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Probiotic evaluation of *Bacillus subtilis* PZ014383 and application of its keratinase in eco-friendly hide dehairing

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Abstract

The current study focused on the assessment of probiotic potential and biotechnological applicability of the *Bacillus subtilis* (*B. subtilis*) PZ014383 strain by means of various *in vitro* analyses. The strain demonstrated the ability to withstand simulated digestive stress, with viability in simulated gastric and intestinal conditions being 72.51% after 3 hours at pH 2.0 and 60.71% in 0.3% bile salts, respectively. Survival in the presence of increasing concentrations of phenol decreased from 8.62 to 7.68 log CFU/mL at 0.6% phenol. Antibiotic testing showed susceptibility to meropenem, moxifloxacin, and rifampicin, whereas several beta-lactams, including ceftriaxone and cefotaxime, produced no inhibition zones. The cell-free supernatant (CFS) demonstrated selective antimicrobial activity with a 12.33 mm inhibition zone against *Staphylococcus aureus (Staph. aureus)* (ATCC 259233) and no inhibition of *Escherichia coli (E. coli)* (ATCC 25922). The tested strain tolerated 8% NaCl and displayed favorable surface characteristics, including 89.27% auto-aggregation and 73.3% hydrophobicity, with co-aggregation with *E. coli* being 19.34%. The γ-hemolysis result showed the absence of hemolytic activity. The keratinase enzyme produced by this strain demonstrated the ability to dehair hides effectively, serving as a more environmentally friendly biotechnological alternative to the chemical process.

Keywords: Probiotic evaluation; *Bacillus subtilis*; Antimicrobial activity; Hide dehairing

1. Introduction

Safer approaches to protecting human health are needed as inappropriate antimicrobial use continues to increase the burden created by multidrug-resistant pathogens. Probiotic bacteria may offer such a strategy by enhancing immune function, aiding digestion, and supporting intestinal health [1]. A probiotic is defined as a viable microorganism whose administration benefits its host. Species from the genera *Lactobacillus* and *Bifidobacterium* are therefore widely marketed in formulations designed to promote gastrointestinal health [2, 3]. Effectiveness in practice, together with production feasibility, nevertheless limits broader application. Manufacturing is hindered by their slow growth and microaerophilic-to-anaerobic physiology [4]. Additional concerns encompass poor shelf stability, heat sensitivity, and limited resistance to gastric acid, motivating investigation of candidates with greater probiotic efficiency [4, 5]. *Bacillus* sp. offers an alternative because numerous species within the genus possess advantageous features [6, 7]. Spore formation characterizes these organisms, and reports also place them among dominant members of the gut microflora in healthy

DOI:10.62184/nhjbas.jnh20020264

Research Article

individuals [7]. Trials and preclinical studies support the ability of *B. subtilis* to prevent or treat diarrhea arising from several etiologies [8, 9]. Its safety record in people is reinforced by generally recognized as safe (GRAS) status under the Food and Drug Administration (FDA), together with inclusion on the European Food Safety Authority's Qualified Presumption of Safety (QPS) list [10]. Proposed mechanisms of *B. subtilis* probiotic activity comprise antimicrobial-compound production, immunomodulation, intestinal barrier maintenance, and symbiosis involving the gut microbiota [10]. These properties warrant examining different ecological sources when seeking novel *B. subtilis* strains able to meet rising market demand for probiotic supplements.

One of the main conditions of an effective probiotic is survival under unfavorable physicochemical conditions of the upper gastrointestinal tract; these conditions consist of low pH of the stomach and high concentration of bile salts of the small intestine [11]. In the absence of adequate resistance to these stresses, viable cells will not reach the colon in amounts sufficient to cause colonization or manipulation of the gut microbiota [11, 12]. An early criterion at which candidate probiotics are filtered is thus acid and bile tolerance. Safety is also paramount to strains that are to be used by a human or an animal. Candidate strains should be free of transmissible antibiotic resistance genes and virulence features that may pose a threat to the host [13]. Clinical testing of clinically relevant antibiotics assists in distinguishing between intrinsic resistance, which is non-transferable, and inherent and acquired, which may be horizontally transferred to pathogenic bacteria. Absence of hemolytic activity is another necessary safety condition since hemolysis is related to pathogenic potential [12, 13]. It should therefore be preclinical testing that integrates functional testing with rigorous safety testing, such as antibiotic susceptibility profiling and hemolytic activity testing, prior to a strain being deemed effective and safe in functional foods or dietary supplements.

Industrial applications also offer incentives to study *B. subtilis* since it has biotechnological potential due to its production of valuable enzymes, including keratinase. Keratin is the structural protein that comprises various animal appendages, including feathers, hair, wool, nails, and horn [14]. Worldwide accumulation reaches about 40 million metric tons of keratinous material each year and creates substantial environmental pressure [15]. Beyond chicken feathers, processing sheep skin and bovine hide generates about 0.2 million tons of hair and 0.06 million tons of wool, respectively [14]. Tanneries remove hair using lime-sulfide and other aqueous, toxic, and dehairing agents [16]. Wastewater containing high chemical loads and recalcitrant pollutants creates environmental problems and economic losses [17]. Applying microbial keratinase during dehairing offers a potentially economical, greener route that can reduce energy demand [18]. Abdel-Latif *et al*. [18] demonstrated that a cold-active keratinase produced by the psychrophilic strain *Penicillium oxalicum*, at a dehairing temperature of 10 - 20°C, reduced the energy costs of dehairing by 60% compared to the traditional chemical methods.

The enzyme was capable of full goat skin dehairing in 20 hours, and its activity was enhanced by the presence of various agents, demonstrating the potential for industrial use of the enzyme to dehair the goat skin in a sustainable and low-temperature bioconversion process [18]. In contrast to the traditional method, the use of keratinase as a treatment method to remove hair produced toxin-free hydrolysate, which could be used in a variety of commercial feeding products [16]. Thus, it is important to run such a study, hopefully to reveal a local strain of *B. subtilis* that is able to fulfill the main criteria of probiotics, tolerance to gastrointestinal stress, and safety in terms of antibiotic resistance, and also exhibits industrially benefit-relevant production of keratinases to be utilized in environmentally friendly dehairing. It involved a scientific assessment of strain viability in a gut-like environment and testing of the efficiency of dehairing on a hide sample. By fulfilling the increasing demand for effective probiotic supplements as well as addressing the urgent need for sustainable management of the waste of the keratin industry, this investigation studied a dual-purpose, safe, and green solution, which will help to reduce the use of toxic chemicals and contribute to the sustainability of human health and the environment.

2. Materials and methods

2.1. Evaluation of *B. subtilis* probiotic properties

This study used the *B. subtilis* strain (PZ014383), which had previously been obtained from an agricultural soil sample in Assiut, Egypt, and assessed the probiotic potential of the

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Research Article

strain using various *in-vitro* assays as detailed below.

2.1.1. Acid and bile salt tolerance test

Bile and acid tolerance were assessed using methods modified from Wang *et al*. [1] and Shah *et al*. [2]. Nutrient broth containing 0.3% (w/v) bile salts was adjusted to pH 2.0 with 1N hydrochloric acid (HCl) and then inoculated with an overnight culture. In parallel, control tubes containing nutrient broth at pH 6.5 without bile salts were inoculated with the same culture. During the incubation period at 37°C, samples were taken at the intervals 0, 1, 2, and 3 h, and the total bacterial count (TBC) was enumerated. Serial dilutions were plated on nutrient agar and incubated at 37°C for 24 h to determine bacterial counts (CFU/mL) for each test condition. Each condition was tested independently 3 times. The survival rate (%) was calculated using equation (1) provided below.

$$\textbf{Survival rate}\ (\%) = \frac{\text{CFU/mL at time (t)}}{\text{CFU/mL at time (0)}} \times 100 \quad \textbf{(1)}$$

2.1.2. Phenol resistance test

Evaluation of phenol tolerance used a procedure adapted from Jena *et al*. [19] and Somashekaraiah *et al*. [20]. Separate overnight cultures were introduced into nutrient broth containing phenol at concentrations of 0.4% and 0.6%. Following incubation for 24 h at 37°C, serial dilutions from each culture were spread on nutrient agar. Viable cells were subsequently quantified by the plate count method and reported as log CFU/mL.

2.1.3. Antibiotic sensitivity test

Pure colonies were cultured in nutrient broth for 24 h at 37°C. A sterile cotton swab was then used to distribute the resulting suspension uniformly over nutrient agar. Antibiotic discs were placed after the inoculated surface had dried. Susceptibility to 21 antibiotics was determined by disc diffusion. The tested discs were Spiramycin (100 µg/disc), Cefuroxime Na (30 µg/disc), Moxifloxacin (5 µg/disc), Clindamycin (2 µg/disc), Cefoperazone (105 µg/disc), Cephalexin (30 µg/disc), Oxacillin (1 µg/disc), Cefadroxil (30 µg/disc), Aztreonam (30 µg/disc), Vancomycin (30 µg/disc), Rifampicin (5 µg/disc), Meropenem (10 µg/disc), Ceftriaxone (30 µg/disc), Cefixime (5 µg/disc), Cefoperazone (75 µg/disc), Cloxacillin (5 µg/disc), Cefotaxime (30 µg/disc), Nitrofurantoin (300 µg/disc), Ciprofloxacin (5 µg/disc), Norfloxacin (10 µg/disc), and Rifamycin SV (30 µg/disc). Uniform contact between every disc and the agar was maintained. Inhibition-zone diameters were measured after incubation for 24 h at 37°C [21].

2.1.4. Antimicrobial activity test

Before testing, *B. subtilis* was propagated in nutrient broth. An active culture at 2% (v/v) was added to individual 100 mL Erlenmeyer flasks containing 50 mL of nutrient broth. The inoculated medium was incubated for 48 h at 37°C according to Khedkar *et al*. [22]. Cultures were then centrifuged at 10,000 rpm for 15 min at 4°C to recover cell-free supernatants (CFS). Their antimicrobial effects against the test pathogens were determined by agar-well diffusion [23].

The pathogen panel investigated included the Gram-positive *Staph. aureus* (ATCC 259233) and the Gram-negative *Ps. aeruginosa* (ATCC 27853) and *E. coli* (ATCC 25922). The strains were obtained from the Genetic Engineering and Biotechnology Research Institute, City of Scientific Research and Technological Applications, New Borg El-Arab, Alexandria, Egypt. Each pathogen was grown in nutrient broth as a pure culture and incubated for 24 h at 37°C. A sterile swab was used to evenly spread the bacterial suspension on nutrient agar. Agar wells of 7 mm diameter were loaded with 100 µL of CFS. After refrigeration for 2 h to permit diffusion, the plates were incubated at 37°C for 24 h before the inhibition zones were measured.

2.1.5. NaCl tolerance test

Salt tolerance was examined by exposing the *B. subtilis* strain to NaCl at 2%, 4%, 6%, and 8% (w/v). After sterilization, tubes containing each NaCl concentration received 1% (v/v) overnight culture and were incubated for 24 h at 37°C. Culture turbidity provided the measure of growth. The scoring system assigned (++) to maximum growth, (+) to normal growth, and (-) when growth was absent, following Hoque *et al*. [24].

2.1.6. Cell surface properties

2.1.6.1. Auto-aggregation ability test

The auto-aggregation of the strain was assessed according to Zuo *et al*. [25], with slight modifications to the growth medium. The strain was cultured in nutrient broth at 37°C for 16 h. After centrifugation at 6,000 × g for 10 min, the cell pellets were washed twice and resuspended in phosphate-

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Research Article

buffered saline (PBS, pH 7.4) to an optical density (OD_{600}) of 1.0 using a spectrophotometer (JENWAY 7315). Following incubation at 37°C for 2 h, 100 µL of the upper suspension was transferred to a new tube containing 1.9 mL of PBS, and the OD_{600} was measured. The auto-aggregation (%) was calculated using equation (2):

$$\textbf{Auto} - \textbf{aggregation}(\%) = \left(1 - \frac{OD_{600}\text{ of the upper suspension}}{OD_{600}\text{ of the total bacterial suspension}}\right) \times 100 \qquad \textbf{(2)}$$

2.1.6.2. Co-aggregation ability test

The auto-aggregation protocol was also followed when preparing cell suspensions for assessment of co-aggregation. A 1 mL volume of target-strain suspension was mixed under sterile conditions with 1 mL of *E. coli* (ATCC 25922) suspension. Optical density at 600 nm (OD_{600}) was read without delay and assigned as A0. After 2 h of incubation at 37°C, OD_{600} was measured once more and recorded as At. Equation (3) supplied in the study of Nagaoka *et al*. [26] was used to determine co-aggregation (%):

$$\textbf{Co} - \textbf{aggregation}(\%) = \frac{(A0 - At)}{A0} \times 100 \qquad \textbf{(3)}$$

2.1.6.3. Hydrophobicity test

Assessment of cell-surface hydrophobicity in *B. subtilis* strain (PZ014383) employed xylene extraction as specified in the study of Zuo *et al*. [25]. Cells grown for 24 h in nutrient broth were harvested through centrifugation at 6,000 × g for 5 min. Two washes with 50 mM K_2HPO_4 buffer (pH 6.5) preceded adjustment of the bacterial suspension to an optical density at 600 nm (OD_{600}) of 0.5 ± 0.05. Xylene (0.6 mL) was then added to a 3 mL aliquot, followed by vortexing for 180 s. Phase separation proceeded for 1 h at room temperature, after which OD_{600} of the carefully collected aqueous phase was recorded. Hydrophobicity (%) was obtained through equation (4) presented below:

$$\textbf{Hydrophobicity}\ (\%) = \frac{(A0 - A1)}{A0} \times 100 \qquad \textbf{(4)}$$

Whereas A0 = initial absorbance, and A1 = final absorbance.

2.1.7. Hemolytic activity test

The procedure for hemolytic screening of transferred bacterial colonies from nutrient agar, following 48 h of growth at 37°C, onto blood agar. A clear halo indicated beta-hemolysis, while alpha-hemolysis produced a greenish halo. Colonies showing no visible surrounding zone were categorized as gamma-hemolytic [27, 28].

2.2. Buffalo hide preparation and dehairing methods

2.2.1. Buffalo hide sample preparation

A hide of an Egyptian buffalo (*Bubalus bubalis*) was obtained from a local butcher shop in Assiut, Egypt. The hide was collected from the neck region, cured, and dried before preparation for the experiment. Once dry, it was divided into 5 × 5 cm pieces for the subsequent dehairing treatments [29].

2.2.2. Dehairing using *B. subtilis* enzyme and chemicals

A comparison of 3 treatment conditions assessed keratinase as an environmentally preferable replacement for conventional chemical dehairing. The keratinase enzyme used in this study was produced by *B. subtilis* strain (PZ014383), which exhibited probiotic characteristics, and the experiment followed procedures similar to those described by Chie *et al*. [29]. For enzymatic treatment, hide pieces were exposed to a (10%, v/v) keratinase enzyme solution. The pieces assigned to the chemical method were placed in distilled water containing (5%, w/v) calcium oxide and (2%, w/v) sodium sulfide; control pieces received distilled water alone under identical conditions. Exposure lasted 24 h at 30°C for all groups, with gentle agitation at 150 rpm. Hair loosened during treatment was then removed manually.

2.3. Statistical analysis

Triplicate experiments generated the values reported as mean ± standard deviation (SD). Analysis of variance (ANOVA) was performed in Statistix 8.1 software [30]. Treatment means were compared by the Least Significant Difference (LSD) test, using $p < 0.05$ as the significance level.

3. Result and discussion

3.1. Acid and bile salt tolerance test

B. subtilis (PZ014383) was evaluated for survival during 3 h of simulated gastrointestinal exposure (Figure 1). At pH 2.0, survival was 90.37±1.78% after 1 h, 81.36±2.38% after 2 h, and 72.51±2.81% after 3 h. Exposure to 0.3% bile salts produced survival values of 82.73±1.50%, 76.12±4.13%, and 60.71±6.35% after 1, 2, and 3 h, respectively. Under bile

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Research Article

conditions, 1 h and 2 h did not differ significantly ($p<0.05$), whereas the decline at 3 h was significant. The strain thus retained more than 60% survival after 3 h, indicating strong acid and bile tolerance. This behavior satisfies common probiotic-selection expectations that a strain withstands gastric acidity and intestinal bile before providing benefits *in vivo*. Resistance to acid and bile is widely used as an *in vitro* predictor of gastrointestinal survival for *Lactobacillus*, *Bifidobacterium*, and selected *Bacillus* strains [31]. Under comparable conditions, other *Bacillus* spp. retained only 35.92-49.56% viability at pH 2.0 and 52.69-87.91% viability in bile salts [32], illustrating strain-dependent variation in gastrointestinal stress tolerance.

The improved *B. subtilis* tolerance is due to inherent stress-response mechanisms, and this strain probably utilizes σB (sigma B)-mediated regulons, which induce survival during acid and osmotic stress [33]. Moreover, enzymes like the bile salt hydrolase (BSH) can also be a factor contributing to bile tolerance, as conjugated bile salts are hydrolyzed and their toxicity is reduced [34]. The progressive decrease in viability is a natural response of the cell to long-term environmental stress, but the fact that the viability remained more than 60% after 3 h speaks volumes of the strength of this strain. Taken together, these results suggest the possibility of *B. subtilis* (PZ014383) to be used as a probiotic in functional foods and dietary supplements, and that high gastrointestinal survival is necessary to provide the health benefits [35].

3.2. Phenol resistance test

To survive in the gut, bacteria must tolerate phenol. Gut bacteria deaminate aromatic amino acids from dietary proteins and thereby generate phenolic compounds [36-38]. The results showed that *B. subtilis* growth was suppressed more as phenol concentration increased. TBC dropped from 8.62±0.13 log CFU/mL at 0.0% phenol to 8.25±0.16 at 0.4% phenol concentration and to 7.68±0.13 at 0.6% phenol concentration. As shown in Figure 2, the results showed significant differences as the treatments changed (LSD, $P<0.05$). The presence of phenol resulted in a concentration-dependent loss of viable cells, suggesting that the growth of the bacteria was increasingly suppressed. There are many experimental studies and research articles that have documented the high antibacterial activity of phenolic acids and other related compounds in many bacterial species [39-40].

B. subtilis is also attracted to lower concentrations of phenol (μM) and is repelled by higher concentrations (mM). This response is due to specific chemoreceptors, wherein McpA is the main repulsion mediator and McpC and HemAT are attraction mediators [41]. The results suggest that stress at higher phenol concentrations is consistent with the reduced TBC.

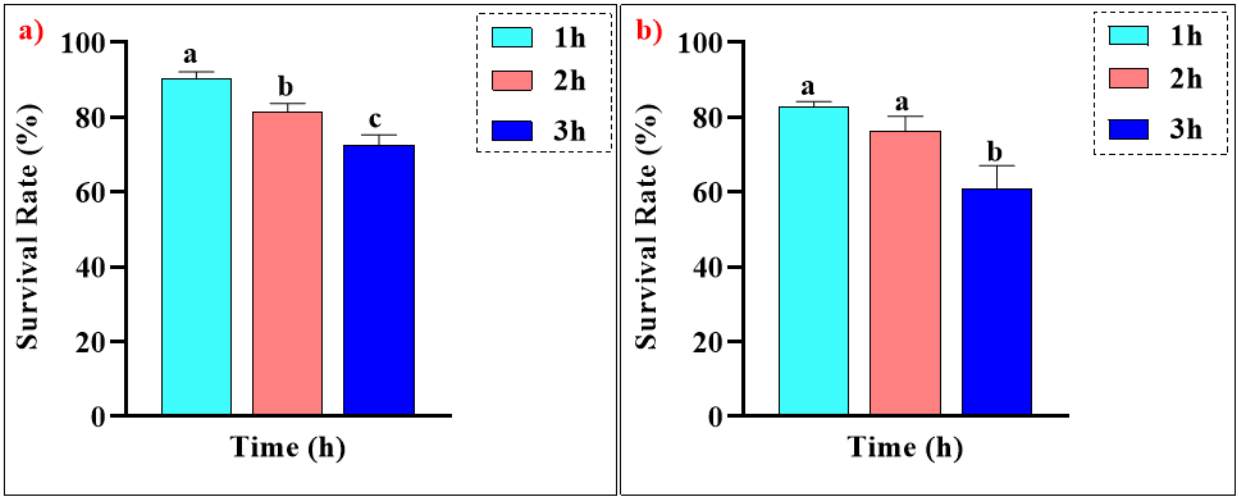

Figure 1. Survival rate (%) of *B. subtilis* (PZ014383) under simulated gastrointestinal conditions. **A**: Acidic conditions (pH 2.0); **B**: Bile salts (0.3%). Values are reported as mean±SD (n=3). Different superscript letters (a-c) indicate significant differences between bars ($P<0.05$, LSD test).

New Horizons Journal of Basic and Applied Sciences, 2026, Vol. X, Iss. X, 118-132

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Research Article

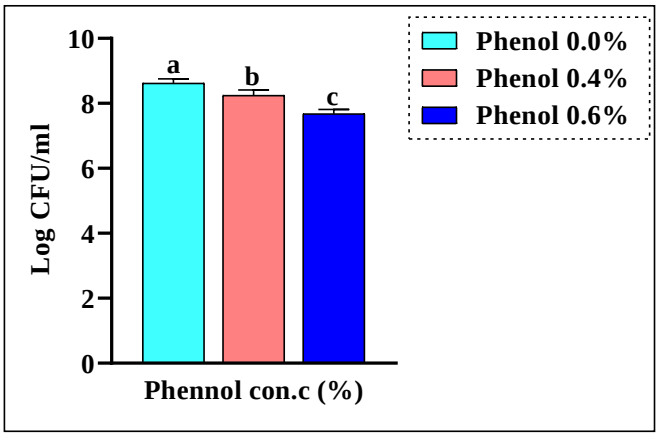

Figure 2. Effect of phenol concentration on log CFU/mL of *B. subtilis* (PZ014383). Values are presented as mean±SD (n=3); different superscript letters (a-c) denote significant differences among bars ($P<0.05$, LSD test).

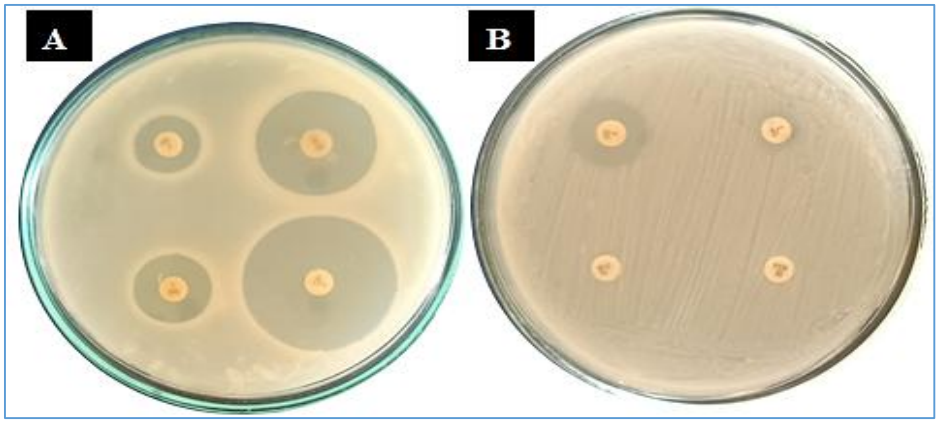

Figure 3. Panel (**A**) showed clear inhibition zones consistent with effective growth suppression, whereas panel (**B**) showed 3 discs without inhibition zones.

Table 1. Antibiotic susceptibility profile of *B. subtilis* strain based on disc diffusion assay.

Antibiotic (µg/Disc)	Diameter of inhibition zone (mm)	Antibiotic (µg/Disc)	Diameter of inhibition zone (mm)
Spiramycin (SP 100)	17.33±1.53 **I**	**Meropenem (MEM 10)**	45.67±3.21 **S**
Cefuroxime Na (CXM 30)	16.33±1.53 **I**	**Ceftriaxone (CRO 30)**	00±00 **R**
Moxifloxacin (MXF 5)	31±1.00 **S**	**Cefixime (CFM 5)**	00±00 **R**
Clindamycin (DA 2)	16.67±1.53 **I**	**Cefoperazone (CFP 75)**	15±1.00 **R**
Cefoperazone (SCF 105)	30±1.73 **S**	**Cloxacillin (OB 5)**	11±1.00 **R**
Cephalexin (CL 30)	25.67±1.53 **S**	**Cefotaxime (CTX 30)**	00±00 **R**
Oxacillin (OX 1)	00±00 **R**	**Nitrofurantoin (F 300)**	16±1.00 **I**
Cefadroxil (CFR 30)	12.33±0.58 **R**	**Ciprofloxacin (CIP 5)**	34±1.00 **S**
Aztreonam (ATM 30)	18±2.00 **I**	**Norfloxacin (NOR 10)**	27±1.73 **S**
Vancomycin (VA 30)	19±1.00 **I**	**Rifamycin SV (RF 30)**	25.67±1.53 **S**
Rifampicin (RD 5)	27.67±2.52 **S**		

Note: Sensitive (**S**) (> 21 mm); Intermediate (**I**) (16- 20 mm), and Resistant (**R**) (< 15 mm), according to that described by [21].

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Research Article

3.3. Antibiotic sensitivity test

Disc diffusion analysis (Figure 3) demonstrated a varying response of *B. subtilis* (PZ014383) to different classes of antibiotics. Intrinsic, non-transferable antibiotic resistance may help probiotic *B. subtilis* survive in the gastrointestinal tract following antibiotic treatment [42-45]. As depicted in Table 1, *B. subtilis* (PZ014383) demonstrated the following resistances and susceptibilities: high susceptibility to meropenem, moxifloxacin, ciprofloxacin, norfloxacin, rifampicin, rifamycin SV, cephalexin, cefoperazone (SCF 105); intermediate susceptibility to spiramycin, cefuroxime, clindamycin, aztreonam, vancomycin, nitrofurantoin; and resistance to the β-lactam antibiotics oxacillin, cefadroxil, ceftriaxone, cefixime, cefoperazone (CFP 75), cloxacillin, and cefotaxime.

The *B. subtilis* strain exhibited a selective resistance towards β-lactam antibiotics, while maintaining susceptibility to a number of important classes of antibiotics. Probiotic *B. subtilis* was demonstrated to be resistant to β-lactam antibiotics while exhibiting susceptibility towards fluoroquinolones, like ciprofloxacin [46]. Ecological origin and genetic diversity may account for variation among *B. subtilis* strains; marine isolates, for example, can carry distinct antimicrobial-resistance determinants [47]. Research demonstrating the resistance patterns of *Bacillus* spp. reveals that strains isolated from raw milk exhibited susceptibility to meropenem, while demonstrating a variety of resistance patterns among the tested strains [48]. Overall, the pattern appears intrinsic and potentially non-transferable, supporting the strain's safety for probiotic use.

3.4. Antimicrobial activity test

Probiotic products may suppress pathogens through peptides, exopolysaccharides, hydrogen peroxide, organic acids, and bacteriocins [49, 50]. Here, cell-free supernatant (CFS) obtained from *B. subtilis* (PZ014383) exhibited selective antimicrobial activity across the pathogen panel. *Staph. aureus* (ATCC 27853) showed the greatest inhibition, and *Ps. aeruginosa* (ATCC 259233) ranked second; *E. coli* (ATCC 25922), however, showed no measurable response, as summarized in Table 2 and shown in Figure 4. Gram-negative bacteria restricts penetration by many antimicrobial agents, helping to explain the reduced susceptibility and selective pattern observed during the present assays [51].

3.5. NaCl tolerance test

Some bacterial species exhibit growth inhibition by NaCl [24]. Bacteria can be found in nature, industry, and the upper section of the small intestine, where they come into direct contact with osmotic stress [52]. In the current research, *B. subtilis* was able to grow in nutrient broth with 8% NaCl. Maximum growth (++) occurred at 2%, 4%, and 6% NaCl, whereas normal growth (+) was recorded at 8% NaCl (Figure 5). The strain therefore tolerated moderate salinity, although growth remained optimal at the lower NaCl concentrations. This is in accordance with the most recent studies on the moderate halotolerance of *B. subtilis*, where *B. subtilis* NA2 grew *in vitro* at up to 8% NaCl before growth declined, matching the sustained viability at 8% salinity and maximal growth at lower NaCl concentrations reported by Gul *et al*. [53]. This is in agreement with the results of the present study, which showed that *B. subtilis* is able to cope with osmotically stressed environments that have low to moderate concentrations of salt.

Table 2. Antagonistic effect of *B. subtilis* (PZ014383) cell-free supernatant (CFS) against some pathogenic bacteria.

Target strain	Pathogenic tested organisms		
	***Ps. aeruginosa* (ATCC 259233)**	***Staph. aureus* (ATCC 27853)**	***E. coli* (ATCC 25922)**
	Diameter of inhibition zones (mm)		
B. subtilis (PZ014383**)**	7.33±2.52 ^{b}	12.33±1.53 ^{a}	0.00±0.00 ^{c}

Note: Data are presented as mean±SD. Different letters indicate statistically significant differences according to the LSD test ($P \leq 0.05$). The inhibition zone was calculated by subtracting the well diameter (7 mm) from the total measured diameter.

New Horizons Journal of Basic and Applied Sciences, 2026, Vol. X, Iss. X, 118-132

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Research Article

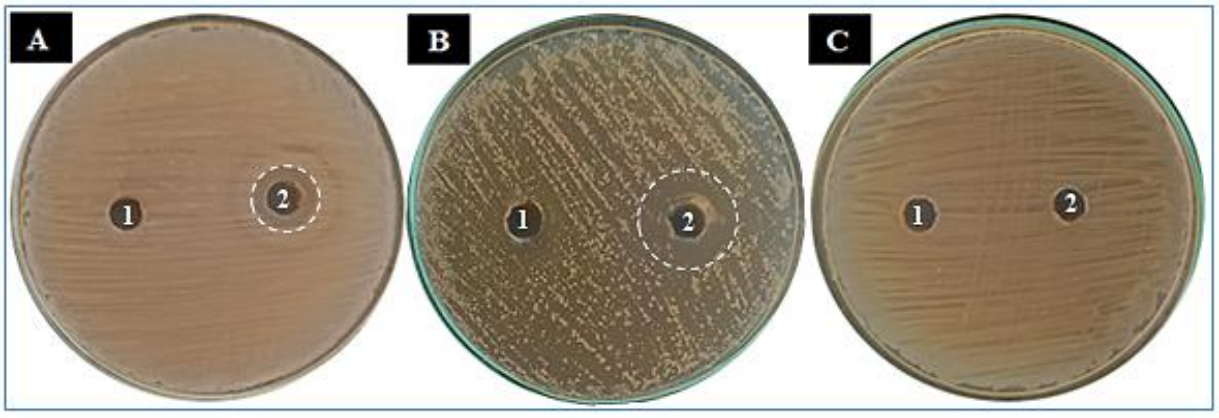

Figure 4. Antimicrobial activity of *B. subtilis* cell-free supernatant (CFS) against (**A**) *Ps. aeruginosa* (ATCC 259233), (**B**) *Staph. aureus* (ATCC 27853), and (**C**) *E. coli* (ATCC 25922), showing (**1**) control and (**2**) CFS-treated zones.

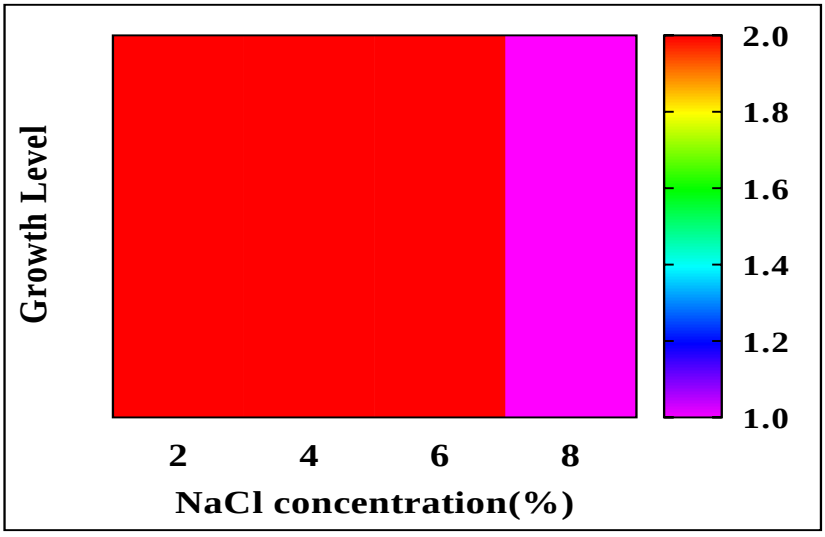

Figure 5. *B. subtilis* (PZ014383) growth response to NaCl stress in nutrient broth with different concentrations of sodium chloride. Red color indicates high growth (++), whereas purple color indicates lower growth (+).

3.6. Cell surface properties

Cell-surface characteristics of *B. subtilis* (PZ014383) were assessed as possible indicators of probiotic function. As shown in Figure 6, the strain displayed high auto-aggregation (89.27 ± 0.63%) and hydrophobicity (73.3 ± 1.85%) but low co-aggregation (19.34 ± 1.11%). Auto-aggregation permits cells of the same species to form clusters, which may support adhesion to the intestinal mucosa [54] and reduce removal through intestinal peristalsis, making it a desirable probiotic trait [55]. The auto-aggregation value of *B. subtilis* PZ014383 resembles that reported for other probiotic *Bacillus* strains, including *B. subtilis* GM1 (~90.25% after 24 h). Strong self-clustering may therefore be common among resilient probiotic *Bacillus* strains and may reflect surface properties that favor host interaction [56].

High cell-surface hydrophobicity has been linked to increased adhesion to host epithelial layers and is a desirable probiotic trait because it aids in gut colonization. Hydrophobic interactions ease contact with the mucosal layer and may help in the competitive inhibition of gut pathogens. Recent studies have corroborated these findings; Probiotic *Bacillus* spp. have shown hydrophobicity above 50% together with improved *in vitro* adhesion, suggesting that more hydrophobic strains may localize more effectively within the host [57]. The combination of high auto-aggregation and high hydrophobicity indicates that *B. subtilis* PZ014383 has

DOI:10.62184/nhjbas.jnh20020264

Research Article

favorable surface properties for adhesion and self-association, which may support persistence at the intestinal mucosa. This interpretation agrees with evidence linking strong hydrophobic and auto-aggregative traits to adhesion, colonization, and competitive exclusion [58].

The low co-aggregation of *B. subtilis* PZ014383 (~19%) indicates that under the conditions tested, direct interaction with pathogenic *E. coli* (ATCC 25922) was limited under the tested conditions. Probiotic co-aggregation can prevent pathogen colonization, as they may provide a physical barrier and a locally inhibitory environment [59]. This type of competitive exclusion is beneficial, as it helps clear infections [60]. This can also apply in contexts where there is a competitive environment between pathogenic and beneficial microorganisms [61].

The weak co-aggregation of *B. subtilis* PZ014383 with *E. coli* indicates that the direct mechanism of cell-cell interaction is not likely the primary method of inhibiting pathogens. Instead, *B. subtilis* PZ014383 may be able to inhibit pathogens by occupying adhesion sites as a result of its intrinsic properties, such as the combination of high hydrophobicity (73.3%) and a strong tendency to auto-aggregate (89.27%), which may help to form stable aggregates at the epithelial cell surfaces of the host. These properties promote persistence and enable the formation of biofilms, and may limit pathogen attachment and regulate host physiology [62].

3.7. Hemolytic activity test

A safe probiotic must not have hemolytic activity, lysis of red blood cells (RBCs) with release of the intracellular contents, including hemoglobin [63, 64]. *B. subtilis* (PZ014383) in the blood-agar test did not form any clear or greenish colonies. This lack of hemolysis would be γ-hemolysis, as seen in Figure 6. The finding supports the non-virulent nature of the strain, its biosafety, and its potential application in biotechnology. A cited non-hemolytic (γ-hemolytic) *B. subtilis* strain has been previously reported as being safe in industrial and probiotic use [64].

3.8. Dehairing treatments

Keratinase obtained from *B. subtilis* (PZ014383) provided effective enzymatic dehairing and represents an environmentally preferable substitute for the chemical method. This outcome extends the growing microbiological evidence supporting microbial-enzyme use by the leather industry. Complete hair removal was achieved by conventional chemical dehairing (Sample A), although the treatment also darkened the grain surface and damaged collagen. Those changes reveal clear disadvantages for finished-leather quality as well as environmental sustainability. In comparison, application of the *B. subtilis* keratinase (Sample B) removed hair effectively without disrupting the collagen matrix, leaving the structural framework of the hide visibly intact. No hair was released from the pieces exposed only to distilled water (Sample C). The control therefore confirms that the observed dehairing resulted exclusively from the enzymatic or chemical interventions, as shown in Figure 7.

Recent research has proven more and more that microbial keratinases can be used in the dehairing process, as the *B. subtilis* ES5 keratinases have been demonstrated to entirely debilitate goat and sheep hides with soft and smooth grain surfaces and leave undamaged hides featuring smooth grain surfaces, unlike the traditional lime-sulfide processes, which leave hides hard and chemically modified, as reported by Alamnie *et al*. [65]. These findings are in line with the current findings, and this supports the idea that keratinases can be used effectively to remove hair with minimum effects on the structure of hide. In addition, microbial keratinases have been cited to make a significant reduction in the environmental pollution related to the manufacture of leather. The enzymatic process of dehairing with *B. subtilis* keratinase significantly reduced biochemical oxygen demand (BOD), chemical oxygen demand (COD), and other pollution indicators in comparison with chemical methods, which is the significant ecological benefit of the study by Alamnie *et al*. [65]. The fact that enzyme-based dehairing is reported to have environmental benefits underlies the notion that dehairing is not only a preservation of leather quality but also a way to achieve the goals of the industry in regard to greener processing.

A useful point of comparison is the rate and mechanism of keratinase activity. In the study of Tian *et al*. [66], high-expression keratinases from *B. subtilis* SCK6 achieved complete dehairing during a short (6 h) incubation under optimized conditions, and histological examinations of enzyme treated pelts have revealed normal fiber orientation

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and the preservation of dermal collagen [66]. Although the current study does not directly address the issues of the kinetics of dehairing or histological examinations, the qualitative preservation of collagen is consistent with earlier studies. Nonetheless, there are some limitations. More recent mechanistic studies suggest that enzyme penetration into hides can be slow and pH-sensitive, potentially limiting dehairing efficiency and producing variable performance among hides [67]. Therefore, the biological dehairing process would potentially benefit from optimization of the dehairing enzymes as well as other process variables such as pH and ionic strength.

Overall, the current findings demonstrated the use of *B. subtilis* PZ014383 keratinases as microbial and biotechnological alternatives to traditional leather dehairing methods. Keratinases strip the hair from the hide while leaving the underlying structure intact. Additionally, *Bacillus* spp. keratinases can also reduce the environmental pollutants generated during leather processing. Although enzyme activity and hide penetration still vary, advances in enzyme engineering and process optimization may enable further development of industrial-scale enzymatic dehairing.

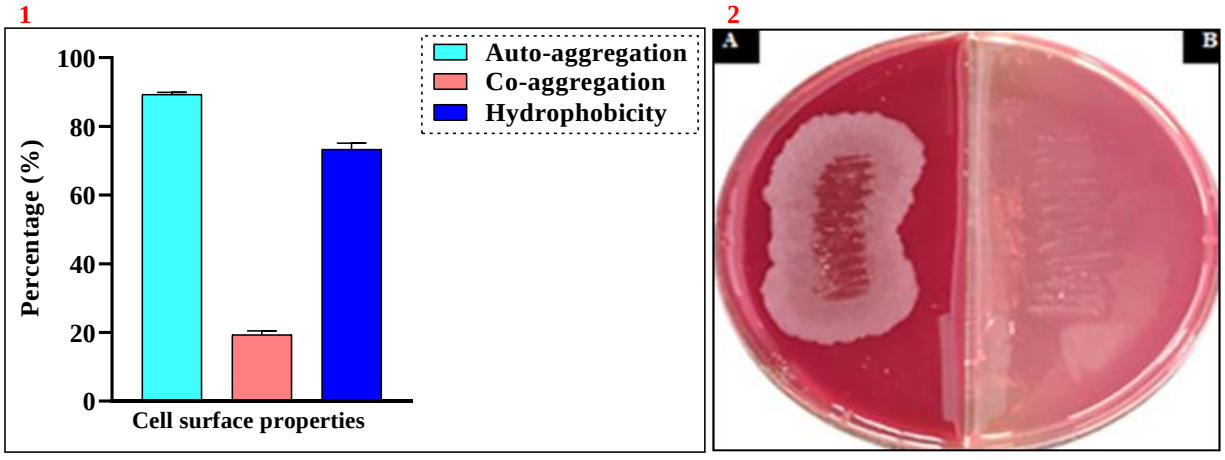

Figure 6. (**1**) Cell-surface properties of *B. subtilis* (PZ014383), showing the percentages obtained in the auto-aggregation, co-aggregation, and hydrophobicity assays. Values are reported as mean±SD (n=3). (**2A**) Absence of hemolysis (γ-hemolysis) on blood agar and (**2B**) absence of growth on MacConkey agar.

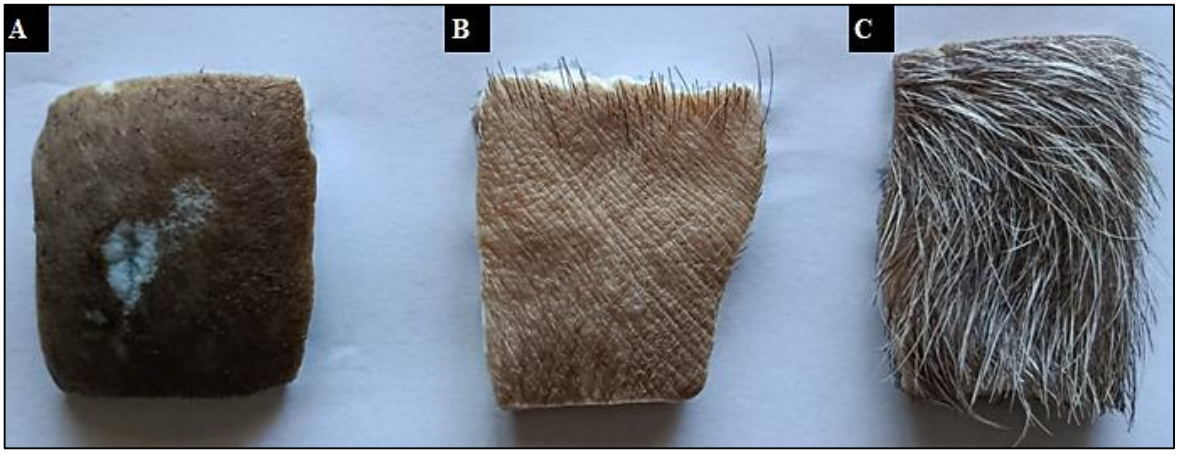

Figure 7. Comparison of dehairing treatments with **(A)** chemical treatment, **(B)** *B. subtilis* (PZ014383) enzyme treatment, and **(C)** control (distilled water treatment).

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4. Conclusion

Probiotic evaluation of the *B. subtilis* strain PZ014383 was performed, demonstrating its ability to survive digestive stress under simulated gastric and intestinal conditions; in addition, it displayed favorable surface characteristics. Moreover, the cell-free supernatant demonstrated selective antimicrobial activity against *Staph. aureus* and no inhibition of *E. coli*. Regarding the trial of application in eco-friendly hide dehairing, the ability to dehair hides was clear, serving as a more environmentally friendly biotechnological alternative to the chemical process. Further studies should evaluate its industrial potential.

Authors' contributions

E-A.W.E.S.: Conceptualization, formal analysis, methodology, writing original draft, review & editing, data curation, visualization, validation. **S.G.A.:** Formal analysis, writing original draft, review & editing. **E.K.B.:** Formal analysis, writing original draft, review & editing. **M.A.:** Formal analysis, writing original draft, review & editing. **H.E-G.:** Conceptualization, formal analysis, methodology, writing original draft, review & editing, data curation, visualization, validation.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be available upon request.

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