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Original Article

Identification of Probiotic Strains from *Adesmia Cancellata* Microbiomes for Vitamin B6 Production: A New Approach to Developing Functional Foods

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Abstract

Probiotics are beneficial bacteria that produce specific metabolites and influence the host's health. Isolates from the intestinal microbiome of *Adesmia cancellata* were evaluated for probiotic properties. Then, quantitative and qualitative analyses of vitamins B2, B3, and B6 production in the cell-free supernatants (CFS) of probiotic strains from gut microbiota, local dairy products, and human breast milk were conducted using thin-layer chromatography (TLC), spectrophotometry, spectrofluorophotometry, and high-performance liquid chromatography (HPLC). Additionally, the probiotic strains effects on eukaryotic cells were examined to assess toxicity against human alveolar basal epithelial adenocarcinoma cells (A549) and their ability to adhere. Among the eight strains, DDMiCC12c, DDMiCC2Bk, and DDMiCC2D showed notable probiotic function in gut microbiota. DDMiCC2Bk demonstrated the strongest biofilm formation and the highest co-aggregation against *Salmonella typhimurium*. DDMiCC12c was the most resistant to acidic and bile conditions and exhibited co-aggregation with *Bacillus subtilis*. DDMiCC2D showed the highest auto-aggregation and co-aggregation against *Staphylococcus aureus*. Spectrofluorophotometric analysis confirmed the production of vitamin B2 in CFS, ranging from 5 to 10 mg/mL, while vitamin B6 concentrations were 15 to 20 mg/mL. HPLC analysis revealed the highest production of vitamin B6 in DDMiCC2D. SUBCC57 was the producer of vitamins B2 and B3. The selected probiotic strains maintained high viability for A549 cells, with SUBCC57 showing the lowest adhesion and SUBCC2 showing the highest adhesion to A549 cells. Incorporating probiotics into food products is a promising strategy to enhance both health and innovation in the food industry.

Keywords: *Adesmia Cancellata*, B Vitamins, Gut Microbiome, Probiotic Characteristics, Probiotic Metabolites, *Tenebrionidae*

Introduction

The use of fermented foods goes back thousands of years and is common across many cultures around the world. Ancient civilizations recognized their health benefits, which helped shape the modern understanding of "beneficial bacteria" and their role in maintaining gastrointestinal health. In the past, bacteria were mainly seen as threats to human, animal, and crop health. However, recent advances in microbiology have significantly changed this view. Technologies such as metagenomics and 16S

rRNA sequencing enable scientists to identify and analyze the diverse microbial communities within the human gut. These methods provide detailed insights into the complex relationships between gut microbes and their hosts. This growing research field has led to the emergence of a new scientific discipline focused on understanding how specific microbes benefit their hosts' health. Each microbial group can have unique effects and requires different study approaches. The concept of "beneficial bacteria" is closely linked to probiotics—live microorganisms that, when given in adequate amounts, offer health benefits. Probiotics have led to the development of new functional products designed to address various health issues [1]. Important features of probiotics include their ability to survive stressful conditions, adhere to host surfaces and harmful microbes, and maintain a good safety profile. Additionally, probiotics support health by producing essential metabolites such as vitamins, short-chain fatty acids (SCFAs), and bacteriocins [2, 3]. Probiotics are a diverse group, including lactic acid bacteria (LAB) such as Lactobacillus and Bifidobacterium, as well as non-lactic acid bacteria like *Bacillus cereus* and *Bifidobacterium lactis*. They also include yeasts such as *Saccharomyces cerevisiae* and *Saccharomyces boulardii*, as well as fungi like *Aspergillus niger* and *Aspergillus oryzae*. The most common probiotics originate from Lactobacillus and Bifidobacterium. These microbes are sensitive to oxygen and high temperatures [4]. Since humans cannot produce vitamin B on their own, they must obtain it from a balanced diet or supplements. Probiotics can naturally enrich the diet by biosynthesizing various B vitamins, including B1 (thiamine), B2 (riboflavin), B3 (niacin), B5 (pantothenic acid), B6 (pyridoxine), B9 (folic acid), and B12 (cobalamin). Many probiotic strains from dairy, animal, and plant sources, as well as those found in the human digestive system, can produce these vitamins [5]. Probiotic bacteria not only produce B vitamins, but also engage in symbiotic relationships, where different strains exchange vitamins for mutual benefit—for example, one producing vitamin B12 and another producing vitamin B6, each using the other's product. This cooperation increases the availability of essential nutrients in the gut. Probiotics act as natural microfactories, supporting human nutrition by forming vital B vitamins, promoting a balanced diet, and potentially improving health through their metabolic activities [4]. Folic acid, an essential B vitamin, is notably produced in large amounts by *Lactobacillus plantarum*, especially when para-aminobenzoic acid (pABA) is present in the medium. This vitamin is essential for nucleotide synthesis, DNA replication, and repair, thereby supporting crucial cellular and metabolic functions. *L. plantarum* is especially valued for its high folate production, making it useful for nutritional enrichment in fermented foods. Cobalamin (vitamin B12), necessary for nervous system function and blood formation, is produced only by bacteria. Probiotic strains that can synthesize cobalamin include *Limosilactobacillus reuteri* and *Propionibacterium shermanii*. Some Clostridium species, along with *L. reuteri*, also help release cobalamin during sourdough fermentation [6]. Thiamine (vitamin B1) is another important nutrient produced during grain fermentation by *Lactobacillus sanfranciscensis*. Several bacteria, including *Escherichia coli*, *Salmonella typhimurium, Bacillus subtilis*, *Lactobacillus plantarum*, and *Lactobacillus reuteri*, also contribute to thiamine production, further enhancing the nutritional value of fermented foods [7]. When introducing live probiotics into the diet, safety checks are essential. Regulations in the European Union, the United States, and Canada require detailed assessments before approving probiotics for use in food. These typically include the origin and isolation

history, proper identification, absence of pathogenicity, lack of toxicity or antibiotic resistance, and testing for toxins and harmful activities. This strict process helps ensure only safe strains are used in food products. A recent study examined eight probiotic strains from the intestinal microbiome of *Adesmia cancellata* (family *Tenebrionidae*), evaluating their safety, surface properties, and resilience under stress [8]. This ongoing research aims to find and characterize new probiotic candidates with beneficial effects and good safety profiles.

Materials and Methods

Bacterial Strains Used in this Study

The strains include Gram-positive cocci (DDMiCC12Bs, DDMiCC2D, and DDMiCC9B), Gram-negative bacilli (DDMiCC12Bk, DDMiCC12c, DDMiCC2Bk, and DDMiCC5D), and Gram-negative cocci (DDMiCC2A), which were isolated from the insect microbiome of the *Tenebrionidae* family in the Dasht Desert. Experiments were conducted to identify probiotic traits. Then, cell-free supernatant (CFS) of probiotic strains isolated from the gut microbiome of cockroaches, probiotic strains isolated from local dairy products include *Lactobacillus plantarum* (SUBCCC4) and SUBCCC57 (Gram-positive bacilli) and *Enterococcus faecium* (SUBCC2) (data not shown), along with a strain isolated from human breast milk, *Enterococcus faecalis* (SUBC156) [9], were assayed to produce vitamin B groups [10].

Probiotics Characteristics

To identify potential probiotic bacteria, tests were conducted for low pH (3) and 0.3% bile salt (ox-bile) tolerance, antagonistic activity using freeze-dried supernatant (against *S. typhimurium*, *P. aeruginosa* (ATCC 27853), *P. vulgaris, S. aureus*, *B. subtilis*, *E. coli*, *K. pneumoniae* (ATCC 13883))[11], antibiotic sensitivity (penicillin 10 mcg, gentamicin 10 mcg, ampicillin 10 mcg, chloramphenicol 30 mcg, erythromycin 15 mcg, tetracycline 30 mcg, novobiocin 5 mcg) [12], hemolytic activity [13], gelatin hydrolysis [6], biofilm formation [14], autoaggregation, and coaggregation [15]. All assays were performed in triplicate to evaluate the probiotic potential.

Biochemical and Molecular Identification of Selected Strains

The strains from the insect microbiome, which exhibit significant probiotic traits, were first identified using Gram staining and biochemical tests, including catalase, oxidase, and lactose fermentation, in conjunction with the standard methods outlined in Bergey's Manual of Systematic Bacteriology [16].

Genomic DNA is extracted from overnight cultures of the identified strains: SUBCC57, DDMiCC12C, and DDMiCC2D, and used to amplify the 16S rRNA gene sequences with specific primers: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The DNA samples underwent a pre-denaturation step at 98 °C for 3 min, followed by 35 cycles of amplification, each consisting of denaturation at 98 °C for 40 s, annealing at 60 °C for 45 s, and extension at 72 °C for 90 s. After a final extension at 72 °C for 5 min, the PCR products are stored at 4 °C and submitted for sequencing.

Production of the Vitamin Group B

Analysis by Spectrophotometer

A dilution series of vitamins B2, B3, and B6 was prepared to generate a standard curve (vitamin B2: $y = 207.76x + 2.4792$, $R^2 = 0.9868$; vitamin B3: $y = 33.841x - 3.2031$; vitamin B6: $y = 33.967x - 7.157$, $R^2 = 0.993$). The absorption spectra for each vitamin were measured across a wavelength range of 200 to 700 nm. The peak absorption values recorded

were 458 nm for vitamin B2, 262 nm for vitamin B3, and 292 nm for vitamin B6.

Twenty-five milliliters of overnight culture were centrifuged at 6000 rpm for 10 minutes, and CFS was sterilized using a 0.22-µm filter. The absorption spectrum of the samples was measured in the wavelength range of 200 to 700 nm. Finally, the maximum absorption intensity of the standard samples was compared to determine the amount of vitamin produced.

Analysis Using the Spectrofluorophotometer

A dilution series was prepared using a standard vitamin solution. The absorbance values of these standards were measured with a spectrophotometer and recorded as data. The emission spectra were obtained over the range of 200 to 700 nm for vitamin B6 ($y = 45392x + 1962.6$, $R^2 = 0.9998$) and vitamin B2 ($y = 42417x + 2645.6$, $R^2 = 0.9997$). Subsequently, the CFS of the samples was prepared for analysis. The emission and absorption wavelengths were measured at specific intervals to evaluate each vitamin. The intensity of the emission wavelengths from the control was compared to the test to determine the amount of vitamin produced.

Evaluation of Production Using TLC

To evaluate vitamin B production using the thin-layer chromatography (TLC) method, a silica gel chromatography sheet (60 F254) with a thickness of 0.2 mm and dimensions of 6 × 8 cm was used. CFS from the samples, along with a control solution containing vitamins B2, B3, and B6, was loaded onto the sheet in a volume of 20 µl, while the control sample was loaded with 5 µl. The samples were spaced 50 mm apart to prevent overlap. The mobile phase consisted of butanol, chloroform, acetic acid, ammonium, and deionized water in specific proportions: 7:6:5:1:1 [17]. The chromatographic sheet was immersed in the mobile phase for 30 to 40 minutes. After this period, the sheets were examined under UV light to observe the separated bands [18]. Under the same conditions, the Retardation Factor (Rf) was calculated for each strain as the ratio of the distance traveled by the solute to the distance traveled by the solvent.

Analysis of Vitamins B_2 and B_3 Production Using HPLC

The production of vitamins B_2 and B_3 was analyzed using High-Performance Liquid Chromatography (HPLC) under the following conditions: column: C-18 (150 x 4.6 mm, 5 µm); mobile phase: A mixture of methanol and buffer in a ratio of 4:96; 210 nm. To prepare the buffer, dissolve 15.6 grams of potassium phosphate dibasic in 940 mL of HPLC-grade water. Add 5 mL of triethylamine to the solution to adjust the pH to 3 using orthophosphoric acid. Control samples of vitamins B2 and B3 were prepared to establish standard curves. Vitamin B2 concentrations of 10, 20, and 30 ppm, and vitamin B3 concentrations of 50, 100, 200, and 1,000 ppm, were injected into the HPLC system to generate the curves. After designing the standard curves (vitamin B2 : $y = 0.0031x + 1.1683$, $R^2 = 0.992$; vitamin B3 : $y = 0.015x - 17.007$, $R^2 = 0.9999$), and determining the retention times and peak areas for each standard, CFS(s) were injected into the HPLC (flow rate; 0.8 mL/min, injection volume; 20 µl, LOD B2=2.69 ppm; LOD B3=11.77 ppm, LOQ B2=8.98 ppm; LOQ B3=39.23 ppm). The concentrations of vitamins B2 and B3 in each sample were then quantified based on the peak areas [19]. Vitamin B6 production was analyzed using high-performance liquid chromatography (HPLC) with a C-18 column (Inertsil ODS-3, 250 × 4.6 mm, five µm). The mobile phase was a 5:95 mixture of methanol and water, and vitamin B6 was detected at an absorbance of 254 nm. Vitamin B6 was prepared and injected along with a dilution series of 10^{-1}, 10^{-2}, and 10^{-3} ppm to establish a standard curve (vitamin B6; $y = 0.0491x +$

6.2923, R^2 = 0.9999). The standard curve was created by determining retention times and calculating the area under the observed peaks. After that, 80 µl of each sample was injected into the HPLC system (flow rate: 0.1 mL/min, injection volume: 80 µl), and the concentration of vitamin B6 was quantified based on the area under the corresponding peaks.

Toxicity of Probiotics by MTT Assay

Twenty mL of overnight cultures of probiotic strains were centrifuged at 4,000 rpm for 10 min. The CFS containing bacterial metabolites was transferred to a new tube, and the bacterial cells were washed three times with sterile phosphate-buffered saline (PBS). A dilution series of the probiotic strain mixture was prepared (10^8, 10^6, and 10^5 CFU/mL) using a culture medium containing metabolites. Once the A549 cells reached 80% confluence in the flask, 4.5 × 10^4 cells were seeded into each well of a 96-well plate. The volume in each well was adjusted to 100 µl with DMEM-F12 supplemented with 5% FBS. The plate was incubated for 24 hours at 37°C in a humidified atmosphere with 5% CO_2. Subsequently, 100 µl of the bacterial cell mixture was added to each well containing eukaryotic cells, with three replicates per condition—a nutrient broth without bacteria served as a control. After 24 hours of incubation, the wells were drained three times with PBS. Next, 20 µl of MTT at a concentration of 5 mg/mL was added to each well, followed by a four-hour incubation. Finally, 150 µl of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals formed during the MTT assay. The optical absorbance of the samples was measured at 490 nm. The percentage of cell survival was calculated by dividing the absorbance of the treated cells by that of the control cells and then multiplying by 100 [20].

Kinetics of Acidification and Survival in the Refrigerator

The palette was prepared from 10 mL of an overnight culture of probiotic strains (10^7 CFU/mL) and inoculated into 10 mL of pasteurized milk. The milk was incubated at 37 °C for 48 hours. A pH assay monitored the acidification kinetics during this incubation period. After 21 days of refrigerated storage at 4 °C, the viability of the probiotics was assayed [21].

Addehsin Assay

A549 cells were cultured in DMEM-F12 medium in 24-well plates at a density of 2 × 10^5 cells per well. After incubating for 24 hours at 37 °C with 5% CO_2, the cells were treated with probiotic strains SUBCC2, DDMiCC2Bk, and SUBCC156 at a concentration of 2 × 10^8 CFU/mL, using a multiplicity of infection (MOI) of 1:1,000. Incubation continued for an additional 2 hours. Unattached bacteria were removed by washing the wells with phosphate-buffered saline (PBS). Afterward, 100 µl of PBS and 100 µl of trypsin were added to each well. The dilutions were cultured on nutrient agar to assess bacterial adhesion. For control wells, pellets of probiotic strains without eukaryotic cells were added at a volume of 1 mL. Cell adhesion was evaluated by measuring the percentage of adherent cells remaining after trypsin treatment.

Results

Probiotics Characteristics

The resistance of the strains to ampicillin was also assessed, and the CFS of DDMiCC9B, DDMiCC2D, and DDMiCC12B showed the most growth inhibition halo diameters in the presence of erythromycin. In contrast, other strains displayed the largest growth inhibition halos when exposed to chloramphenicol (Figure S1). None of the strains hydrolyzed gelatin. Among the eight strains tested for probiotic traits, DDMiCC9B and DDMiCC5D

showed the ability to produce alpha hemolysis. In contrast, six strains (DDMiCC12c, DDMiCC2Bk, DDMiCC12Bs, DDMiCC2D, DDMiCC12Bk, and DDMiCC2A) exhibited gamma hemolysis. Further studies were conducted on these six strains. DDMiCC2Bk exhibited the highest biofilm formation, reaching 90%, whereas DDMiCC12Bs and DDMiCC2A showed the lowest levels at 33%. The average biofilm formation for the other strains ranged between 44% and 50%. The DDMiCC12C (27%), DDMiCC2D (35%), and DDMiCC2Bk (24.1%) showed the highest levels of auto-aggregation, while DDMiCC2A (11.6%) and DDMiCC12Bs (13.18%) exhibited the lowest levels. The co-aggregation results showed that DDMiCC2Bk had the highest co-aggregation with *S. typhimurium* at 66.6%. DDMiCC12C was observed with *B. subtilis* at 51.1%, while the lowest co-aggregation was noted with *E. coli*. The co-aggregation percentages for DDMiCC2D ranged from 53% with *E. coli* to 56% with *S. aureus*. After three hours of incubation at pH 3, the highest survival rates were 100% for both DDMiCC12C and DDMiCC2D, while the lowest, at 26.1%, was observed for DDMiCC2Bk. The survival percentage of the strains treated with 0.3% bile salt (ox-bile) showed that DDMiCC12C had a 100% survival rate, while DDMiCC2D and DDMiCC2Bk demonstrated the lowest and highest survival rates, respectively. CFS concentrations of 0.1 µg/mL of SUBCC2 and 0.25 µg/mL of SUBCC4 inhibited the growth of *P. aeruginosa*. A concentration of 0.1 µg/mL of SUBCC57 inhibited the growth of *K. pneumoniae*. For CFS of SUBCC156, DDMiCC12c, and DDMiCC2D, a concentration of 0.2 µg/mL was effective in inhibiting the growth of *K. pneumoniae*, *B. subtilis*, and *E. coli*. In DDMiCC2Bk, a concentration of 0.1 µg/mL was found to inhibit the growth of *B. subtilis*. Furthermore, concentrations of 0.5 µg/mL of both DDMiCC12c and DDMiCC2D were lethal to *E. coli*, effectively killing the bacteria, while 0.2 µg/mL of SUBCC2 inhibited the growth of *P. aeruginosa*. CFS concentrations of 1 µg/mL of DDMiCC2Bk and SUBCC57 were bactericidal against *K. pneumoniae*, meaning they killed the bacteria. No activity was observed against *S. typhimurium*, *P. vulgaris*, or *S. aureus* (Table S1).

Production of Vitamins

Spectrophotometric Analysis

The concentrations of vitamins B2 (riboflavin), B3 (niacin), and B6 (pyridoxine) produced by various microbial strains were measured using spectrophotometry. DDMiCC2Bk exhibited the highest production of vitamin B2. Conversely, the CFS of CUBCC57 had the lowest levels of vitamin B2. However, measuring vitamins B3 and B6 with this method was inconclusive, as the spectrophotometric readings for these vitamins showed negative peaks at their characteristic wavelengths.

Spectrofluorophotometric Quantification

To quantify vitamins B2 and B6 in CFS, they were assayed using a spectrofluorophotometer, which measures the fluorescence emitted by these vitamins upon excitation. This method provided quantitative data. The concentrations of vitamin B2 across the CFS from different strains ranged from 5 to 10 mg/mL, while vitamin B6 levels were higher, ranging from 15 to 20 mg/mL. These results confirm the presence of these vitamins in the microbial cultures, with vitamin B6 generally produced in greater amounts than vitamin B2 (Figure 1).

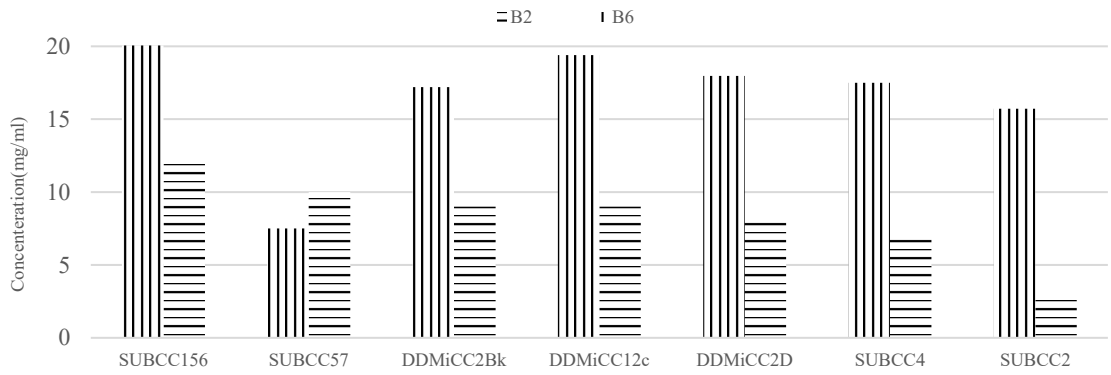

Figure 1: Spectrofluorophotometric assay. The highest production of vitamins B2 and B6 was observed in SUBCC156 using this method. Conversely, SUBCC2 and SUBCC57 showed the lowest production levels of vitamins B2 and B6, respectively

Thin Layer Chromatography (TLC) Analysis

Thin-layer chromatography was used to qualitatively confirm the presence of vitamins B2, B3, and B6 in the CFS of the samples. The control standards for these vitamins showed retention factor (Rf) values of 0.48 for vitamin B2, 0.61 for vitamin B3, and 0.53 for vitamin B6. The Rf values for vitamins B2 and B6 in the CFS of all strains were consistent, indicating stable production. The vitamin B3 band was detected in all samples except for SUBCC2, where no corresponding band was observed (Figure S2).

HPLC Analysis

The production of vitamin B6 in CFS of probiotic strains was measured using high-performance liquid chromatography (HPLC). Strains SUBCC2 and DDMiCC2D produced the highest amounts at 138.6 ppm and 126.73 ppm, respectively, while the CFS of DDMiCC2Bk showed the lowest production at 11.78 ppm. Regarding vitamin B2 synthesis, the highest production was observed in the CFS of SUBCC57, with a concentration of 12 ppm. For vitamin B3, SUBCC57 showed the highest production, yielding 100 ppm. The lowest vitamin B3 output was detected in the CFS of DDMiCC12c at 17.9 ppm (Figure 2).

Biochemical and Molecular Identification of Probiotic Strains

Three bacterial strains isolated from insect microbiomes, exhibiting probiotic traits, were identified as Gram-negative bacilli: DDMiCC2D, DDMiCC12c, and DDMiCC2Bk. These strains shared similar biochemical profiles, characterized by positive motility and negative results for the indole test and hydrogen sulfide (H_2S) production. All three strains produced gas and fermented glucose, lactose, and sucrose, without generating hydrogen sulfide. Based on these biochemical features and referencing Bergey's Manual of Systematic Bacteriology, the strains are likely members of the family Enterobacteriaceae, specifically within the genus Enterobacter.

In contrast, the strain SUBCC57 displayed distinct characteristics; it was a rod-shaped, Gram-positive bacterium capable of producing exopolysaccharides and fermenting glucose. Molecular identification via 16S rRNA gene sequencing: Approximately 1,500 base pair sequences from each strain were analyzed against the NCBI database, DDMiCC12c. The sequence exhibited 99.11% similarity and 99% query coverage with *Enterobacter cloacae*. It has been submitted to the Genomic Database under accession number OR521051.1,

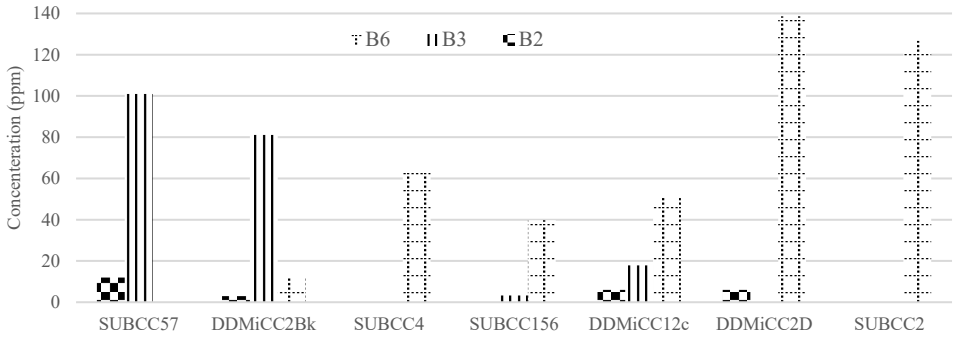

Figure 2: Vitamin B assay of probiotic strains using HPLC. The strains showed differences in vitamin B production among various bacteria. DDMicc2D produces the highest amount of vitamin B6, while DDMiCC2Bk produces the lowest, approximately one ppm. For vitamin B2 production, *Leuconostoc mesenteroides* (SUBCC57) showed the highest output at approximately 3.5 ppm, while DDMi2Bk has the lowest. In vitamin B3 production, SUBCC57 has the highest, and SUBCC156 has the lowest, approximately 3.5 ppm

DDMiCC2Bk showed 98.68% similarity and 100% query coverage with *Enterobacter hormaechei*. This sequence was submitted to the NCBI Database with accession number OR512421.1. SUBCC57 was 99.67% similar and 98% query coverage with *Leuconostoc mesenteroides*. It was submitted to the NCBI Database with accession number OR512420.1.

Toxicity by MTT Assay

Figure 3 demonstrates that eukaryotic cells exposed to probiotics at a concentration of 10^5 CFU/mL exhibit viability rates ranging from 40% to 100%. The highest viability rates are associated with the strains SUBCC2, DDMiCC2Bk, SUBCC57, DDMiCC12c, and DDMiCC2D. In contrast, the strains SUBCC156 and SUBCC4 have lower cell viability. At a higher probiotic concentration of 10^8 CFU/mL, the lowest and highest survival rates were observed for the strains SUBCC2 and SUBCC4, with viability rates of 75% and 100%, respectively.

Kinetics of Acidification and Survival During Refrigerated Storage

After inoculation and incubation for 48 hours, the pH of the milk decreased, as shown in Figure 4. Specifically, the pH values for DDMiCC12c and DDMiCC2Bk dropped from 6.7 to 6.0 during this period. Following 21 days of storage at 4 °C, cultures on nutrient agar revealed the presence of DDMiCC2D, DDMiCC12c, SUBCC156, and SUBCC57. In contrast, no growth was detected for DDMiCC2Bk, SUBCC4, or SUBCC2. These results demonstrate that DDMiCC2D, DDMiCC12c, SUBCC156, and SUBCC57 remained viable after extended refrigerated storage, while others (DDMiCC2Bk, SUBCC4, and SUBCC2) did not survive under the same conditions. The observed reduction in pH after inoculation led to increased acidity due to microbial metabolism.

Addehsin Assay

The adhesion results of the probiotic strains are shown in Figure 5. The adhesion levels of these strains ranged from 4.5% to 12%, with SUBCC2 exhibiting the highest adhesion and SUBCC156 demonstrating the lowest.

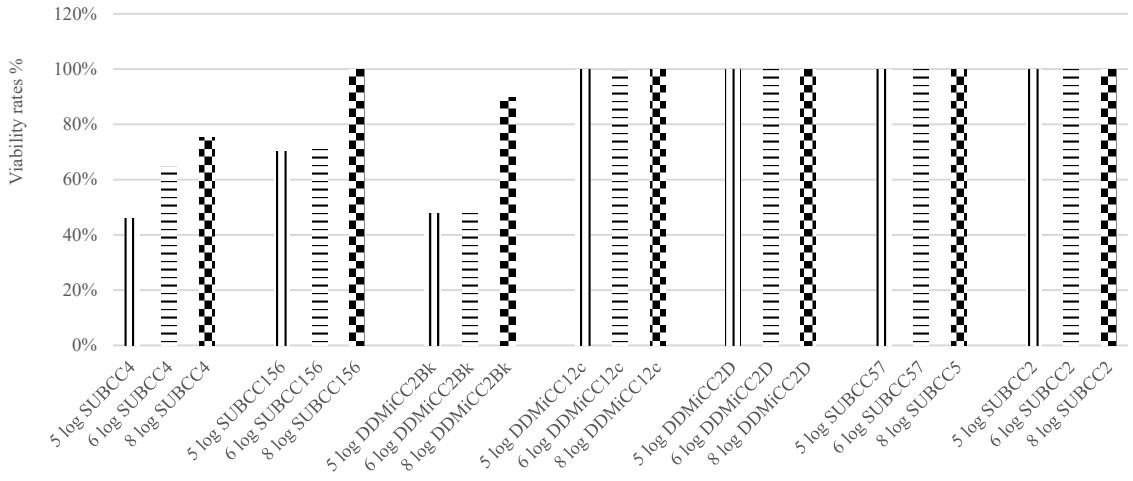

Figure 3: Viability rates of probiotic strains. The viability rates of different dilutions of the probiotic strains were measured, with percentages ranging between 75% and 100%, indicating reasonable survival rates

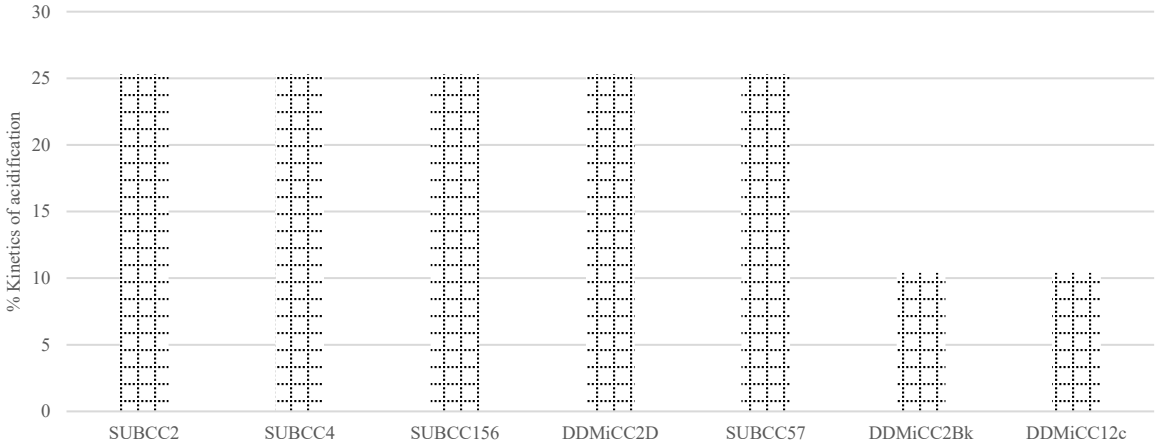

Figure 4: pH reduction in milk after incubation.The percentage decrease in the pH of milk after incubation with the probiotic strains for 48 hours was also evaluated, reflecting their acidification activity and fermentation potential

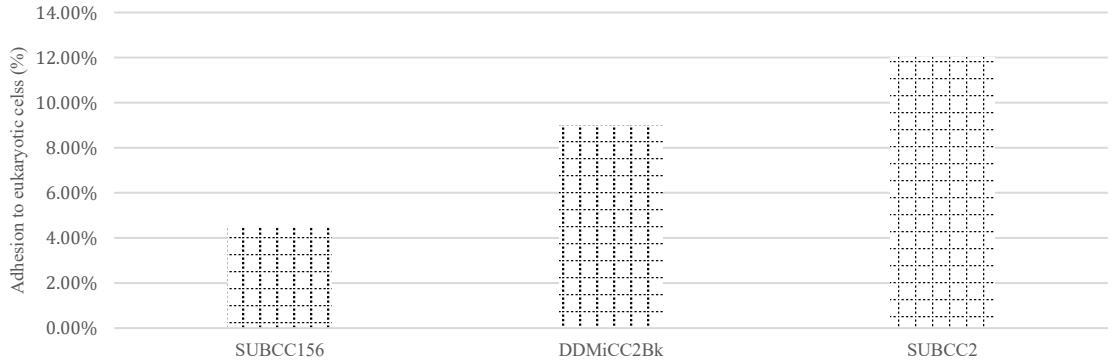

Figure 5: Adhesion of Probiotic Strains to A549 Cells. The adhesion ability of the probiotic strains to A549 cells was assessed, showing significant variability among strains. This adhesion capacity is crucial for their potential probiotic efficacy

Discussion

The probiotic traits of strains isolated from insect guts were assessed based on safety, cell surface properties, and behavior under stress conditions. The safety assessment mainly focused on antibiotic resistance profiles. To be considered safe as a probiotic, a strain should display low levels of antibiotic resistance. In this study, all tested strains showed resistance to ampicillin but were sensitive to chloramphenicol and tetracycline. These results are consistent with those reported by Sirkar *et al.*, who found that two *L. pentosus* strains used as probiotics were sensitive to cephalexin, cefradine, cloxacillin, nitrofurantoin, and norfloxacin, yet resistant to cefuroxime, mecillinam, and nalidixic acid [22]. Similarly, Kezili Eldirim examined the antibiotic sensitivity of Lactobacillus strains isolated from cow's milk. The study showed that 38.9% of the samples were resistant, while 61.1% were sensitive. Notably, six strains were resistant to tetracycline, but all were susceptible to vancomycin and chloramphenicol [23]. To further ensure safety, the strains' hemolytic activity and production of biogenic amines were assessed. Ngampuak *et al.* found that strains VN2 and VN3 exhibited alpha-hemolytic activity, whereas VN5 and VN7 showed gamma-hemolytic activity and were identified as probiotics [24]. The ability to hydrolyze gelatin was tested to evaluate the safety of probiotics. Gelatinase is an enzyme that breaks down proteins, such as gelatin, collagen, casein, and hemoglobin. Although the harmful effects of this enzyme have not been conclusively proven, its expression may be linked to genes involved in cell lysis [17]. This enzyme can damage host tissues by digesting fibrin, which could promote the spread of invading bacteria [18]. In the present study, none of the strains degraded gelatin even after extended incubation, confirming their relative safety as potential probiotics. Similarly, Adogna *et al.* studied six strains and found that none produced gelatinase [25]. Another key feature of bacterial cells is their ability to form biofilms, which enables them to adhere to surfaces in the environment. Probiotic biofilms attach to mucus and can prevent pathogen biofilm formation by competing for nutrients. For successful colonization of the digestive tract, it is essential to evaluate the cell surface traits of different strains, focusing on autoaggregation and coaggregation abilities. Co-aggregation acts as a defense mechanism for the host, helping to prevent pathogen invasion. Auto-aggregation of probiotic bacteria is also crucial for biofilm formation, boosting persistence and function within the host. In this study, auto-aggregation ranged from 11% to 35%, while co-aggregation varied from 45% to 66%. These results differ from those reported by Haghshenas *et al.*, indicating significant variations [26]. The thresholds of 20% for auto-aggregation and 50% for co-aggregation were established based on the average percentages and observed differences. DDMiCC12C, DDMiCC2A, DDMiCC12Bk, and DDMiCC2Bk showed the highest auto-aggregation rates at 35%, 27%, 25%, and 24%, respectively. These strains also had co-aggregation abilities, indicating their potential for colonization and pathogen exclusion. Bacterial strains encounter various environmental stresses depending on their location within the body. For example, probiotics in the stomach must resist highly acidic conditions, which can threaten their survival and function. Therefore, acid resistance is a crucial factor when selecting probiotic strains for industrial applications. A 2023 study by Li *et al.* showed that strains KGC1201 and KCTC3109 maintained approximately 97% survival after 3 hours at pH 3, demonstrating their ability to withstand acidic environments [27]. Understanding a strain's aggregation properties and environmental resilience is important for its effective use in the gut. The results highlight

significant differences among strains, revealing the need to select probiotic candidates. To be effective, probiotic strains must survive passage through the gastrointestinal tract and establish themselves in the gut. Bile salt resistance is therefore crucial for selecting effective probiotics. The findings agree with those reported by Silver *et al.* [28]. Another important trait of probiotics is their ability to produce antibacterial substances. Soundharrajan *et al.* [29] measured antimicrobial activity using a diffusion agar method. The results showed that the probiotics produced compounds effective against *P. aeruginosa*, *K. pneumoniae*, *B. subtilis*, and *E. coli*. Probiotics produce various metabolites with biological effects, such as bacteriocins and vitamins. Vitamins can be analyzed both qualitatively and quantitatively using different methods. Spectrophotometers and spectrofluorometers are common tools for measuring interactions with light. The spectrophotometric analysis indicated that strains SUBCC57, SUBCC156, and DDMiCC2Bk were capable of producing vitamin B2. Additionally, spectrofluorometry detected vitamins B2 and B6 in all samples. Thin-layer chromatography (TLC) is a useful technique for vitamin analysis, especially when comparing multiple samples rapidly and cost-effectively. We used TLC to find suitable separation methods for hydrophilic vitamins. Previous studies, such as those by Simple *et al.* have utilized TLC to measure vitamins, and we confirmed the production of vitamins B2, B3, and B6 using this method [30]. High-performance liquid chromatography (HPLC) is regarded as the standard method for vitamin analysis due to its precision, sensitivity, and compatibility with various detectors. This aligns with methods used by Wazed *et al.* to quantify B vitamins in vegetables [19]. In this study, HPLC was employed to evaluate the production of B vitamins by probiotic strains. Out of the eight strains tested, three produced vitamin B3 and six produced vitamin B6. These results demonstrate that vitamin B production varies among strains within Lactobacillus (LAB) and Enterococcus species, which is crucial for their roles in gut health and potential applications in fermentation and probiotics.

The production of B vitamins, such as B2, B3, and B6, by these bacteria is important because these vitamins serve as cofactors for metabolism, support energy production, and are essential for maintaining gut health and systemic nutrition. *E. faecium* species are known to contribute to vitamin production in the gut, although their abilities differ among strains. Similarly, the *Leuconostoc* and *Lactobacillus* genera are recognized as producers of various vitamins B and are used industrially to enhance the vitamin content in fermented foods. This strain-specific profile of vitamin production indicates that these bacteria could be optimized as natural, cost-effective sources for vitamin biosynthesis in food and probiotic applications. The significant variation from 1 ppm to 170 ppm (B6) and from approximately 3.5 mg/mL to 65 mg/mL (B2) highlights the importance of screening and selecting bacterial strains for targeted vitamin enrichment [31].

To evaluate the safety of probiotic bacteria, their toxicity was tested against eukaryotic cells using the MTT assay. The results showed that all strains were non-toxic, except for SUBCC156, SUBCC4, and DDMiCC2Bk. In a related study, Porkayasta *et al.* examined the cytotoxicity of probiotic isolates and found that all isolates displayed cytotoxic activity against cancer cell lines. Among the three cancer cell lines, the highest cytotoxicity was observed against A549 cells, followed by HeLa and AGS cells [32]. Probiotics must adhere to intestinal mucosal cells to remain stable within the gut and interact effectively with the host. This adherence enables probiotics to directly influence the intestinal immune response, improve epithelial cell function, and

compete with pathogens for space and resources. These interactions are essential for maintaining gut health, supporting digestion, and promoting a balanced microbiome. A study by Song *et al.* evaluated the adhesion of probiotics to epithelial cells and found that probiotics have a significant ability to compete with *H. pylori* for cell adherence. This competitive adhesion can help prevent *H. pylori* from becoming pathogenic. The study indicated that probiotic concentrations between 450 and 1,200 CFU/mL effectively adhered to A549 cells [10]. Despite their health benefits, some probiotic strains have slow fermentation kinetics, which make them unsuitable as primary fermentation starters in the dairy industry. This feature is important when assessing their suitability for industrial use. Several researchers have noted that probiotic bacteria from the genera *Bifidobacterium* and *Lactobacillus* are typically used as supplementary cultures rather than as primary starters in the production of fermented dairy products [33, 34]. To evaluate the potential application of these strains in dairy products, their survival in pasteurized milk stored at 4 °C was monitored over a 21-day period. According to Patrignani and Ciroli, fermented probiotic products should contain a live cell count exceeding 6 log CFU/g at the time of consumption, as recommended by the International Dairy Federation (IDF, 1992). Achieving this standard ensures an adequate daily intake of live bacteria [35, 36].

Conclusion

This study evaluated the probiotic characteristics of eight strains isolated from the intestinal microbiome, focusing on three specific strains: DDMiCC12c, DDMiCC2Bk, and DDMiCC2D. Among these, DDMiCC2Bk demonstrated the highest biofilm formation and coaggregation ability against *S. typhimurium*. In contrast, DDMiCC12c exhibited the most significant resistance to acidic and bile conditions, along with notable aggregation ability against *B. subtilis*. SUBCC156 produced the highest levels of vitamins B2 and B9, as determined by the spectrofluorometric method. High-performance liquid chromatography (HPLC) analysis revealed that SUBCC2 had the most remarkable capacity for vitamin B6 production, while SUBCC57 excelled in synthesizing vitamins B2 and B3. Finally, cytotoxicity assessments indicated that the selected probiotic strains maintained high viability in eukaryotic cells, demonstrating their safety and potential for further application.

Ethical Statement

In this study, written informed consent was obtained from all lactating participants prior to the collection of breast milk samples, which were subsequently analyzed to detect probiotic bacterial strains. Furthermore, probiotic isolates derived from the insect microbiome were obtained through systematic sampling conducted in the Dasht Desert, Semnan Province. These procedures were performed in accordance with the memorandum of understanding approved by the Semnan Province Environmental Department (No. 1401/1342, dated 6/8/2022).

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Conflict of interest

The authors would like to declare that they have no competing interests.

Authors' Contributions

A.R. performed experiments, prepared the figures, and wrote the first draft of the manuscript; S.D.A. and S.A. conceived and supervised the study. All authors have read and approved the manuscript.

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