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# Assessment of Probiotic Properties and Characterization of Some Proteolytic Bacterial Strains Isolated from Different Types of Milk

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## Abstract

Milk is a rich source of nutrients for many microorganisms. Fifty-four bacterial strains from buffalo, sheep, cow, and camel milk were isolated on an MRS agar medium. The isolates were divided into 23 LAB isolates and 31 non-LAB isolates based on catalase, spore formation, Gram staining, and lactic acid formation tests. The probiotic investigations were carried out on the proteolytic isolates. The probiotic isolates (CAL1, BA4, and CA3) were chosen for morphological and biochemical tests. According to the findings, Bacilli BA4, CA3, and *Lactococcus* CAL1 can be used as probiotic proteolytic strains.

**Keywords:** Isolation, LAB, Milk, Probiotics, Proteolysis

**Full-length article** **Corresponding Author, e-mail: elawadysalem.4222@azhar.edu.eg*

## 1. Introduction

Microorganisms thrive in nutrient-rich environments, with milk from mammals being a rich medium for most of them, indicating their diverse metabolic needs. Milk is a nutritious growth medium for many bacteria, containing carbs, fat, casein, protein, vitamins, and minerals [1]. Lactic acid bacteria (LAB) were initially isolated from milk, and it was later shown that LAB exists naturally in raw milk as indigenous microflora [2]. It is also naturally present in milk-based products [3]. LAB are Gram-positive, non-spore-forming bacteria employed as starter cultures, producing lactic acid as a primary by-product of sugar fermentation [4]. Anaerobic or facultative aerobic rods or cocci of lactic acid bacteria are widely distributed in the natural world and naturally occur as native microflora in raw milk, which plays a crucial role in many food and feed fermentations [5]. LAB species, including *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Enterococcus*, and *Streptococcus*, are frequently found in fermented dairy products [6].

Probiotics are living microorganisms that improve the health of a host when provided in appropriate quantities. [7]. The beneficial benefits of probiotic strains are determined by their capacity to survive via a passageway in the stomach, establish themselves in the gastrointestinal tract (GIT), and outcompete pathogens [8]. Potent probiotic isolates must possess specific characteristics, such as the ability to thrive and form colonies under various environmental conditions [9]. The ability of probiotic bacteria to survive in the gastrointestinal tract depends on their properties, including resistance to bile salts and stomach acidity [10]. LAB, or bifidobacteria, are usually beneficial bacteria related to probiotic behavior [11]. Also, *Bacillus* sp. has demonstrated exceptional probiotic potential because of the resistance of the spores to heat and other environmental factors, including pH and pressure [12]. Most commercial *Bacillus* probiotics consist of *B. subtilis*, *B. polyfermenticus*, *B. clausii*, some *B. cereus*, *B. coagulans*, *B. pumilius*, and *B. licheniformis* [13]. The present study aimed to isolate probiotic proteolytic bacteria from raw milk.

## 2. Materials and methods

### *2.1. Sample collection*

Eight samples of different types of milk (buffalo, sheep, cow, and camel) and two samples of each type were collected from different regions (Mansoura, Assiut, and Aswan). Each sample was collected in a sterile bottle, sent in an ice box to a laboratory under aseptic conditions, and refrigerated at 4°C until processing.

### *2.2. Bacterial isolation and purification*

Each sample was cultured on MRS agar medium by the spread plate method and incubated at 37°C for 72 h under aerobic and anaerobic conditions. Following incubation, all colonies that appeared on the plates were selected for purification using the streak plate technique on MRS agar plates.

### *2.3. Examination of bacterial isolates*

#### *2.3.1. Gram staining*

All purified bacterial isolates were stained with Gram-staining and then studied under a light microscope

using an oil immersion lens to determine the shape of the bacterial cells and confirm their purity.

***2.3.2. Catalase test***

On a glass slide, a loopful of 24-hour culture cultured on MRS agar was emulsified with drops of $H_2O_2$ (3%). The liberation of oxygen as gas bubbles indicates the presence of the catalase enzyme [14].

***2.3.3. Spore formation***

A tube of MRS broth medium was inoculated with growth from the agar medium, in which sporulation was suspected to occur. The tube was placed in a water bath at 80 ºC along with an uninoculated broth containing a thermometer, and the water level in the bath was higher than the broth level. The inoculated tube was incubated for 10 minutes, starting when the thermometer reached 80 ºC, cooled, and incubated at 37 °C for 48 h. After incubation, the growth (turbidity) tubes indicated endospore formation [15].

***2.3.4. Lactic acid production on MRS agar medium***

The streak plate technique was used to spread pure culture colonies of each isolate on MRS agar medium supplemented with 0.5% CaCO3, and the plates were then incubated at 37 °C for 48 hours. The translucent zone surrounding the growth on the chalked agar plates indicates the presence of lactic acid produced by the bacterial isolates [16].

***2.4. Screening for proteolytic activity***

Each isolate was streaked on a skim milk agar plate to assess its proteolytic activity. For 48 h at 37 °C, plates were incubated under aerobic and anaerobic conditions for non-LAB and LAB isolates, respectively. After incubation, the transparent or intensely opaque zones around the growth were assessed as positive for proteolytic activity [17].

***2.5. In vitro characterization of probiotic properties***

Isolates that showed proteolytic activity were examined for their probiotic characteristics in the following ways:

***2.5.1. Acid and bile salt tolerance test***

The tolerance of the isolates to both acidic pH values and bile salts was assessed according to [18], with a few minor modifications. The isolates were cultured overnight and inoculated with (1%, v/v) in MRS broth medium adjusted to pH value 2.0, while others were supplemented with 0.3% bile salts. The pH of the MRS broth medium was adjusted to 6.5, and another without bile salts was considered the control for bile salts and pH condition. The samples were incubated at 37 °C for intervals of (0, 1, 2, and 3 h). The biomass (CFU/ml) of each culture was determined on an MRS agar plate after incubating at 37 °C for 24 h. The survival rate (%) was calculated using the following formula:

$$\textbf{Survival Rate}\ (\%) = \frac{\text{Biomass at a time (t)}}{\text{Biomass at the initial time (0)}} \times 100$$

***2.5.2. Phenol tolerance test***

The isolates' phenol tolerance was assessed according to [18]. Overnight-grown isolates were added to the MRS broth medium, which was supplemented with 0.4% and 0.6%, v/v, phenol. Following 24 h of incubation at 37 °C, the cultures were diluted serially and then spread on MRS agar plates. The plate count method calculated the cell viability (log CFU/ml).

***2.5.3. Antibiotic susceptibility test***

Eight isolates were tested for susceptibility to seven different commercially available antibiotic discs (Oxoid™) using the disc diffusion technique. Penicillin G (10µg/disc), cefotaxime (30µg/disc), oxacillin (1µg/disc), vancomycin (30µg/disc), nitrofurantoin (300µg/disc), erythromycin (15µg/disc), and streptomycin (10µg/disc) were put on the surface of MRS agar plates. After 24 h of incubation at 37 °C, the diameter of the inhibition zone for each antibiotic was determined according to [19].

***2.5.4. Auto-aggregation ability test***

The auto-aggregation tests were conducted according to [20]. In the MRS broth medium, bacterial isolates were cultured for 16 hours at 37 °C. After centrifuging the cells at 6000 g for 10 min, the pellets were washed twice and mixed with phosphate-buffered saline (PBS, pH 7.4) until the optical density ($OD_{600}$) reached 1.0. Following a two-hour incubation period at 37 °C, 100 µl of the upper suspension was moved to a different tube containing 1.9 ml of PBS, and the $OD_{600}$ was determined. The auto-aggregation (%) was calculated using the following formula:

$$\textbf{Auto} - \textbf{aggregation}(\%) = 1 - \left(\frac{\text{OD600 of upper suspension}}{\text{OD600 of total bacterial suspension}}\right) \times 100$$

***2.5.5. Co-aggregation ability test***

Similar to the auto-aggregation test, cell suspensions were prepared for co-aggregation. In a cuvette, 1 ml of each isolate's cell suspension and the pathogen strain *Pseudomonas aeruginosa* (ATCC9027) were mixed. The $OD_{600}$ was then measured right away (A0). The combination was incubated at 37 °C for two hours, and then the $OD_{600}$ was measured again (At). The co-aggregation (%) was calculated using the equation in [21]:

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

***2.5.6. Hydrophobicity test***

The hydrophobicity of the isolates was evaluated using xylene extraction, as described in [20]. The bacteria were grown in MRS broth for 24 hours. The cells were then separated by centrifuging at 6000 g for 5 minutes and being washed twice with 50 mM $K_2HPO_4$ buffer (pH 6.5).

The absorbance at 600 nm ($A_{600}$) was set to 0.5± 0.05, and then 3 ml of bacterial suspension and 0.6 ml of hydrocarbon (xylene) were mixed and stirred for 180 seconds. After incubating at room temperature for one hour, the aqueous phase was removed, and its $A_{600}$ was determined. The hydrophobicity (%) was calculated using the following formula:

$$\textbf{Hydrophobicity}\ (\%) = \frac{(\mathrm{A0} - \mathrm{A})}{\mathrm{A0}} \times 100$$

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

### *2.6. Morphological and biochemical examination*

The bacterial isolates (CA3 isolated from cow milk, BA4 isolated from buffalo milk, and CAL1 isolated from camel milk) were identified by examining their morphological features and biochemical tests. They were;

1. Growing up on different media
2. Scanning Electron Microscope (SEM)
3. Indole test
4. Methyl red test
5. V-P test
6. Citrate utilization test
7. Motility test
8. $H_2S$ production test
9. Gas production from glucose
10. Carbohydrate fermentation test

### *2.7. Statistical analysis*

Triplicate measurements were used to determine mean values and standard deviations (SD). The statistical study was carried out using a one-way analysis of variance (ANOVA). The significance ($p < 0.05$) of the differences between the mean values was assessed using Duncan's multiple-range test. The statistical analysis was conducted using IBM SPSS Statistical software (version 22.0).

## 3. Results

### *3.1. Isolation and purification of bacterial isolates*

Raw milk samples from buffalo, sheep, cows, and camels were collected from 8 different farmers in Mansoura, Assiut, and Aswan (cities in Egypt) for the isolation of probiotic bacteria. Fifty-four bacterial isolates were isolated and purified on an MRS agar medium. The most isolated bacteria were from cow milk, then buffalo, sheep, and camel milk, with 38.89%, 24.08%, 22.22%, and 14.81%, respectively.

### *3.2. Examination of bacterial isolates*

In this study, a total of 54 isolates were divided into 23 LAB isolates and 31 non-LAB isolates according to Gram staining, catalase test, spore formation, and lactic acid production on a chalk MRS agar medium, as shown in (Table and Fig. 1). Notably, all the isolates found in the study were Gram-positive.

### *3.3. Screening for proteolytic activity*

The bacterial isolates that showed proteolytic activity on a milk agar plate are listed in (Table 2). The results showed that the opaque zone appeared with 14 isolates of LAB and the clear zone with seven isolates of non-LAB, as shown in (Fig. 2).

### *3.4. In vitro* characterization of probiotic properties

#### *3.4.1. Acid tolerance test*

The results demonstrated a significant decline in the survival rate at pH 2.0 with time. All isolates survived in MRS broth medium pH 2.0 in the first hour, except for isolates SP1, CA8, and CA10. Only BA4, CA3, and CAL1 stayed in the same medium after the second hour. Finally, only two of the eight bacterial isolates, BA4 and CA3, could survive for three hours at pH 2.0. As demonstrated in **(**Table 3), the BA4 strain exhibited a higher ($p < 0.05$) survival rate than the CA3 strain.

#### *3.4.2. Bile salt tolerance test*

The data indicated that the growth rate over time has decreased significantly ($p < 0.05$). The only strain that didn't grow in the MRS broth medium with 0.3% bile salts in the first hour was the SP1 strain. The CA2, CA8, and CA10 isolates could not tolerate bile salts when the seven bacterial isolates were left in the same medium for an extra hour, as shown in (Table 4). Also, the result showed the most adaptable isolate in the bile salt at a concentration of 0.3% for three hours was the CAL1 strain, with a survival rate of 76.59%, followed by isolates BA4, CA3, and CA1 with 63.90%, 50.37%, and 11.21%, respectively.

#### *3.4.3. Phenol resistance test*

The CFU/ml of the bacterial isolates were determined using MRS agar plates following a 24-hour incubation in MRS broth medium that included 0.4% and 0.6% phenol. Comparing the phenol concentrations to the MRS control without phenol, an inhibitory impact was seen, as shown in (Fig. 3). A viable count ranging from 2.53 to 7.05 log CFU/ml was observed with phenol 0.6% and from 4.36 to 7.39 log CFU/ml with phenol 0.4%, while the viable count range was 4.65 to 9.40 log CFU/ml without phenol. The obtained data showed a significant decrease in the log CFU/ml of bacterial isolates at phenol 0.4% in all the isolates except CA10. In contrast, the result showed a significant change in the log CFU/ml of all isolates at phenol 0.6% compared to the control (0.0%, phenol).

#### *3.4.4. Antibiotic susceptibility test*

All bacterial isolates were resistant to penicillin G 10µg, oxacillin 1µg, erythromycin 15µg, streptomycin 10µg, and cefotaxime 30µg. On the other hand, all the isolates were sensitive to vancomycin 30µg, except the isolate CA2, which was intermediate. In addition, all the isolates were intermediate to nitrofurantoin 300µg, except the isolate CA2, which was resistant. Finally, the bacterial strains showed varied antibiotic susceptibility, which is documented in (Table 5).

#### *3.4.5. Confirmatory assays*

Auto-aggregation, co-aggregation, and hydrophobicity assays were conducted for isolates (CA3, BA4, and CAL1), which were the most potent isolates with the probiotic properties mentioned above.

##### *3.4.5.1. Auto-aggregation assay*

The three isolates exhibited auto-aggregation activity with a significant difference *(*$p < 0.05$) after 2 h of incubation at 37°C. The isolate BA4 showed the highest auto-aggregation activity after 16 hours of static cultivation in an MRS broth medium at 37 °C, as shown in (Table 6).

##### *3.4.5.2. Co-aggregation assay*

The co-aggregation ratios between three isolates (CA3, BA4, and CAL1) and *Pseudomonas aeruginosa* (ATCC9027) are shown in (Table 6). Isolate CA3 showed a significantly high *(*$p < 0.05$) co-aggregation ability with *Pseudomonas aeruginosa* compared to other isolates.

##### *3.4.5.3. Hydrophobicity assay*

Hydrocarbon (xylene) was used to assess the cell-surface hydrophobicity of the bacterial isolates. The

hydrophobicity test showed no significant difference ($p < 0.05$) between isolates BA4 and CAL1. On the other hand, the three isolates had hydrophobicity greater than 85%, as indicated in (Table 6).

### *3.5. Morphological and biochemical identification*

For morphological and biochemical testing, the isolates BA4, CA3, and CAL1 were chosen based on their potent probiotic features (Fig. 4 and 5), which show the morphological results. At the same time, (Table 7) illustrates the biochemical results for selected isolates.

## *4. Discussion*

Milk has a high nutritional content, and combined with high water activity at a near-neutral pH, it stimulates the growth of numerous microbes [22], leading to a diverse bacterial community. Raw milk is an excellent source of probiotics, as documented in [23]. Previously, different LAB and non-LAB strains were isolated from raw milk, as recorded in [24–30]. In the present study, lactic acid bacteria (LAB) and non-LAB isolates were isolated from various types of raw milk (buffalo, cow, sheep, and camel milk) in an MRS agar medium. According to [31] and [32], LAB were Gram-positive, catalase-negative, and non-spore-forming isolates. Also, LAB isolates were determined based on a clear zone around the colonies in a chalk agar medium, which indicated the dissolution of CaCO3 by lactic acid (Fig.1). The current results are in agreement with [16,31], which found the transparent area in an MRS agar medium provided with 1% CaCO3 around the growth of LABs after the incubation period. In contrast, Gram-positive, catalase-positive, spore-forming, and non-lactic acid-releasing isolates in chalk agar medium were classified as non-LAB.

Bioactive peptides are defined as those that consist of specific protein fragments that show biological activity and may be beneficial in promoting health [33]. Protein hydrolysis (or breakdown) to get these peptides can come from various sources, but milk-derived peptides are currently the most crucial source [34]. Fifty-four bacterial isolates were tested for their ability to hydrolyze proteins in skim milk (SM) agar medium. The results showed transparent (highly proteolytic) and opaque (weakly proteolytic) zones in SM agar with non-LAB and LAB isolates, respectively. LAB are weakly proteolytic and possess a comprehensive proteinase/peptidase system capable of hydrolyzing oligopeptides into small peptides and amino acids [35]. On the other hand, *Bacillus sp.* can produce exopeptidase and endopeptidase enzymes [36], leading to highly proteolytic action. The findings align with previous studies [37] and [17], which recorded transparent and opaque SM agar zones due to bacterial isolates' proteolytic activity.

The probiotic bacteria are beneficial for human health. Consequently, probiotic tests were performed for LAB isolate CAL1, which showed the highest proteolytic activity, and 7 non-LAB isolates, which also showed proteolytic activity in SM agar plates, as documented in (Table 2). Survival of bacterial strains in low pH conditions is a more accurate indication of the ability of strains to survive passage through the stomach [38]. So, the probiotic strain *L. plantarum* can be found in various parts of the human gastrointestinal tract, such as the stomach and intestine [39]. The pH of the human stomach fluctuates between 1.5 and 4.5, and the acidity has the most detrimental impact on bacterial growth and viability [40]. *In vitro*, survival tests at 2.0 pH were conducted using human stomach juice simulation, a commonly used method for quickly screening probiotic qualities in bacteria [41–43]. The study reveals that only (BA4, CA3, and CAL1) isolates can survive for 2 hours in simulated gastric juice at pH 2.0, with 71.31%, 63.50%, and 10.36% survival rates, respectively

Bile salt tolerance is a crucial selection factor for probiotic isolates to survive in the small intestine [32]. The resistance of probiotic microorganisms to bile salts is associated with the activity of bile salt hydrolase, which reduces the inhibitory effect of bile by hydrolyzing conjugated bile salts [44,45]. The isolates (BA4, CA1, and CA3) grew with 0.3% bile salt with 63.905%, 11.21%, and 50.37% survival rates, respectively. These results are somewhat consistent with those [41,46], where they observed that *Bacillus* strains could grow in bile salt at 0.3%. Compared to the previous study [47], isolate CAL1 had a 76.59%. According to [48], bacterial strains' intolerance toward bile salts is due to their ability to cause disturbances in cellular homeostasis, causing disintegration of the lipid bilayer and integral proteins of cell membranes, leading to leakage of bacterial content and, eventually, cell death.

Phenol tolerance is required for isolates to survive in gastrointestinal conditions because gut bacteria can deaminate aromatic amino acids received from food proteins, which may generate phenols [49–51]. The results in (Fig. 3) prove that bacterial isolates in the present study can survive in phenol at concentrations between 0.4% and 0.6%. There are several reports of tolerance of LAB and spore-forming strains to phenol, as documented in [18,52–54]. Strains must be resistant to a variety of drugs and must not carry any antibiotic-resistant genes [55,56]. The antibiotic-resistance characteristics of probiotic bacteria are considered helpful for survival in the gastrointestinal system after antibiotic therapy [57,58]. In the current study, all isolates were resistant to penicillin G, oxacillin, erythromycin, and streptomycin.

Depending on the previous examinations, the bacterial isolates (CA3, BA4, and CAL1) were selected to complete the characterization of probiotics. Auto-aggregation allows microorganisms of the same species to create self-forming groups, and this process is commonly connected with microorganisms adhering to the intestinal mucosa [59]. Subsequently, it inhibits the body from eliminating bacterial strains through peristalsis, and the auto-aggregation of bacterial isolates on the intestinal epithelial lining is a desired characteristic [60]. The current results show that after 2 hours of incubation, all isolates showed an excellent aggregation phenotype greater than 90%. Similarly, co-aggregation potential enables probiotic cells to establish a barrier that limits pathogenic colonization by producing an environment with a high concentration of inhibitory chemicals [61]. The presence of beneficial and harmful bacteria in the environment shows the possibility of competition, which might lead to infection removal from the eukaryotic host [62].

**Table 1:** Differentiate between LAB and non-LAB isolates by their morphological shape, catalase test, spore formation, and lactic acid production on a chalk agar plate

| Isolation source | Region | Morphological shape | | Catalase test | | Spore formation | | Lactic acid production | | Examination of isolates' results | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Bacilli | Cocci | +ve | -ve | +ve | -ve | +ve | -ve | LAB | Non-LAB |
| Buffalo Milk | Mansoura A | 1 | 4 | 2 | 3 | 1 | 4* | 3 | 2 | 3 | 2* |
| | Mansoura B | 5 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| | Total | 6 | 7 | 6 | 7 | 5 | 8 | 7 | 6 | 7 | 6 |
| Sheep milk | Mansoura A | 3 | 2 | 3 | 2 | 3 | 2 | 2 | 3 | 2 | 3 |
| | Mansoura B | 4 | 3 | 4 | 3 | 4 | 3 | 3 | 4 | 3 | 4 |
| | Total | 7 | 5 | 7 | 5 | 7 | 5 | 5 | 7 | 5 | 7 |
| Cow Milk | Assiut A | 7 | 2 | 7 | 2 | 7 | 2 | 2 | 7 | 2 | 7 |
| | Assiut B | 9 | 3 | 7 | 5* | 8 | 4 | 4 | 8 | 4 | 8* |
| | Total | 16 | 5 | 14 | 7 | 15 | 6 | 6 | 15 | 6 | 15 |
| Camel milk | Aswan A | 1 | 2 | 1 | 2 | 1 | 2 | 2 | 1 | 2 | 1 |
| | Aswan B | 0 | 5 | 2 | 3 | 0 | 5 | 3 | 2 | 3 | 2 |
| | Total | 1 | 7 | 3 | 5 | 1 | 7 | 5 | 3 | 5 | 3 |
| Total of all isolates | | 30 | 24 | 30 | 24 | 28 | 26 | 23 | 31 | 23 | 31 |

Note: (*) Refers to non-LAB isolates that were negative for spore-forming and catalase tests.

**Table 2.** Proteolytic activity of bacterial isolates on skim milk agar medium

| Isolation source | No. of Isolate | Code of Isolate | Proteolytic Result |
|---|---|---|---|
| Buffalo Milk | 3 | BAL 1 | $^{o}$+ |
| | 5 | BAL 3 | $^{o}$+ |
| | 6 | BAL 4 | $^{o}$+ |
| | 8 | BAL 6 | $^{o}$+ |
| | 9 | BAL 7 | $^{o}$+ |
| | 10 | BA 4 | +$^{c}$ |
| Sheep Milk | 14 | SP 1 | +++$^{c}$ |
| | 18 | SPL 2 | $^{o}$++ |
| | 19 | SPL 3 | $^{o}$++ |
| | 20 | SPL 4 | $^{o}$+ |
| | 21 | SPL 5 | ++$^{o}$ |
| Cow Milk | 26 | CA 1 | +$^{c}$ |
| | 27 | CA 2 | +$^{c}$ |
| | 28 | CA 3 | ++$^{c}$ |
| | 34 | CAL 2 | ++$^{o}$ |
| | 35 | CA 8 | +$^{c}$ |
| | 37 | CA 10 | +++$^{c}$ |
| Camel Milk | 48 | CAL 1 | $^{o}$+++ |
| | 52 | CAL 3 | $^{o}$+ |
| | 53 | CAL 4 | $^{o}$+ |
| | 54 | CAL 5 | $^{o}$+ |

+, Small zone; ++, Medium zone; +++, Large zone. **(+$^{O}$)** Opaque zone on skim milk agar plate; **(+$^{C}$)** Clear zone on skim milk agar plate

**Table 3.** The survival rate of bacterial isolates at an acidic pH value of 2.0

| Isolates | Survival Rate (%) | | |
|---|---|---|---|
| | 1h | 2h | 3h |
| BA4 | 88.52±2.46 $^{Aa}$ | 71.31±2.46 $^{Ab}$ | 63.11±1.88 $^{Ac}$ |
| SP1 | 0±0 $^{Da}$ | 0±0 $^{Da}$ | 0±0 $^{Ca}$ |
| CA1 | 29.44±10.2 $^{Ba}$ | 0±0 $^{Db}$ | 0±0 $^{Cb}$ |
| CA2 | 14.22±3.72 $^{Ca}$ | 0±0 $^{Db}$ | 0±0 $^{Cb}$ |
| CA3 | 81.03±2.57 $^{Aa}$ | 63.50±1.09 $^{Bb}$ | 54.26±1.25 $^{Bc}$ |
| CA8 | 0±0 $^{Da}$ | 0±0 $^{Da}$ | 0±0 $^{Ca}$ |
| CA10 | 0±0 $^{Da}$ | 0±0 $^{Da}$ | 0±0 $^{Ca}$ |
| CAL1 | 35.74±4.67 $^{Ba}$ | 10.36±3.24 $^{Cb}$ | 0±0 $^{Cc}$ |

**Table 4.** The survival rate of bacterial isolates at 0.3% bile salts

| Isolates | Survival Rate (%) | | |
|---|---|---|---|
| | 1h | 2h | 3h |
| BA4 | 92.71±1.51 $^{Aa}$ | 84.43±0.99 $^{Ab}$ | 63.90±4.02 $^{Bc}$ |
| SP1 | 0±0 $^{Ea}$ | 0±0 $^{Da}$ | 0±0 $^{Ea}$ |
| CA1 | 45.79±4.28 $^{Ca}$ | 23.36±4.28 $^{Cb}$ | 11.21±4.86 $^{Dc}$ |
| CA2 | 13.66±2.85 $^{Da}$ | 0±0 $^{Db}$ | 0±0 $^{Eb}$ |
| CA3 | 71.21±3.47 $^{Ba}$ | 63.63±3.93 $^{Bb}$ | 50.37±4.59 $^{Cc}$ |
| CA8 | 11.45±4.77 $^{Da}$ | 0±0 $^{Db}$ | 0±0 $^{Eb}$ |
| CA10 | 14.27±3.68 $^{Da}$ | 0±0 $^{Db}$ | 0±0 $^{Eb}$ |
| CAL1 | 96.80±1.07 $^{Aa}$ | 81.91±2.13 $^{Ab}$ | 76.59±3.19 $^{Ac}$ |

The values represent the mean ± SD of n = 3.
$^{(a-c)}$ Different lowercase superscripts for the same row's mean values indicate a significant difference ($p < 0.05$).
$^{(A-E)}$ Different uppercase superscripts for the same column's mean values indicate a significant difference ($p < 0.05$).

**Table 5.** Antibiotic susceptibility profile of the bacterial isolates

| Antibiotic | Concentration (µg/disc) | Bacterial Isolates | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | BA4 | SP1 | CA1 | CA2 | CA3 | CA8 | CA10 | CAL1 |
| | | Diameter of inhibition zone (mm) | | | | | | | |
| **Penicillin G** | **10** | $10^{R}$ | $8^{R}$ | $8^{R}$ | $0^{R}$ | $9^{R}$ | $8^{R}$ | $8^{R}$ | $13^{R}$ |
| **Cefotaxime** | **30** | $9^{R}$ | $9^{R}$ | $10^{R}$ | $10^{R}$ | $0^{R}$ | $0^{R}$ | $9^{R}$ | $15^{R}$ |
| **Oxacillin** | **1** | $0^{R}$ | $0^{R}$ | $0^{R}$ | $0^{R}$ | $0^{R}$ | $0^{R}$ | $0^{R}$ | $0^{R}$ |
| **Vancomycin** | **30** | $21^{S}$ | $25^{S}$ | $21^{S}$ | $16^{I}$ | $23^{S}$ | $21^{S}$ | $21^{S}$ | $24^{S}$ |
| **Nitrofurantoin** | **300** | $16^{I}$ | $17^{I}$ | $16^{I}$ | $15^{R}$ | $16^{I}$ | $16^{I}$ | $19^{I}$ | $17^{I}$ |
| **Erythromycin** | **15** | $11^{R}$ | $0^{R}$ | $10^{R}$ | $12^{R}$ | $10^{R}$ | $10^{R}$ | $9^{R}$ | $12^{R}$ |
| **Streptomycin** | **10** | $9^{R}$ | $8^{R}$ | $10^{R}$ | $0^{R}$ | $8^{R}$ | $10^{R}$ | $14^{R}$ | $10^{R}$ |

According to the values given by [19], the values are Sensitive (S) (≥ 21 mm), Intermediate (I) (16–20 mm), and Resistant (R) (≤ 15 mm).

**Table 6**. Auto-aggregation, co-aggregation with *Pseudomonas aeruginosa* (ATCC9027), and hydrophobicity of bacterial isolates (CA3, BA4, and CAL1)

| Isolates | Auto-aggregation (%) | Co-aggregation (%) | Hydrophobicity (%) |
|---|---|---|---|
| CA3 | 91.24±0.55 [c] | 20.14±0.52 [a] | 89.77±1.35 [b] |
| BA4 | 96.22±0.46 [a] | 13.54±1.02 [b] | 97.48±0.66 [a] |
| CAL1 | 93.15±0.16 [b] | 8.96±0.06 [c] | 97.60±0.02 [a] |

The values represent the mean ± SD of n = 3. [(a-c)] Different lowercase superscripts for the same column's mean values indicate a significant difference ($p < 0.05$).

**Table 7**. Morphological shapes, biochemical tests, and carbohydrate fermentation of the selected bacterial isolates

| Morphological shapes | | Isolates | | |
|---|---|---|---|---|
| | | **BA4** | **CA3** | **CAL1** |
| **The shape of the cell under a light microscope** | | Diplobacilli | Diplobacilli | Diplococci |
| **Size of a cell under SEM** | - Length: | 2.1µm | 2.6µm | 0.5µm |
| | - Width: | 0.75µm | 1.07µm | 0.5µm |
| **Gram staining** | | + | + | + |
| **Biochemical Tests** | | | | |
| Indole test | | - | - | - |
| M.R test | | + | - | + |
| V.P test | | + | + | - |
| Citrate test | | - | - | - |
| Catalase test | | + | + | - |
| Motility test | | + | + | - |
| $H_2S$ Production test | | - | - | - |
| Gas production from glucose | | - | - | - |
| Type of fermentation | | Homo | Homo | Homo |
| **Carbohydrate Fermentation** | | | | |
| Glucose | | + | + | + |
| Lactose | | - | - | + |
| Sucrose | | + | + | + |
| Fructose | | + | + | + |
| Starch | | + | - | + |
| Sorbitol | | + | - | + |
| Mannitol | | + | - | + |

+, Positive result; −, Negative result.

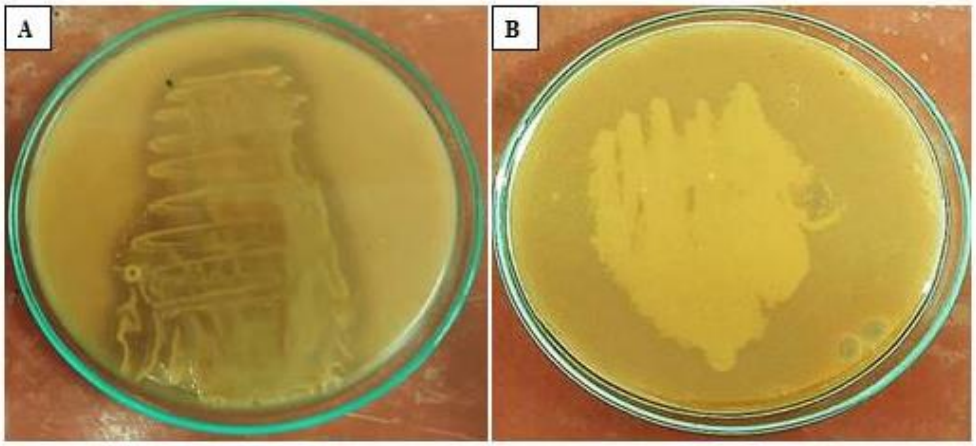


**Figure 1.** A clear zone on MRS agar medium with 0.5% CaCO3 added to it in the plate (A) illustrates the production of lactic acid. Plate (B), which shows no change, means that no lactic acid is being produced.


**Figure 2.** Screening for proteolytic activity on the skim milk agar medium, showing an opaque zone and clear zone on plate (A) and (C), respectively, as positive results, while plate (B) showed a negative result for proteolysis.

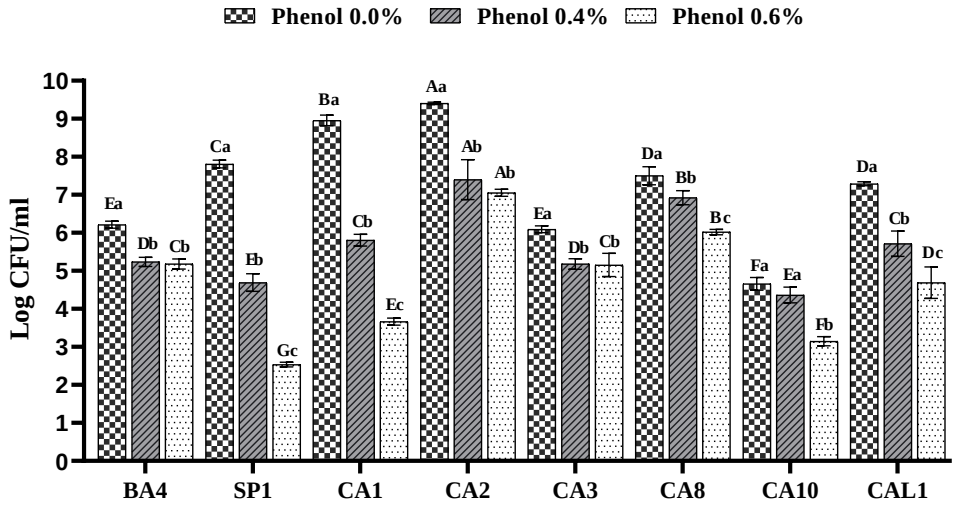


**Figure 3:** Log CFU/ml of bacterial isolates at 0.4% and 0.6% phenol. The data given are the mean and the standard deviation of n =3. Means followed by different (a-c) lowercase letters between the different concentrations of phenol at the same isolate and different (A-E) capital letters between the same concentrations of phenol at different isolates are significantly different ($p < 0.05$).

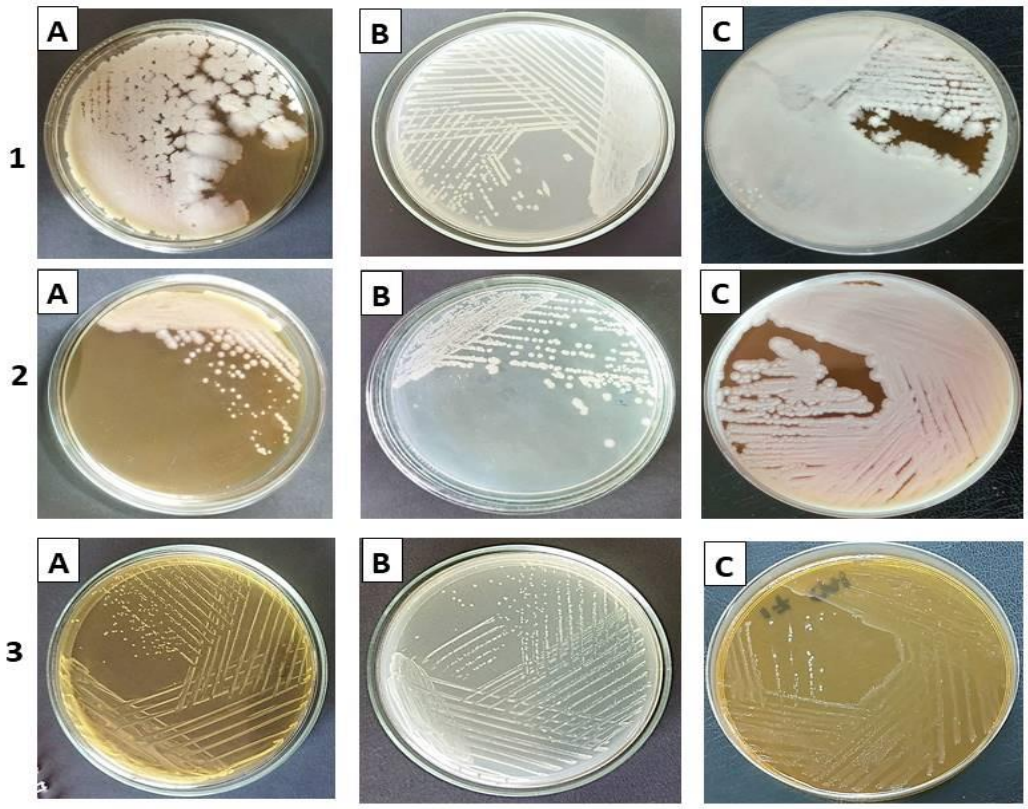


**Figure 4.** Growth of bacterial isolates on different media and colony morphology following incubation for 24 h: (1). *Bacillus* BA4, (2). *Bacillus* CA3, and (3). *Lactococcus* CAL1 on (A) MRS agar, (B) Nutrient agar, and (C) M17 agar.

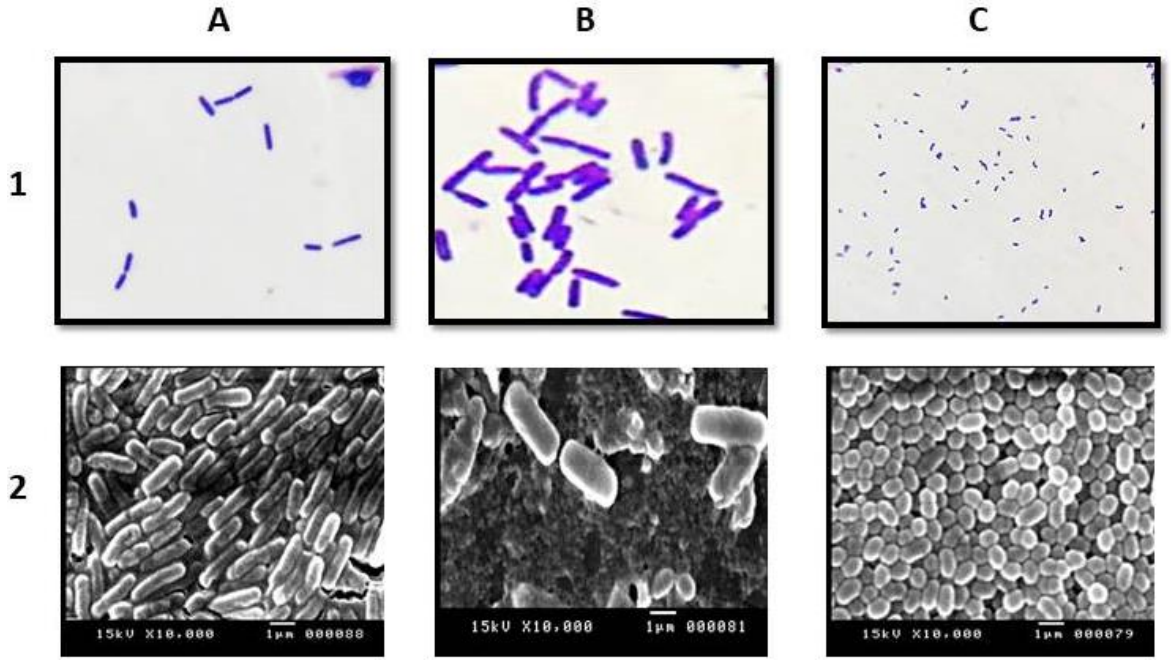


**Figure 5. (1)** Gram staining ; (2) SEM of the bacterial isolates: (A) *Bacillus* BA4, (B) *Bacillus* CA3, and (C) *Lactococcus* CAL1

The present study showed significant changes in the co-aggregation between bacterial isolates and *Pseudomonas aeruginosa* (ATCC9027), which is lower than 30%. According to the study of [63], the current study revealed varying levels of co-aggregation across the various species, implying that these abilities are strain-dependent. The difference in co-aggregation between bacterial isolates and pathogenic bacteria is most likely due to physical reasons (e.g., electrical charge variations) [62].

Probiotics' capacity to adhere to epithelial cells correlates with their hydrophobicity, a crucial characteristic of their cell surface features [64]. In the present study, all isolates showed excellent adhesion toward xylene, greater than 85%. The large percentage of bacterial adhesion to xylene demonstrates the cell surface's hydrophobic character [63].

Morphological and biochemical characterizations were performed for three bacterial isolates (BA4, CA3, and CAL1), which were selected based on their probiotic properties. In different agar media (MRS, Nutrient, and M17), the colonies of isolate BA4 were irregularly cream-colored, fuzzy white, and white with jagged edges. In contrast, the colonies of isolate CA3 were circular (yellow, white, and off-white), and the colonies of isolate CAL1 were white circular colonies in all media types. The identical form of bacterial isolate BA4 was seen on the MRS agar plate in the investigation [65]. Similar to the findings of [66], the biochemical examination revealed that bacterial isolates may ferment glucose without producing gas.

## 5. Conclusions

Milk contains probiotics from lactic acid bacteria (LAB) and non-LAB strains. Most LABs have proteolytic activity, producing opaque regions on SM agar surfaces, while non-LABs produce clear zones. In the future, we will focus on the production, purification, and immobilization of protease enzymes from Bacilli BA4 and CA3 strains and *Lactococcus* CAL1 strain, which can be used in various industrial processes.

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