivangi, Devi, Ragul, and Shetty (2020) was employed. In summary, the isolates were streaked on DNase agar plates and incubated at 37 °C for 48 h for clear zone analysis.

2.4.3. Antibiotic susceptibility

The antibiotic susceptibility of *Bacillus* strains was assessed using the disk diffusion method, following the protocol described by Dabiré, Somda, Somda, Mogmenga, et al. (2022). Ten different antibiotic disks were used, including amoxicillin-clavulanic acid (30 μg), amikacin (30 μg), chloramphenicol (30 μg), ampicillin (10 μg), erythromycin (15 μg), ceftriaxone (30 μg), gentamicin (10 μg), penicillin G (10 μg), nitrofurantoin (30 μg), and vancomycin (5 μg). A 1 mL sample of each *Bacillus* strain, at a concentration equivalent to 0.5 McFarland, was spread onto Muller-Hinton agar plates using sterile beads. The plates were allowed to dry for 1 h before placing the antibiotic disks on the surface of the inoculated agar. After incubating the plates for 24 h at 37 °C, the diameters of the zones of inhibition around the antibiotic disks were measured. This allowed for the classification of each strain as susceptible (S), intermediate (I), or resistant (R) to the tested antibiotics.

2.5. Cholesterol removal

The ability of the isolates to remove cholesterol was assessed following the method outlined by Shivangi et al. (2020). Overnight cultures of the test isolates, with a concentration of 10^9 CFU/mL, were inoculated into MRS broth supplemented with 0.3% ox bile and 10 mg/mL cholesterol. The samples were then incubated for 24 h at 37 °C. The amount of cholesterol in the samples was quantified using the *o*-phthalaldehyde method, with spectrophotometric measurements taken at a wavelength of 550 nm.

2.6. Antioxidant effect

The antioxidant activity of *Bacillus* strains was evaluated using various methods, including DPPH radical scavenging (RS), hydroxyl RS, superoxide anion RS, and lipid peroxidation inhibition. These methods were conducted following the protocols described by Shivangi et al. (2020). Cells from the test isolates, cultured in MRS broth for 18 h, were collected by centrifugation (9000 × rpm, 5 min). The cell pellets were washed three times with PBS (pH 7.2) and adjusted to a concentration of 10^9 CFU/mL using the same buffer.

2.6.1. DPPH-RS effect

The DPPH assay was performed by combining 1 mL of bacterial cells with a freshly prepared 0.2 mM solution of DPPH and incubating the mixture in the dark for 30 min. The absorbance was recorded at 517 nm. Carbinol was used to prepare the blank sample, and the scavenging effect of the strains was calculated using the following formula:

$$\text{Scavenging effect } (\%) = [(1 - A_{\text{sample}})/A_{\text{blank}})] \times 100$$

2.6.2. Hydroxyl-RS effect

The hydroxyl-RS assay was performed by mixing 1 mL of 0.1% O-phenanthroline, 1 mL of 2.5 mM $FeSO_4$, 1 mL of 20 mM H_2O_2, 1 mL of 0.02 mM PBS (pH 7.4), and 0.5 mL of bacterial cells. The mixture was incubated at 37 °C for 90 min, after which the absorbance was measured at a wavelength of 536 nm. The RS activity was measured using the following formula:

$$\text{Scavenging effect } (\%) = [(A_{\text{sample}} - A_{\text{blank}})/(A_{\text{control}} - A_{\text{blank}})] \times 100$$

2.6.3. Superoxide anion RS effect

The assay involved combining 2.8 mL of Tris-HCl buffer (0.05 M, pH 8.2) and 0.1 mL of 0.05 M pyrogallic acid with 1 mL of the sample. This mixture was incubated in the dark at 25 °C for 4 min. To stop the reaction, 1 μL of 8 M HCl was added, and the absorbance was measured at 325 nm. A blank reaction mixture, without the sample, was also prepared. The ability of the strains to scavenge superoxide radicals was calculated accordingly.

$$\text{Scavenging effect } (\%) = [(1 - A_{\text{sample}})/A_{\text{blank}})] \times 100$$

2.6.4. Lipid peroxidation inhibition

The inhibitory effect of *Bacillus* strains on linoleic acid peroxidation was quantified using the thiobarbituric acid (TBA) method. In brief, 500 μL of sample, 200 μL of ascorbic acid (0.01%), 200 μL of $FeSO_4$ (0.01%), 500 μL of PBS (pH 7.4), and 1000 μL of linoleic acid emulsion were mixed together and incubated for 12 h at 37 °C. After incubation, 200 μL of trichloroacetic acid (4%), 2000 μL of TBA (0.8%), and 200 μL of butylated hydroxytoluene were added to the reaction mixture and incubated for an additional 30 min at 100 °C. The solution was then cooled and extracted with chloroform (2 mL), and the absorbance was

measured at a wavelength of 532 nm using the following formula:

Inhibition effect (%) = [(1 − A $_{sample}$)/A $_{blank}$)] × 100

2.7. Anti-pathogenic effect

2.7.1. Minimum inhibitory concentration

The MIC of CFS from *Bacillus* species was determined using the double broth dilution method. *Bacillus* strains were inoculated into tryptic soy broth and incubated at 37 °C for 24 h with shaking. The cultures were then centrifuged (10,000 × rpm, 4 °C, 15 min), and the CFS was filtered through a 0.22 μm syringe filter. The CFS was diluted two-fold in TSB, resulting in a concentration from 100% to 0.78%. Each dilution (50 μL) was added to a 96-well microtiter plate, followed by the addition of bacterial suspension (10^6 CFU/mL). The plate was shaken for 5 min and incubated at 37 °C for 24 h. The MIC was determined as the lowest concentration of CFS that showed no visible bacterial growth (Park et al., 2023).

2.7.2. Biofilm formation and degradation

The biofilm inhibition and degradation were assessed using a crystal violet assay (Park et al., 2023). The CFS underwent a serial two-fold dilution process, ranging from 100% to 0.78%. Bacterial cultures were also diluted to 10^6 CFU/mL. In a 96-well plate, 50 μL of CFS and 50 μL of bacterial culture were added per well. After incubation at 37 °C for 24 h, the cell suspensions were discarded, and wells were rinsed with water. Crystal violet solution (1%) was added for staining, and after 30 min, the staining solution was extracted. OD at 570 nm was measured using a microplate reader.

To assess the impact of CFS on mature biofilm degradation, a bacterial suspension (10^6 CFU/mL) was added (100 μL) to each well of a 96-well microtiter plate. After 24 h of incubation at 37 °C, the cell suspensions were removed. CFS, diluted in two-fold increments (100%–0.78%), was added (100 μL) to each well. For each strain, a control group was prepared using TSB instead of CFS. After an additional 24 h of incubation at 37 °C, the plates were rinsed twice to remove any remaining biofilms. The remaining biofilms were measured using the previously mentioned method. The rate of biofilm formation was determined accordingly:

Biofilm formation rate (%) = [OD(treatment)/OD(control)] × 100

2.7.3. Cell surface properties

To assess the hydrophobicity and auto-aggregation capacity of CFS-treated pathogens, the methods described by Park et al. (2023) were followed. A 20 mL volume of CFS (1/2 MIC) was mixed with a bacterial culture (20 mL; 10^7 CFU/mL). For the control sample, TSB was used instead of CFS. The combined cultures were incubated at 37 °C for 24 h, followed by centrifugation at 10.000 × rpm and 4 °C for 5 min. The cells were then rinsed twice with PBS (pH 7.4) and resuspended in the same buffer to reach an OD of 0.4 ± 0.05 at 600 nm (referred to as OD_i). To evaluate cell surface hydrophobicity, a 2 mL cell suspension was mixed with 500 μL of chloroform. After vortexing for 2 min, the mixture was incubated for 15 min at room temperature. The OD of the aqueous phase was then measured at 600 nm (referred to as OD_t) to determine hydrophobicity.

Hydrophobicity (%) = (1-OD_t/OD_i) × 100

To examine the auto-aggregation of *L. monocytogenes*, the combined culture was subjected to two rounds of washing using PBS. The OD of the culture was then adjusted to 0.4 ± 0.05 at a wavelength of 600 nm (this value is referred to as ODi) in a volume of 4 mL of PBS, followed by incubation at 37 °C for 24 h. After the bacterial strain settled, the absorbance of the clear layer was measured at a wavelength of 600 nm (referred to as OD_t) to obtain the auto-aggregation:

Auto-aggregation capacity (%) = (1-OD_t/OD_i) × 100

2.7.4. Adhesion ability

Bacterial strain (~10^7 CFU/mL) was mixed with an equal volume of CFS (1/2 MIC) in glass tubes. TSB was used as a substitute for CFS in the control group. The tubes were positioned at a 30° angle and incubated at 37 °C for 24 h. Planktonic cells that did not adhere were carefully removed from the tubes. The tubes were washed with PBS by adding 5 mL of PBS and vortexing to detach the cells. Adhered cells were then centrifuged at 10.000 × rpm for 5 min at 4 °C, and the supernatant was removed. PBS was used to resuspend the collected cells, and the absorbance of the adhered cells was measured at a wavelength of 600 nm (ODa). The adhered cells were combined with the planktonic cells and underwent the same washing process as described earlier. The total cells were reconstituted in 1 mL of PBS, and their absorbance was measured at a 600 nm wavelength (ODt). The adhesion ability was calculated using the formula below: (Park et al., 2023):

Adhesion ability (%) = (OD_a/OD_t) × 100

2.7.5. Extracellular polymeric substance (EPS) production

The CFS (1/2 MIC; 5 mL) was mixed with 5 mL of bacterial culture (~10^7 CFU/mL). Instead of CFS, the control was subjected to a treatment with TSB. The mixed cultures were then incubated at 37 °C for a duration of 24 h. Following this, the cells were centrifuged at 9000 × rpm for 10 min at 25 °C, after which the supernatant was carefully discarded. The pellet was resuspended in a NaCl solution (1.5 M; 1 mL), and the cells were re-centrifuged (5000 × rpm, 10 min, 25 °C). Sixty μL of supernatant were mixed with 4 mL of sulfuric acid and 60 μL of 5% phenol. The mixture was then vortexed and incubated (30 °C, 10 min) and its absorbance was measured at a wavelength of 490 nm. The rate of EPS production was calculated as below (Park et al., 2023):

EPS production (%) = (OD_t /OD_c) × 100

where, OD_t and OD_c are the optical density of treatment and control, respectively.

2.7.6. Morphological changes by scanning electron microscopy (SEM)

The effect of CFS on the cell wall of *L. monocytogenes* was assessed using SEM. Bacteria were activated by incubating them in Muller-Hinton broth at 37 °C for 24 h. A 4MIC dilution of CFS was prepared and mixed with the microbial suspension in a 1:1 ratio. The mixture was then incubated at 37 °C for 18 h and centrifuged (6000 × rpm, 5 min) to remove the supernatant. The remaining pellet was washed with PBS and centrifuged again under the same conditions. A 2.5% glutaraldehyde fixative was added to the sample and stored at 4 °C for 2 h. The sample was washed three times with sodium cacodylate buffer (0.1 M, pH = 7.4) for 15 min each and dehydrated with alcohol (various concentrations), with each wash followed by centrifugation for 15 min. Finally, the sample was coated with gold and imaged using an SEM device (Ghazanfari, Fallah, Vasiee, & TabatabaeiYazdi, 2023).

2.7.7. Gene expression

The expression of biofilm-associated genes was analyzed using qRT-PCR, following a modified method developed by Jalil Sarghaleh et al. (2023). The bacterial strain used was *L. monocytogenes* ATCC 19115. Bacterial cultures (5 mL) were diluted to a concentration of 10^5 CFU/mL and mixed with 5 mL of *B. subtilis* GS3 CFS (1/2 MIC). In the control group, TSB was used instead of CFS. After a 24-h incubation at 37 °C, total RNA was successfully extracted using TRIzol™ (Invitrogen™,

Thermo Fisher Scientific, Carlsbad, CA, USA) kit. cDNA synthesis was performed SensiFAST cDNA synthesis kit (Bioline, London, UK). The qRT-PCR process included an initial cycle at 95 °C for 2 min, followed by 40 cycles alternating between 5 s at 95 °C and 15 s at 60 °C.The specificity of the cycles was ascertained using an analysis of the melting curve. The comparative CT ($2^{-\Delta\Delta Ct}$) method was utilized to compute the relative gene expression of *L. monocytogenes* ATCC 19115 when treated with CFS. The primers employed for this calculation are provided in Table 1.

2.8. Statistical analysis

The experiments were conducted three times, with the data being analyzed based on a completely randomized design in factorial arrangement through the Minitab software (version 16). The means were further categorized using the Tukey post-test ($p < 0.05$).

3. Results and discussion

3.1. Strain identification

Seven strains exhibiting rod-shaped, spore-forming characteristics were carefully selected and assessed for their haemolytic and DNase activity to ensure their non-pathogenicity. Out of these, 4 isolates showed negative results for the aforementioned tests and were selected for further evaluation. These strains were subsequently labeled as DS-1, DS-2, DS-3, and DS-4. The bacteria were rod-shaped and Gram-positive. They grew in 5% NaCl and at pH 5.7. They tested positive for catalase, Voges Proskauer, starch hydrolysis, gelatin liquefaction, nitrite reduction, and glucose fermentation. However, they tested negative for indole formation. DS-1 was unique in its ability to grow at 7% NaCl. DS-3 was negative for citrate test compared to other strains. DS-1 and DS-2 did not test positive for xylose and arabinose, while DS-4 did not test positive for arabinose (Table 2).

The nucleic acid characteristics were determined using the 16S rRNA molecular identification method. The strains that were isolated found to be *Bacillus* species. DS-1 was found to have a similarity of 99% to *B. subtilis* strain GS3 (LC768706), while DS-2 had a sequence homology of 100% with *B. cereus* BF2 (MH569091). DS-3 showed a sequence similarity of 98% with *B. velezensis* strain DM (OR364492), and DS-4 was closely related to *B. thuringiensis* strain JB (KJ372208) with a similarity of 99%.

3.2. Stress tolerance

For probiotic strains to be effective in promoting gut health, they must be able to survive the harsh conditions of the GIT, including the acidity of the stomach. Therefore, to determine their ability to withstand the GIT conditions, the strains were exposed to the simulated gastric and intestinal fluids (Fig. 1). It was observed that all four strains exhibited high resistance to simulated gastric juice, with survival rates exceeding 90% after 4 h incubation time. On the other hand, the strains were able to survive and grow in the simulated intestinal fluid, with a survival rate exceeding 145%. Moreover, the cell count in the small intestine was significantly higher than that in the gastric phase (6.26 *vs*. 5.02 log CFU/mL), and DS-4 and DS-1 had generally higher viable cells compared to the other strains. Similarly, a study conducted by Elsadek et al. (2023) found that *B. velezensis*, *Bacillus aryabhattai*, and *Bacillus mojavensis* had survival rates exceeding 90% and 140% under simulated gastric and intestinal conditions, respectively. The ability to withstand acidity is a crucial criterion for selecting a probiotic strain. This characteristic serves as an indicator of the strain's potential to endure the gastric and duodenal juices.

Moreover, bacteria growth is prevented by the presence of bile in the duodenal part of the small intestine. This is due to the fact that the cell membrane of bacteria is composed of lipids and fatty acids that are sensitive to bile salts. Nonetheless, the ability to resist these substances is crucial for the survival and development of bacteria in the intestinal tract, making it an essential requirement for probiotic strain selection. To determine bile tolerance, researchers often use a 0.3% oxgal bile solution, which is similar to human bile juice and is considered a critical concentration for evaluating bile-tolerant probiotics (Vasiee, Falah, Behbahani, & Tabatabaee-Yazdi, 2020). Fig. 2 illustrates the results of the bile salts tolerance test. The strains exhibited a decline in their viable cell counts (from 5.47 to 5.37 log CFU/mL) throughout the exposure period ($p > 0.05$). Nevertheless, the DS-1 strain demonstrated a higher

Table 1
Primers used in this study.

Gene	Primer
flaA	Forward CTGGTATGAGTCGCCTTAG
	Reverse CATTTGCGGTGTTTGGTTTG
inlB	Forward AAGCAMGATTTCATGGGAGAGT
	Reverse TTACCGTTCCATCAACATCATAACTT
sigB	Forward GATGATGGATTTGAACGTGTGAA
	Reverse CGCTCATCTAAAACAGGGAGAAC
agrA	Forward ATGAAGCAAGCGGAAGAAC
	Reverse TACGACCTGTGACAACGATAAA
hly	Forward AACCAGATGTTCTCCCTGTA
	Reverse CACTGTAAGCCATTTCGTCA
prfA	Forward CGGGAAGCTTGGCTCTATTTG
	Reverse GCTAACAGCTGAGCTATGTGC
plcB	Forward CAGGCTACCACTGTGCATATGAA
	Reverse CCATGTCTTCYGTTGCTTGATAATTG

Table 2
Morphological, physiological and biochemical features of *Bacillus* strains.

Characteristics	DS-1	DS-2	DS-3	DS-4
Gram	+	+	+	+
Shape	rod	rod	rod	rod
Growth at 5% NaCl	+	+	+	+
Growth at 7% NaCl	+	–	–	–
Growth at pH 5.7	+	+	+	+
Catalase	+	+	+	+
Voges Proskauer	+	+	+	+
Formation of indole	–	–	–	–
Hydrolyzed starch	+	+	+	+
Gelatin liquefaction	+	+	+	+
Nitrate reduction	+	+	+	+
Citrate test	+	+	–	+
Fermentation of xylose	–	–	+	+
Fermentation of arabinose	–	–	+	–
Fermentation of glucose	+	+	+	+

+ means positive, - means negative.

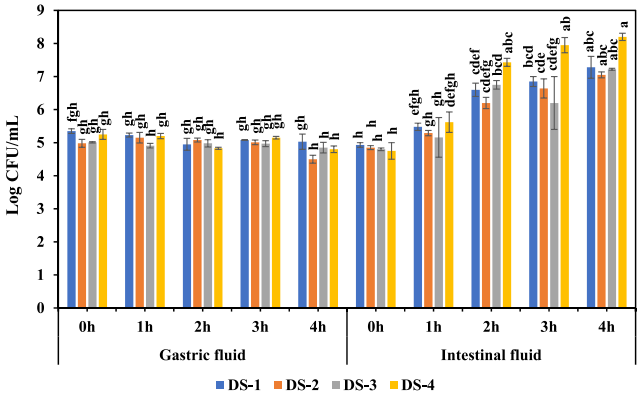

Fig. 1. Survival (log CFU/mL) of the strains over exposure to simulated gastric and intestinal fluids. Different letters indicate significant differences between samples at $p < 0.05$.

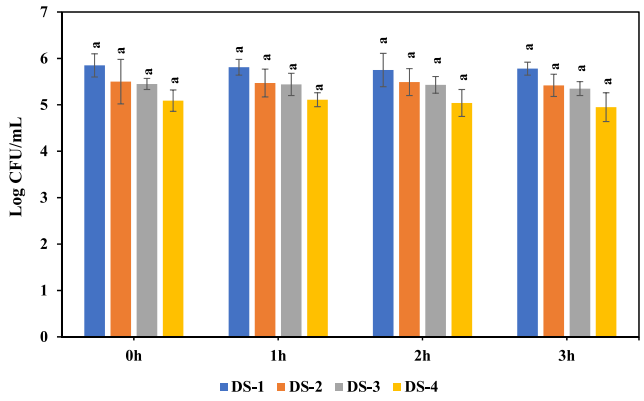

Fig. 2. Survival (log CFU/mL) of the strains after exposure to (0.3%) bile salts for different times. Different letters indicate significant differences between samples at $p < 0.05$.

level of resilience when exposed to bile salts. Overall, after being subjected to bile salts (0.3%) for 3 h, the survival rates for the strains were found to be 98.80%, 98.54%, 98.16%, and 97.24% for DS-1, DS-2, DS-3, and DS-4, respectively. Consistent with our findings, the *B. cereus* strains isolated from traditional fermented African locust bean seeds exhibited remarkable bile tolerance, displaying over 83% viability even after being incubated for 3 h in MRS solution containing 0.4% bile salts (Nwagu et al., 2020). These bacteria have, therefore, the capability to survive in environments rich in bile, which is essential for them to reach and grow in the intestinal tract and provide benefits to the host.

Phenols are known to be toxic metabolites that are released *via* bacterial deamination of certain aromatic amino acids obtained from both dietary and endogenous proteins during the digestion process. These compounds possess bacteriostatic activity (Vizoso Pinto et al., 2006). Following a 24-h incubation period in a medium containing 0.4% phenol, DS-1 demonstrated the maximum viability (62.80%), whereas DS-4 displayed the minimum viability of 32.60% (Fig. 3). Similarly, the *Bacillus* strains *B. dakarensis*, *B. cereus*, *B. benzoevorans*, *B. subtilis*, *B. cabrialesii*, and *B. tequilensis* were found to be generally moderately tolerant to 0.4% phenol (Dabiré, Somda, Somda, Compaoré, et al., 2022). Bacteria that possess a tolerance to phenols are more likely to survive compared to those that do not have such tolerance.

Assessing a probiotic's effectiveness heavily relies on its ability to withstand varying internal body temperatures as the bacteria can experience shock upon ingestion (Elsadek et al., 2023). Additionally, during microbial growth, exposure to saline stress can result in a decrease in turgor pressure and water efflux, negatively impacting the cell's overall physiology and the biosynthesis of vital molecules (Nwagu et al., 2020). As presented in Fig. 4, the strains are capable of surviving at 15 °C (89.86% viability) and 45 °C (87.20% viability). Additionally, they exhibit varying degrees of tolerance towards different concentrations of sodium chloride; as the NaCl levels increased from 1 to 5%, the survival rate of the strains decreased from 94.44% to 81.23%. DS-1 was generally more halotolerant and thermostable (90.55% viability) than its counterparts. The tolerance of lactic acid bacteria to saline and thermal stresses has been reported in the literature (Nwagu et al., 2020; Pundir, Kashyap, & Kaur, 2013). The high tolerance of the strains, particularly DS-1 to the GIT stresses could be attributed to the spore-forming ability of the bacteria, which creates a protective membrane consisting of four layers (Pedraza-Reyes, Ramírez-Ramírez, Vidales-Rodríguez, & Robleto, 2012).

3.3. Adhesion properties

To ensure that the isolated strains are effective, their adhesion was also examined (Fig. 5). For probiotics to function properly, they must first be able to adhere to the digestive tract of the host. Adhesion can be determined indirectly through hydrophobicity and auto-aggregation activities (Alizadeh Behbahani, Noshad, & Falah, 2019; Barzegar; Alizadeh; Behbahani, & Falah, 2021; Saboktakin-Rizi, AlizadehBehbahani, Hojjati, & Noshad, 2021). DS-1, DS-2, DS-3, and DS-4 displayed high cell surface hydrophobicity values of 76.20%, 70.80%, 89.30%, and 84.60%, respectively ($p < 0.05$). *B. tequilensis*, a strain isolated from chicken, exhibited a hydrophobicity level ranging from 74% to 94% with n-hexadecane, and between 65% and 70% with xylene, as reported by Parveen Rani et al. (2016). Similarly, *Bacillus* species isolated from traditional Korean soy sauce displayed a variable hydrophobicity level with xylene, ranging from 25% to 89% (Lee et al., 2017). The hydrophobic nature of the isolated strains might be attributed to the multi-layered protein coat that encapsulates the spores of the *Bacillus* species (Doyle, Nedjat-Haiem, & Singh, 1984).

The auto-aggregation capacity of the isolates was found to be 85.50%, 82.70%, 92.70%, and 79.30% for DS-1, DS-2, DS-3, and DS-4, respectively ($p < 0.05$) (Fig. 5). The findings show similarities with *B. subtilis* (60%) obtained from food waste (Patel, Ahire, Pawar, Chaudhari, & Chincholkar, 2009), as well as *B. velezensis* (95%), *B. aryabhattai* (67%), and *B. mojavensis* (93%), which were isolated from the digestive tracts of *Rhynchocypris lagowskii* (Elsadek et al., 2023). The ability of cells to aggregate contributes to a denser environment in the gut and better adherence to digestive system cells. It is necessary for the auto-aggregation ability to be above 40%, and there is a strong correlation between this ability, hydrophobicity, and adhesion (Elsadek et al., 2023). These strains have shown promising results as a probiotic due to their ability to auto-aggregate and their level of hydrophobicity.

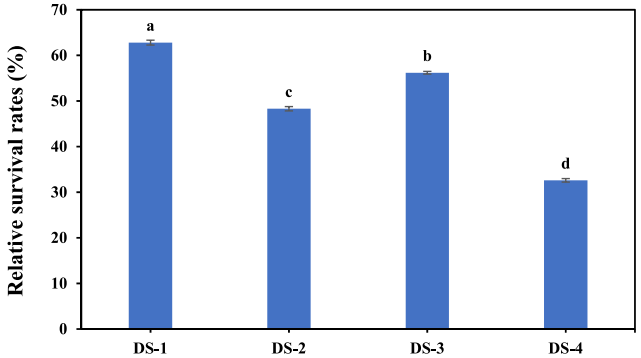

Fig. 3. Relative survival rates of *Bacillus* strains in medium containing 0.4 % (w/v) phenol. Different letters indicate significant differences between samples at $p < 0.05$.

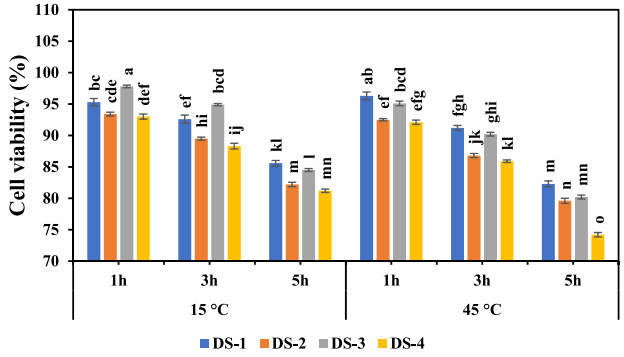

Fig. 4. Cell viability (%) in different concentrations of NaCl upon 24 h of incubation at 15 °C and 45 °C. Different letters indicate significant differences between samples at $p < 0.05$.

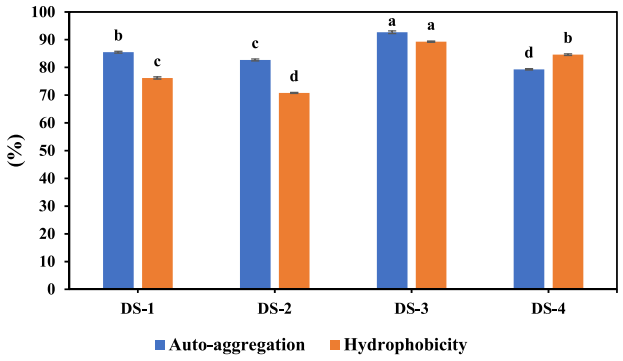

Fig. 5. Cell surface hydrophobicity and auto-aggregation ability (%) of the studied *Bacillus* strains. Different letters indicate significant differences between samples at $p < 0.05$.

3.4. Antibiotic resistance

Probiotic bacteria serve as repositories for antibiotic resistance genes. The potential for these genes to be transferred to pathogenic organisms is not merely theoretical, but a persistent concern, particularly in the context of dietary probiotic consumption. This could potentially catalyze the emergence and proliferation of pathogens that exhibit resistance to commonly utilized antibiotics (Nwagu et al., 2020). The analysis of the antibiogram revealed that the *Bacillus* strains were susceptible to almost all antibiotics, except for nitrofurantoin and penicillin G, to which DS-4 was resistant (Fig. 6). The susceptibility of the strains to antibiotics was observed to follow the order ($p < 0.05$): DS-3 (26.52 mm) > DS-2 (25.64 mm) > DS-1 (23.42 mm) ≥ DS-4 (23.09 mm). Among the strains, DS-1 and DS-3 exhibited the highest sensitivity to erythromycin, with sensitivity diameters of 28.60 mm and 33.70 mm, respectively, while DS-2 and DS-4 showed the highest sensitivity to amoxycillin-clavulanic acid (30.20 mm *vs.* 29.30 mm). Similarly, the study conducted by Lee et al. (2017) found that the probiotic *Bacillus* strains, MKSK-E1, MKSK-J1, and MKSK-M1, which were isolated from Korean traditional soy sauce, showed susceptibility to all antibiotics tested. These included cell wall inhibitors such as cephalexin, ampicillin, vancomycin, and penicillin G, as well as protein synthesis inhibitors like erythromycin, chloramphenicol, tetracycline, and gentamicin (Lee et al., 2017). The high susceptibility of the isolated strains to various antibiotics implies that these *Bacillus* strains may not possess antibiotic resistance genes that could potentially be transferred to pathogenic microorganisms.

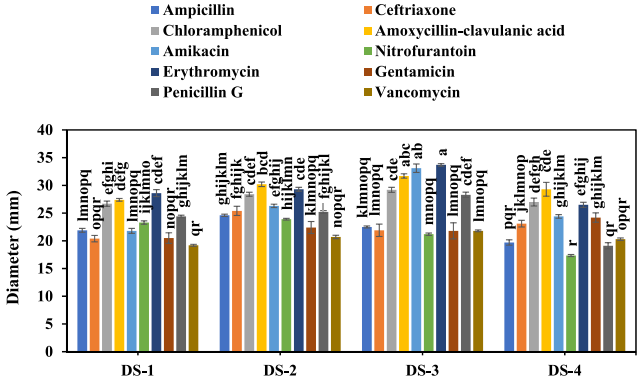

Fig. 6. Antibiotic susceptibility profile of *Bacillus* strains. Different letters indicate significant differences between samples at $p < 0.05$.

3.5. Cholesterol removal

It has been documented that probiotic bacteria possess the ability to lower cholesterol levels (Mousanejadi, Barzegar, AlizadehBehbahani, & Jooyandeh, 2023; Saboktakin-Rizi et al., 2021; Barzegar, AlizadehBehbahani, Mirzaei, & GhodsiSheikhjan, 2023; Zibaei-Rad, Rahmati-Joneidabad, AlizadehBehbahani, & Taki, 2023). The ability to remove cholesterol was observed in DS-1, DS-2, DS-3, and DS-4, with percentages of 59.46%, 32.45%, 48.80%, and 20.12% respectively ($p < 0.05$) (Table 3). These findings may have potential use in preventing particular cardiovascular conditions. Ragul, Syiem, Sundar, and Shetty (2017) reported that certain strains of *Bacillus* isolated from fermented pickle have the ability to decrease cholesterol levels (12–70%). The effectiveness of this property is dependent on the specific strain, and is due to the production of compounds that hinder enzyme activity, such as 3-hydroxy-3-methylglutaryl coenzyme A, bile salt hydrolase activity, and cholesterol assimilation (Vasiee, Falah, Behbahani, & Tabatabaee-Yazdi, 2020).

3.6. Antioxidant effect

The results of antioxidant effect of *Bacillus* strains are provided in Table 3. DS-1 and DS-4 showed the highest and lowest DPPH-RS activity (43.52% *vs.* 23.32%), hydroxyl-RS effect (30.40% *vs.* 15.45%), superoxide anion-RS potential (16.72% *vs.* 11.20%), and lipid peroxidation inhibition (18.79% *vs.* 8.56%), respectively. Similar to our findings, Shivangi et al. (2020) discovered that the *Bacillus* spp. intact cells that were isolated from *Idli* (an acidic fermented food) displayed remarkable DPPH-, hydroxyl-, and superoxide anion-RS activity, as well as lipid peroxidation inhibition ranging from 26 to 41%, 29–31%, 11–13%, and 18–20%, respectively (Shivangi et al., 2020). Additionally, Kadaikunnan, Rejiniemon, Khaled, Alharbi, and Mothana (2015) found that *B. amyloliquefaciens* recovered from silage was impervious to hydrogen peroxide and had high DPPH and hydroxyl RS activities. Similar to humans and animals, bacteria also have their own antioxidant enzymes, with superoxide dismutase being the most crucial as it facilitates the conversion of superoxide into hydrogen peroxide and water. Additionally, the antioxidant activity of probiotic bacteria can be attributed to their metabolites such as glutathione, butyrate, and folate (Wang et al., 2017). The findings confirmed that the isolated strains have the capability to inhibit the oxidation of plasma lipids.

3.7. Anti-pathogenic effect

3.7.1. Antibacterial activity

The ability of CFS to inhibit bacterial growth was assessed by measuring its MIC against the strain *L. monocytogenes* ATCC 19115. The CFS from DS-1, DS-2, DS-3, and DS-4 demonstrated inhibition of *L. monocytogenes* ATCC 19115 by 12.5%, 25%, 25%, and 50% respectively. *Bacillus* spp. are known to produce a variety of bacteriocins with a

Table 3
Cholesterol removal and antioxidant activity of *Bacillus* strains.

Isolates	Cholesterol removal activity (%)	DPPH-RS activity (%)	Hydroxyl-RS activity (%)	Superoxide anion-RS activity (%)	Inhibition of linoleic acid peroxidation (%)
DS-1	59.46 ± 0.65 ^{a}	43.52 ± 0.40 ^{a}	30.40 ± 0.39 ^{a}	16.72 ± 0.75 ^{a}	18.79 ± 1.08 ^{a}
DS-2	32.45 ± 1.25 ^{c}	29.45 ± 1.84 ^{c}	27.32 ± 0.85 ^{ab}	11.45 ± 1.35 ^{a}	14.65 ± 1.65 ^{ab}
DS-3	48.80 ± 0.63 ^{b}	35.76 ± 0.29 ^{b}	23.65 ± 0.61 ^{b}	14.32 ± 0.74 ^{a}	13.20 ± 0.30 ^{ab}
DS-4	20.12 ± 0.84 ^{d}	23.32 ± 0.85 ^{d}	15.45 ± 1.02 ^{c}	11.20 ± 1.25 ^{a}	8.56 ± 1.54 ^{b}

Different letters indicate significant differences between samples at $p < 0.05$.

wide antimicrobial range (Mercado & Olmos, 2022). For example, An et al. (2015) reported that bacteriocin CAMT2, a product of *B. amyloliquefaciens* ZJHD3, demonstrated significant activity against *L. monocytogenes* in pork meat. Furthermore, *B. licheniformis* derived bacillocin 490 indicated a substantial reduction in *Bacillus smithii* growth in buffalo milk (Martirani, Varcamonti, Naclerio, & De Felice, 2002). The anti-listeria activity of bacitracin F103, produced by *B. thuringiensis*, has also been reported in minced beef (Kamoun et al., 2011).

3.7.2. Anti-biofilm activity

The inhibitory effect on biofilm formation is associated with a suppressive impact during the initial phase of pathogen biofilm development. When treated with CFS of DS-1, DS-2, DS-3, and DS-4, the biofilm formation rate of *L. monocytogenes* ATCC 19115 ranged from 7.50% to 60.20%, 11.20%–78.30%, 12.20%–79.30%, and 13.20%–88.30% across a spectrum of 1/8 MIC to 4 MIC, respectively (Fig. 7). It's noteworthy that the CFS from *Bacillus* spp. markedly reduced the formation of biofilm by *L. monocytogenes* ATCC 19115, even at 1/2 MIC; the CFS of DS-1, DS-2, DS-3, and DS-4 inhibited the formation of biofilm by 77.70%, 59.80%, 64.70%, and 51.70% respectively.

Fig. 8 illustrates the impact of the CFS on the degradation of biofilms. The strains' CFS effectively reduced mature biofilm formation of *L. monocytogenes* ATCC 19115, at 4MIC, 2 MIC, MIC, 1/2 MIC, 1/4 MIC, and 1/8 MIC ($p < 0.05$). After treating L. *monocytogenes* ATCC 19115 with CFS of DS-1, DS-2, DS-3, and DS-4, the rate of biofilm formation was observed to be 7.50%–51.20%, 11.60%–69.20%, 10.30%–61.30%, and 12.30%–95.60%, respectively. It was observed that all the *Bacillus* species' CFS were effective in significantly degrading mature biofilms of the pathogen at sub-inhibitory concentrations. It is noteworthy that the rate at which the mature biofilm was degraded was similar to the rate at which the initial formation phase of biofilm was prevented. This shows that the CFS from Bacillus spp. not only inhibited the initial phase of biofilm formation, but it also effectively degraded the mature biofilm.

In line with our results, the research findings indicate that bacin A2, a bacteriocin, exhibited exceptional antimicrobial properties against *S. aureus*, Methicillin-resistant *S. aureus*, *L. monocytogenes*, and *B. cereus*. Bacin A2 was capable of damaging cell membranes and effectively killing Methicillin-resistant *S. aureus* cells. Moreover, bacin A2 efficiently prevented the formation of biofilms of *S. aureus* and Methicillin-resistant *S. aureus* (>0.5MIC), and it also eliminated the cells in the established biofilms (>4MIC) (Liu et al., 2022). Moreover, in a study conducted by Mathur et al. (2018), it was found that the use of nisin, when combined with citric acid and cinnamaldehyde essential oil, exhibited inhibitory effect against the formation of biofilm by *L. monocytogenes* F2635.

Table 4 displays the results of the correlation coefficient analysis between *Bacillus* strains and *L. monocytogenes*. The findings demonstrate a significant relationship between different concentrations of MIC from Bacillus strains and the formation of *L. monocytogenes* biofilm at the 1% and 5% significance levels. Furthermore, the correlation values, which exceeded 0.5, indicate a strong correlation between the various MIC concentrations of *Bacillus* strains.

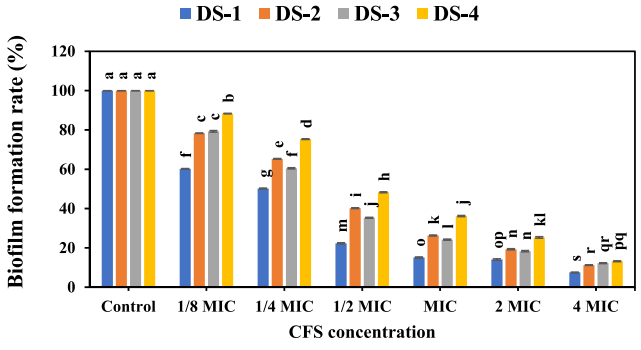

Fig. 7. Biofilm formation rate in the initial stage of biofilm formation by *Listeria monocytogenes* ATCC 19115 treated with the cell-free supernatant (CFS) of *Bacillus* strains. Different letters indicate significant differences between samples at $p < 0.05$.

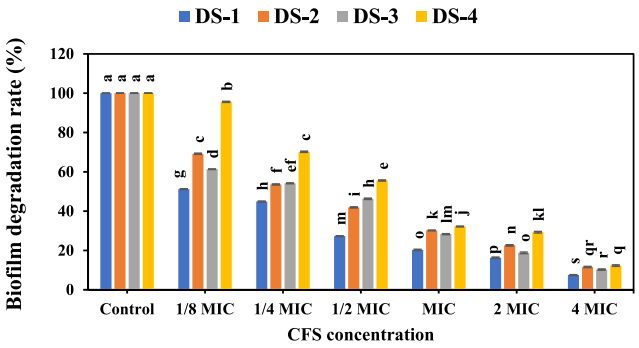

Fig. 8. Biofilm degradation activity of the cell-free supernatant (CFS) of *Bacillus* spp. on matured biofilms of *Listeria monocytogenes* ATCC 19115. Different letters indicate significant differences between samples at $p < 0.05$.

Table 4
Pearson correlation coefficient analysis (Anti-biofilm activity).

4MIC	2MIC	MIC	1/2 MIC	1/4 MIC	1/8 MIC	Concentration
0.866[b]	0.832[a]	0.712[a]	0.748[a]	0.944[b]	1	**1/8 MIC**
0.851[b]	0.768[a]	0.766[a]	0.745[a]	1	0.944[b]	**1/4 MIC**
0.810[a]	0.905[b]	0.923[b]	1	0.745[a]	0.748[a]	**1/2 MIC**
0.871[b]	0.879[b]	1	0.923[b]	0.766[a]	0.712[a]	**MIC**
0.780[a]	1	0.879[b]	0.905[a]	0.768[a]	0.832[a]	**2MIC**
1	0.780[a]	0.871[b]	0.810[a]	0.851[b]	0.866[a]	**4MIC**

MIC: Minimum inhibitory concentration.
[a] Correlation is significant at the 0.05 level.
[b] Correlation is significant at the 0.01 level.

3.7.3. Cell surface characteristics and EPS formation

The initial stage of biofilm formation involves microbial adhesion. The formation of biofilm is greatly influenced by bacterial traits such as cell surface hydrophobicity and auto-aggregation. In Fig. 9, we can observe the variations in cell surface features of *L. monocytogenes* ATCC 19115. The results of the study indicated that using 1/2 MIC of CFS

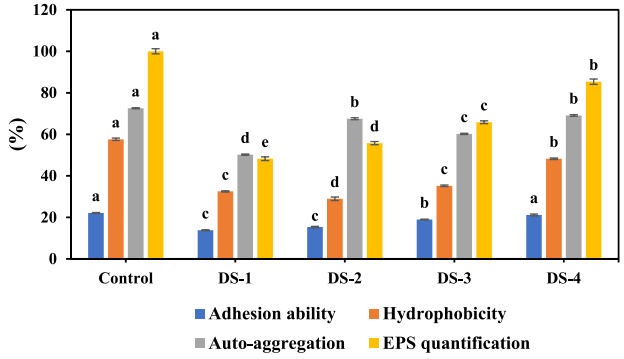

Fig. 9. Changes in cell surface characteristics and extracellular polymeric substance (EPS) quantification of by *Listeria monocytogenes* ATCC 19115 treated with sub-inhibitory concentration (1/2 MIC) of each cell-free supernatant (CFS) of *Bacillus* strains. Different letters indicate significant differences between samples at $p < 0.05$.

significantly decreased the adhesion ability of the pathogen. The ability to adhere varied from 13.81% in DS-1 to 21.18% in DS-4. These figures were notably less than the control sample, which had an adhesion rate of 22.14%. The hydrophobicity and auto-aggregation ability of the pathogen, when treated with CFS, particularly DS-1 CFS, showed a significant reduction in comparison to the control treatment. These effects suggest that the CFS of *Bacillus* spp. can inhibit the initial stage of biofilm formation by the pathogen. In addition, bacterial cells secrete EPS to support and preserve the structural integrity of biofilms during colonization and maturation. When exposed to 1/2 MIC of *Bacillus* species CFS, particularly DS-1, the EPS production rates of *L. monocytogenes* ATCC 19115 were significantly reduced compared to the control (45%–84.04% *vs*. 100%). According to the findings, the biofilm formation process of *L. monocytogenes* ATCC 19115 was significantly impacted by the CFS of *Bacillus* spp., with DS-1 being particularly effective. Consequently, this specific combination was selected for further investigation.

3.7.4. Morphological studies

The surface structure of *L. monocytogenes* ATCC 19115 was analyzed using SEM after treatment with CFS of DS-1. Fig. 10 depicts the morphological changes that occurred in the bacteria before and after treatment. The original structure of *L. monocytogenes* ATCC 19115 was a double coccobacillus (Fig. 10a), but after treatment with CFS, the bacteria underwent several changes in its structure (Fig. 10b). These changes included cell wall wrinkling, indentation, and rupture, as well as cell folding. The CFS also caused intracellular substances to leak outside the cell, which ultimately led to cell lysis. The CFS seemed to target the cell wall and membrane of pathogenic bacteria.

3.7.5. Gene expression

The study examined *L. monocytogenes* ATCC 19115 during the late growth stage with a concentration of 1/2 MIC to observe its biofilm formation. Fig. 11 illustrates the impact of DS-1's CFS on the transcription of genes associated with biofilm formation, including virulence

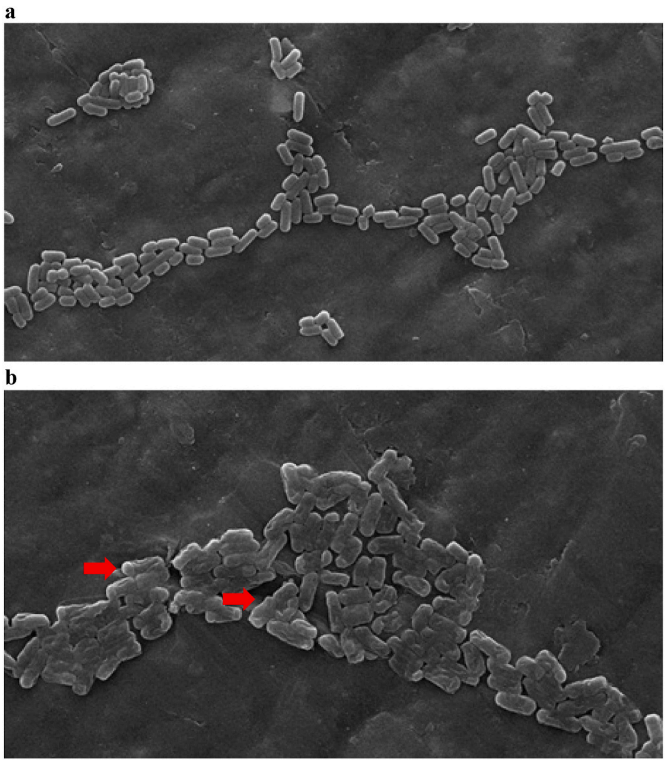

Fig. 10. Scanning electron microscopy (SEM) images of control (a) and treated (b) *Listeria monocytogenes* ATCC 19115 with cell free supernatant (CFS) of *Bacillus subtilis* GS3.

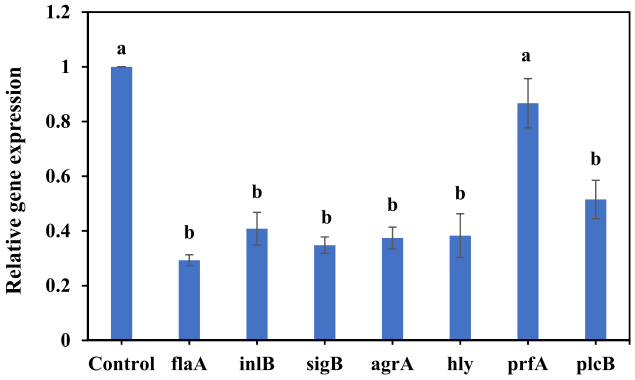

Fig. 11. Gene expression in *Listeria monocytogenes* ATCC 19115 treated with a sub-inhibitory concentration (1/2 MIC) of cell free supernatant (CFS) of *Bacillus subtilis* GS3. Different letters indicate significant differences between samples at $p < 0.05$.

factors (prfA), quorum sensing gene (agrA), stress response factor (sigB), listeriolysin O gene (hly), internalization protein regulatory gene (inlB), phosphatidylcholine phospholipase-encoding gene (plcB), and flagella (flaA). The sigB gene is responsible for regulating the expression of genes that allow *L. monocytogenes* to adapt to environmental stress, as well as some virulence factors that aid in colonization of the GIT and pathogen internalization by the intestinal epithelium (Sibanda & Buys, 2022). The prfA is a transcriptional activator of genes involved in cell invasion, intracellular survival, and spreading to neighboring cells (Marini et al., 2018). The flaA gene is important for *L. monocytogenes* motility and biofilm formation through adhesion. The genes plcB and inlB are instrumental in managing the processes of adhesion and invasion, serving a crucial function in guiding bacteria to enter host cells, propagate among cells, or evade the body's natural immune defenses. Phospholipases, which are encoded by the genes plcA and plcB, aid in ensuring that *L. monocytogenes* does not get trapped within the internalization vacuole. This facilitates its dispersion into the cytoplasm (Jalil Sarghaleh et al., 2023). In the sample that underwent CFS treatment, the expression of sigB, flaA, inlB, agrA, hly, prfA, and plcB genes reduced remarkably by 34.80%, 29.30%, 40.80%, 37.40%, 38.3%, 86.7%, and 51.50%, respectively. Given that CFS curtailed the transcription of plcB, hly, and prfA genes in *L. monocytogenes* ATCC 19115, it is likely that it mitigated the pathogenicity of the bacteria by controlling the secretion of hemolysins and regulating phospholipase function.

4. Conclusions

This research has indicated the probiotic characteristics of *Bacillus* species isolated from dairy sludge. It further demonstrated the antibacterial effect and anti-biofilm formation of the CFS from these strains against *L. monocytogenes* ATCC 19115. The CFS of the species was found to significantly impede the formation of biofilms by *L. monocytogenes* ATCC 19115. This was achieved by reducing the bacterium's ability to adhere to surfaces and its hydrophobicity and auto-aggregation. Furthermore, the rate of EPS production by *L. monocytogenes* ATCC 19115 was also diminished after treatment with CFS. Among the *Bacillus* species studied, DS-1 (*B. subtilis* GS3) exhibited the most potent anti-biofilm property. The CFS of DS-1 significantly downregulated the expression of genes in *L. monocytogenes* ATCC 19115 that are associated with virulence factors, flagella, and quorum sensing. The antibacterial efficacy of the CFS from DS-1 was further confirmed through morphological studies. Hence, the CFS of *B. subtilis* GS3, derived from dairy sludges, exhibit potent antimicrobial and anti-biofilm properties against *L. monocytogenes*. DS-1 could potentially be utilized as an additional or alternative strategy to mitigate infections caused by *L. monocytogenes*. Future studies should investigate the impact of DS-1 on the virulence

factors of *Listeria* and the *in vivo* molecular mechanism through animal studies.

Consent for publication

All authors approved the manuscript for publication.

Availability of data and material

All data relevant to the study are included in the article.

Funding

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Ethical approval

This article does not contain any studies with human or animal subjects.

CRediT authorship contribution statement

Behrooz Alizadeh Behbahani: Conceptualization, Resources, Supervision, Investigation, Methodology, Writing – review & editing. **Mohammad Noshad:** Resources, Supervision, Methodology, Writing – original draft. **Alireza Vasiee:** Investigation, Methodology, Writing – review & editing. **Wolfram M. Brück:** Investigation, Writing – review & editing.

Declaration of competing interest

We declare no conflict of interest.

Data availability

Data will be made available on request.

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