Isolation, Characterization and Safety Assessment of Probiotic Lactic Acid Bacteria from Metata Ayib (Traditional Spiced Cottage Cheese)

Mulugojjam Adugna (mgojjama@gmail.com)
University of Gondar/Debre Tabor University
https://orcid.org/0000-0002-9862-5943

Berhanu Andualem

Research Article

Keywords: Antagonestic activity, Antibiotics susceptibility, Lactic acid bacteria, Lactobacillus species, Metata ayib, Probiotics

Posted Date: February 7th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2556671/v1

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Abstract

(1) Background: Consumption of fermented foods and beverages has health benefits; fermented milk products in particular are thought to contain probiotics. The purpose of this study was to characterize probiotic property of lactic acid bacteria isolates from metata ayib.

(2) Methods: LAB isolates from metata ayib were isolated and purified for characterization of its probiotic activity.

(3) Results: A total of 80 LAB isolates were isolated and purified. The majority of isolates, 67 (83.75%), had bacilli shapes, and 59 (73.75%) were homofermentative. All 80 (100%) isolates were tolerated at pH 3.0 treated for three hours; but only 6 (7.5%) isolates were survived at pH 2.0. After 0.3 and 0.5% bile salt treatment for 24 hours, all 6 (7.5%) LAB isolates survived above 92.09±0.18 and 83.39±0.21%, respectively. All the 6 isolates were identified as *Lactobacillus* species; and all were hydrophobicity positive. *K. pneumoniae* was resistant but *S. aureus* was sensitive to all of six identified LAB isolates cell free supernatant. All 6 isolates were ciprofloxacin, chloramphenicol, cotrimoxazole and erythromycin-sensitive. Vancomycin resistance was present in four isolates. All these six *Lactobacilli* species were non-hemolytic, non-biogenic amine producing, and gelatin hydrolysis negative.

(4) Conclusion: Thus, *Lactobacilli* species isolated from metata ayib may be used as the suitable candidates of probiotics, and bio-therapeutics agents against bacterial infection to humans.

1. Introduction

Live microorganisms that, when given to a host in sufficient quantities, have a positive impact on their host are referred to as probiotics [18]. A specific food item must be traditionally consumed by humans, be regarded as safe for humans consumption, contain strains that have been described using certain methods, be viable at the time of ingestion, and contain at least $10^9$ CFU/ml (g) live cells per daily portion to qualify as a probiotic [18, 24].

Consuming fermented foods and beverages is well recognized to provide health advantages; fermented milk products in particular are anticipated to include probiotics [3], although not all fermented foods may contain probiotics. All of the isolates were not probiotic when [9] investigated the probiotic potential of yeast strains detected in kefir samples. A unique fermented food's probiotic effects must be proven [24], and the isolate's probiotic qualities must be strain-specific [10]. The typical probiotic bacteria are lactic acid bacteria (LAB), according to numerous researchers. Probiotic isolates have obvious health advantages; in addition they also have a variety of biotechnological uses because of the metabolites they produce. Additionally, according to [25], probiotics, prebiotics, and their synergy may eventually replace antibiotics.

Similar to other nations, Ethiopia has a variety of traditionally produced fermented foods and beverages made from diverse milk, meat and plant-based ingredients. One of the traditional fermented dairy foods
that is prepared and consumed in the Amhara region of Northwestern Ethiopia is metata ayib. According to [2, 17, and 20], it has a shelf life of up to one year in semi-solid form and more than ten years when stored in dry form. Traditionally, it has been used to cure a variety of illnesses. Even though metata ayib possesses similar benefits, there is no scientific evidence to support its probiotic potential. Therefore, the objective of this research was to characterize probiotic property of lactic acid bacteria isolates from metata ayib.

2. Materials And Methods

2.1 Isolation of LAB

Isolation of LAB isolates from metata ayib was carried out by using standard procedure [4]. Metata ayib was prepared based on [2, 17] and information acquired from producers, in the microbiology laboratory of the University of Gondar and at its 20th fermentation day sample was prepared and inoculated on MRS agar and incubated at 37°C for 24–48 h. Morphologically different colonies were taken and streaked on MRS agar and incubated under anaerobic conditions at 37°C for 24 h. The colonies were further sub-cultured onto MRS agar plates. The viable cultures were stored in MRS slants at 4°C.

2.2 Preliminary characterization of LAB isolates

Eighty LAB colonies were isolated based on their morphology and biochemical characteristics that included Gram's reaction, catalase test, spore staining, and citrate utilization assay. Colonies with characteristics of Gram positive, none spore forming, catalase and citrate utilization negative were considered as LAB.

2.3 Invitro characterization of probiotic properties

2.3.1 Tolerance to low pH (acid tolerance)

The isolates were cultured individually in 10 ml of anaerobic MRS broth at 37°C overnight. Nine ml of MRS broth were used to dilute one ml of isolates cell suspension, which contained 10⁷ CFU/ml of each overnight-grown culture. The culture was centrifuged for 1–2 minutes at 10,000 rpm. In order to imitate the gastrointestinal environment, the pellet was cleaned twice in sterile phosphate-saline buffer (pH 7.2) and then re-suspended in 10 ml of sterile MRS broth (to give 10⁶ cells/ml). The pH of the broth was then changed to 3.0, 2.5 and 2.0 using 1N HCl. The test tubes were incubated at 37°C for 3 h. Following the proper amount of incubation, 1 ml of the acid challenge suspension was withdrawn and serially diluted in 9 ml of sterile phosphate buffer solution. On MRS agar medium, a 100µl aliquot of serially diluted culture was spread plated. The inoculated plates were placed in anaerobic jars and incubated at 37°C for 24 to 48 hours. The ratio of the number of LAB colonies enumerated on MRS agar following an acid challenge (N1) divided by the initial bacterial number concentration at time zero (N0) was used to compute the percentage of acid challenge survivors [34].
2.3.2 Tolerance to bile salts

Separate isolates of LAB isolates that exhibit high levels of acid tolerance to pH values of 2.0 were cultured overnight in MRS broth at 37°C under anaerobic conditions to test their tolerance to bile salts [21]. Then, each culture was centrifuged at 5000 rpm for 10 min with an initial concentration of $10^6$ CFU/ml. Two sterile phosphate-saline buffer washes were performed on the pellet (PBS at pH 7.2). After that, the cell pellet was once again re-suspended in sterile MRS broth that had 0.3 and 0.5% (w/v) bile salt added to it. For the purpose of assessing the ability of the cells to survive, samples were collected 24 h after the start of the incubation [21]. A parallel positive control of MRS broth without bile salts was established and inoculated with each distinct culture. Following the proper incubation, the amount of bile salt tolerance was assessed by diluting 1 ml of each distinct culture individually in sterile 9 ml phosphate buffer (0.1 M, pH 7.2). On MRS agar medium, a 100 µl aliquot of the culture and its 10-fold serial dilutions were plated. Using an anaerobic jar, inoculated plates were incubated at 37°C for 24 to 48 h in an anaerobic environment. The ratio of the number of LAB colonies counted on MRS agar following bile salt challenge (N1) divided by the initial bacterial number concentration at time zero (N0) and multiplied by 100 was used to compute the percentage of survivors [21].

\[
\text{Survival rate} \, (\%) = \frac{\log \text{CFU} \, N_1 \times 100}{\log \text{CFU} \, N_0}
\]

2.3.3 Cell surface hydrophobicity

The cell surface hydrophobicity of LAB isolates linked with metata ayib that exhibit high levels of acid and bile salt tolerance was examined. It was determined by using qualitative method on a Congo red stain [26]. The bacterial colonies were streaked on MRS agar plates that contained 0.03% (w/v) Congo red, and they were then left to grow anaerobically for 24 h at 37 °C. Consequently, white or translucent colonies were thought to be non-hydrophobic strains, whereas red colonies were thought to be hydrophobic strains.

2.3.4 Antagonestic activity

Hydrophobic LAB isolates associated with metata ayib were cultured separately in test tubes without agitation in 10ml MRS broth. The control was MRS broth that hadn’t been inoculated. Using the well diffusion assay method, the antibacterial activity of centrifuged cell free filtrates of the isolated LAB against standard pathogens *Escherichia coli* ATCC 25922 (*E. coli*), *Klebsiella pneumoniae* ATCC 700603 (*K. pneumoniae*), *Staphylococcus aureus* ATCC 25923 (*S. aureus*) and *Streptococcus pneumoniae* ATCC 49619 (*S. pneumoniae*) were assessed [31]. The pathogenic test bacteria were cultivated in Muller Hinton broth at 37 °C for 24 h while being adjusted to 0.5McFarland. Muller Hinton agar (MHA) was thoroughly dissolved and sterilized before being put into a petri-dish, and after it solidified, 0.1 ml of a fresh culture of pathogenic bacteria were streaked. Then 100 µl of cell-free filtrate was poured into wells that were
mm in diameter. The inoculated plates were then incubated for 24 h at 37°C. The clear zone around the wells was measured to ascertain the antibacterial activity. Using calipers, the inhibition zone's diameter was measured in millimeters, and a clear zone of at least one millimeter was interpreted as positive inhibition [29].

### 2.3.5 Safety assessment of isolates

- **Antibiotic susceptibility tests:** The disc diffusion assay was used to examine the LAB isolates that exhibit high levels of antimicrobial activity against some antibiotics to identify their resistance to antibiotics [16]. Antibiotic discs were used to test the susceptibility of isolates to seven different antibiotics, including bacitracin (10 µg), ciprofloxacin (5µg), chloramphenicol (30µg), Cotrimoxazole (trimethoprim/sulfamethoxazole (1.25/23.75 µg), erythromycin (15µg), Gentamicin (10µg) and vancomycin (30µg). A sterile cotton swab was used to equally spread 100 µl of each prospective LAB's actively developing cultures from mid-log phase cultures onto the surface of MRS agar plates, where they were then left to dry. In order to allow the antibiotics to diffuse, antibiotic discs were placed on the agar surface. The plates were then anaerobically incubated at 37°C for 24 to 48 h. By measuring the width of the inhibition zones surrounding the disks with calipers, each antibiotic's zone of inhibition (measured in millimeters) was classified as susceptible, intermediate or resistant [30].

- **Hemolysis test:** The test isolates were inoculated on 5% sheep blood agar and incubated at 37°C for 48 h, as per [27]. On blood agar, hemolysis could be identified by the presence of β-hemolysis (clear zone around colonies), α-hemolysis (green zone around colonies), or γ-hemolysis (no halo around colonies).

- **Arginine hydrolysis test:** Test tubes containing decarboxylase basal medium and 1 test tube devoid of L-arginine amino acid were each coated with sterile mineral oil. Tubes that contained L-arginine and without L-arginine were inoculated with the test LAB isolate and incubated for 4 days at 35–37°C. Every day, each tube was checked. Because of the decarboxylation of amino acids, alkaline pH becomes red (positive). Acid produced from the fermentation of glucose causes the color yellow (negative). The control tubes that don't contain any amino acids should have yellow color. No growth can be seen in control tubes with red media [19].

- **Gelatin degradation:** inoculums of an 18 to 24 hour old test LAB was stab-inoculated into tubes containing nutrient gelatin. The inoculated tubes and an uninoculated control tube were incubated at 37°C up to a week, and checked every day for gelatin liquefaction. The tubes were immersed in an ice bath for 15 to 30 minutes. Afterwards, tubes were tilted to observe if gelatin has been hydrolyzed. Hydrolyzed gelatin resulted in a liquid medium even after exposure to cold temperature (ice bath), while the uninoculated control medium remained solid [11].

Finally, isolates tentatively identified as potential probiotic LAB was stored at -20°C in MRS broth containing 40% (v/v) glycerol.
2.4 Morphological, biochemical and physiological identification of probiotic LAB isolates

The probiotic LAB isolates were identified with standard procedures according to their morphological, physiological and biochemical characteristics [31, 34 and 29].

2.4.1 Cell morphology

Young pure culture (24 h old) was examined with compound microscope in order to determine shape and cell arrangement after Gram staining procedures followed [31].

2.4.2 Growth at different NaCl concentrations

The tolerance of LAB isolates to various NaCl concentrations was examined. For this purpose, the salt tolerance of the isolates was assessed using 4% and 6% NaCl concentrations. In a similar manner, test tubes containing 5 ml of modified MRS broths with phenol red indicator were made, and each one was inoculated separately with 50 ml of an overnight LAB culture before being incubated for 7 days at 37°C. The change from red to yellow was interpreted as a positive for cell growth [29].

2.4.3 Growth at different temperatures

The growth of LAB isolates at various temperatures was assessed using a 5 ml MRS broth medium containing the phenol red indicator. For each isolate, 100 µl of overnight LAB culture were added to two different tubes. Following inoculation, one test tube was incubated for seven days at 15°C and the other at 45°C. The change of the medium color from red to yellow indicated cell growth [34].

2.4.4 Gas production from glucose

In a modified MRS broth that contained 1% glucose with inverted Durham tubes, CO₂ generation from glucose was determined in order to ascertain if the LAB isolate has homofermentative or heterofermentative ability. The MRS broth (8 ml) was prepared and inoculated separately with 50 µl of overnight culture containing 1% glucose with inverted Durham tubes. The test tubes were incubated for 5 days at 37°C. Gas was detected in Durham tubes, which was interpreted as positive for glucose-induced CO₂ generation [34].

2.4.5 Carbohydrates fermentation

For the carbohydrate fermentation test, 0.1 ml of overnight cultures were inoculated to a test tube containing 5 ml of MRS, the necessary 1% (v/w) amount of sugar (D-arabinose, α-cellulose, fructose, galactose, maltose, manose, TSI and starch), as well as the indicator dye phenol red (0.02 gm/l). The incubation period for each test tube was 24 hours at 37°C. In the fermentation test, a negative control tube without extra sugar was used. It was read as a positive reaction when the broth's color changed to yellow, and it was read as a negative reaction when it stayed red [31].
2.5 Data analysis

Microbial count data for pH and bile tolerance were first transformed to logarithmic values (log10) before statistical analysis. Antagonistic activity, antibiotics susceptibility, percentage of pH and bile tolerated isolates were calculated and analyzed separately by the analysis of variance technique using the one way ANOVA procedure of SPSS software (RRID:SCR_002865). The values was considered to be significantly different at 95% (p < 0.05). Mean separation was carried out using the least significant difference. Hydrophobicity, the morphological and physiological identification of isolates was analyzed with descriptive statistics.

3. Results

3.1 Isolation and preliminary characterization of LAB

Eighty isolates that were Gram positive, non-spore-producing, citrate utilization and catalase negative were collected from the sample of the 20th fermentation day of metata ayib that was manufactured in the laboratory. A total of 67 isolates (83.75%) were bacilli-shaped, although 13 (16.25%) were cocci-shaped. 21 (26.25%) of the total isolates were hetrofermentative, while 59 (73.75%) were homofermentative. For the characterization of their probiotic property, all of these purified isolates were then further grown using the conventional approach on MRS agar.

3.2 Invitro characterization of probiotic properties

3.2.1 Low pH tolerance of isolates

Eighty (100%) isolates were treated for a total of three hours at pH 3.0, where all 80 (100%) of the isolates remained after the initial treatment; following this, the isolates that remained at pH three were treated again for three hours at pH 2.5, where 37 (46.25%) of the isolates remained. Finally, 6 (7.5%) of the isolates that had survived at pH 2.5 remained alive after three hours of treatment at pH 2.0 (Table 1).

3.2.2 Bile salt tolerance of isolates

All the 6 (7.5%) LAB isolates that could survive pH 2.0 were exposed to 0.3 and 0.5% bile salt concentrations overnight, and they all survived above 92.09 ± 0.18 and 83.39 ± 0.21%, respectively (Table 1).

3.2.3 Hydrophobicity of isolates

Six (7.5%) isolates that were resistant to low pH and bile salts underwent a qualitative hydrophobicity test using 0.03% Congo red. All six (100%) LAB isolates, which had shown red color, were deemed to be hydrophobicity positive.
Table 1
Determination of pH (3 h), bile tolerance, and hydrophobicity of LAB isolated in metata ayib

<table>
<thead>
<tr>
<th>LAB</th>
<th>pH tolerance (%)</th>
<th>Bile salt tolerance (%)</th>
<th>Hydrophobicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.0</td>
<td>2.5</td>
<td>3.0</td>
</tr>
<tr>
<td>14</td>
<td>56.7 ± 0.05a</td>
<td>98.61 ± 1.16b</td>
<td>109.86 ± 0.045c</td>
</tr>
<tr>
<td>15</td>
<td>57.49 ± 0.115a</td>
<td>79.43 ± 1.19b</td>
<td>84.43 ± 0.34c</td>
</tr>
<tr>
<td>16</td>
<td>54.71 ± 0.275a</td>
<td>87.46 ± 0.005b</td>
<td>87.99 ± 0.025c</td>
</tr>
<tr>
<td>47</td>
<td>52.4 ± 0.15a</td>
<td>62.36 ± 0.14b</td>
<td>86.79 ± 0.095c</td>
</tr>
<tr>
<td>60</td>
<td>58.08 ± 0.08a</td>
<td>82.65 ± 0.1b</td>
<td>105.11 ± 0.115c</td>
</tr>
<tr>
<td>70</td>
<td>49.73 ± 0.73a</td>
<td>70.71 ± 0.04b</td>
<td>105.2 ± 0.095c</td>
</tr>
</tbody>
</table>

Means with the same superscript letters in a row are not significantly different (P > 0.05).

3.2.4 Detection of antagonistic activity of LAB isolates

The antagonistic activity of each of the six identified LAB isolates cell free supernatant was evaluated against two Gram negative (E. coli, K. pneumoniae) and two Gram positive (S. aureus and S. pneumoniae) pathogenic bacteria (Table 2). K. pneumoniae was resistant to all of the isolates, while S. aureus was sensitive to all LAB isolates.

Table 2
Antagonistic activity of LAB isolates against pathogenic bacteria

<table>
<thead>
<tr>
<th>LAB isolates</th>
<th>Tested pathogenic bacteria with inhibition zone (mm)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>E. coli</td>
</tr>
<tr>
<td>14</td>
<td>0.00 ± 0.00a</td>
</tr>
<tr>
<td>15</td>
<td>12 ± 20b</td>
</tr>
<tr>
<td>16</td>
<td>0.00 ± 0.00cdf</td>
</tr>
<tr>
<td>47</td>
<td>0.00 ± 0.00dacf</td>
</tr>
<tr>
<td>60</td>
<td>4.67 ± 1.15e</td>
</tr>
<tr>
<td>70</td>
<td>0.00 ± 0.00facd</td>
</tr>
</tbody>
</table>
Means with the same superscript letters in a column are not significantly different (P > 0.05).

3.2.5 Safety assessment

Detection of antibiotic susceptibility of LAB strains: All isolates (100%) were ciprofloxacin, chloramphenicol, cotrimoxazole and erythromycin-sensitive (Table 3). Vancomycin resistance was present in isolates 15, 16, 60, and 70, totaling four (66.67%).

<table>
<thead>
<tr>
<th>Antibiotics discs</th>
<th>Concentration (µg/disc)</th>
<th>LAB isolates</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>14 15 16 47 60 70</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>5</td>
<td>S S S S S S S</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10</td>
<td>S R S S S R S</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>10</td>
<td>S R S S S R S</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>15</td>
<td>S S S S S S S</td>
</tr>
<tr>
<td>Cotrimoxazole/trimethoprim/sulfamethoxazole</td>
<td>1.25/23.75</td>
<td>S S S S S S</td>
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<tr>
<td>Chloramphenicol</td>
<td>30</td>
<td>S S S S S S S</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>30</td>
<td>S R R S R R R</td>
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</table>

R, resistant; S, sensitive; the breakpoints for the antibiotic sensitivity/resistant in mm zone of inhibition was done according to [30].

Hemolytic activity

Any of the tested isolates did not show hemolysis (γ-hemolysis (no halo around colonies)).

Decarboxylase activity

The entire tested six LAB isolates were found to be negative for biogenic amine production, as none of them changed the color of decarboxylase broth to red.

Gelatinase activity

All the tested six LAB isolates were found to be negative for gelatin hydrolysis test. Therefore, the test tube that contained nutrient gelatin after inoculated with the test LAB and incubated remained solid.

3.3 Morphological and physiological properties of LAB isolated from metata ayib
All the 6 selected strains were rod shaped, homofermentative, able to grow at 15 and 45 °C, grow at 4 and 6% salt. All tested LAB isolates could ferment sugars including triple sugar iron agar, fructose, starch, lactose, galactose, mannose and maltose but all were negative for D-arabinose and α-cellulose and was identified as Lactobacillus species.

Table 4
Morphological and physiological properties of LAB isolates from metata ayib

<table>
<thead>
<tr>
<th>LAB isolate</th>
<th>Shape</th>
<th>Gg</th>
<th>Carbohydrates fermented</th>
<th>To (°C)</th>
<th>Salt (%)</th>
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</table>

Gg = Gas production from glucose; TSIA = triple sugar iron agar; D-A = D-arabinose; α-C = α-Cellulose; Sta = Starch; Gal = Galactose; Fru = Fructose; Mal = Maltose; Man = Mannose; HF = homo-fermentation; R = Rod shape

4. Discussion

A total of 80 LAB colonies were isolated from metata ayib, and 67 isolates (83.75%) were Gram positive, bacillus shaped, catalase and oxidase negative and non-spore forming bacteria. All 80 LAB isolates were characterized for its probiotic characteristics and six LAB isolates showed probiotic potential. The morphological and biochemical tests revealed that all the six isolates were Lactobacillus species. This result is similar with the result of different researchers who reported probiotic Lactobacillus species from ergo (traditionally fermented sour milk) sample [7]; from curd sample [23]; from Anbaris-traditional Lebanese fermented dairy product [1]; from traditional Ethiopian fermented teff injera dough, ergo and kocho products [29]. Lactobacillus species are the principal members of the intestinal microbiota of vertebrates, including humans, and have generally recognized as safe (GRAS) status. It usually dominates the microbial succession during milk fermentation with rise in acidity [7], and involves in fermentation of various carbon sources and persists in low pH of food environment. Thereby, improves the food quality and safety, the health of the consumers, and uses for the production of various important metabolites. Such microorganisms can be used safely as probiotics and for the production of functional foods.
One requirement for identifying probiotics was the characterization of microorganisms for low pH tolerance, to simulate the human gastric environment (pH 1.5–3.5), and their persistence for three hours. In this study, all 80 (100%) LAB isolates survived at pH 3.0 after treated for three hours, 37 (46.25%) of LAB isolates persisted at pH 2.5 and 6 (7.5%) of LAB isolates persisted at pH 2.0. This result was somewhat higher than that reported by [4], who stated that 44 isolates (44%) were 100% tolerant at pH 3.0 treated for 3 h. 7 isolates (7%) were 100% tolerant at pH 2.5 treated for 3 h, and 16 isolates (16%) were 1–10% tolerant at pH 2.0 treated for 3h; from this 3 Lactobacillus isolates out of 56 survived with 1–10% at pH 2.0 treated for 3 hours. According to the report of [27], none of the tested LAB isolates were survived at pH 2.0. [35] also reported percentage survival of probiotic LAB at pH 3.0, 2.5 and 2.0 to be 90.28 ± 2.03 to 97.11 ± 2.17, 77.98 ± 1.19 to 90.13 ± 1.10 and 57.36 ± 1.63 to 60.15 ± 0.74, respectively. In this work, the six Lactobacillus species isolates had showed persistence at pH 3.0, 2.5 and 2.0 with the range of 84.43 ± 0.26 to 109.86 ± 0.045, 62.36 ± 0.14 to 98.61 ± 1.16, and 49.73 ± 0.73 to 58.08 ± 0.08, respectively. Among the tested 6 LAB isolates, isolate number 60 (survival rate 58.08%±0.08) at pH 2.0; 14 (survival rate 98.61%±1.16) and (survival rate 109.86%±0.045) at pH 2.5 and 3.0, respectively were observed to be the highest. Lactobacillus species are in general, aciduric or acidophilic, and they can tolerate high acidity through a variety of mechanisms, including H+ pumping to an acidic environment, acid end-product efflux, decreased proton permeability to maintain a low intracellular concentration of protons, synthesis of alkali products to neutralize acid produced during extracellular metabolism, repair macromolecules, form biofilms, and preadaptation and cross protection [37].

Bile salt tolerance test is one of the criteria for choosing probiotic, and all the six Lactobacillus species in our study treated with 0.3 and 0.5% bile acid for 24 hours had shown tolerance of 92.09 ± 0.83 to 94.12 ± 0.08% and 87.25 ± 0.09 to 93.20 ± 0.20%, respectively. Similarly [35] reported 91.37 ± 2.66 to 97.22 ± 0.35% tolerance of 0.3% bile salt. Bile salt functions as a biological detergent that emulsifies and solubilizes lipids, playing a crucial role in fat digestion. Bile’s ability to serve as a detergent also confers powerful antibacterial activity, mostly by dissolving bacterial membranes [6].

All the six isolated Lactobacillus species showed positive hydrophobicity. The cell surface hydrophobicity experiment helps to study the colonization and adhesion of probiotic bacteria to epithelial cells in the gastrointestinal tract, which lead to the prevention of colonization by pathogens through their interaction. This result is agreed with the results of [26], who noted that all 12 isolates tested positive for Congo red. [35] also reported that more than 75% hydrophobicity of all eight tested LAB isolates.

Antimicrobial activity of probiotic bacteria is functional property that helps for the exclusion of pathogenic bacteria in the gastro-intestinal tract of its host. Cell free supernatant of the 6 isolated Lactobacillus species was tested against S. aureus, S. pneumoniae, E. coli and K. pneumoniae. All the six (100%) isolated Lactobacillus species cell free supernatant was effective against S. aureus but on the contrary the result of [35], showed that all of the LAB isolates did not show antagonistic activity against S. aureus. Although isolates number 14, 47 and 70 were efficient only against S. aureus, isolates number 15 and 60 were most effective against 3 (75%) pathogens. [29], reported that all four Lactobacillus species showed antagonistic activity against both E. coli and S. aureus with the range of 17.33 ± 0.58 to
20.67 ± 0.58 and 17.00 ± 1.00 to 21.00 ± 1.00, respectively; but in contrary, in this work the highest antagonistic activity of *Lactobacillus* species against *E. coli* and *S. aureus* was 12 ± 20 mm and 15.33 ± 1.15 mm, respectively; in addition in this work only 2 (33.3%) *Lactobacillus* species showed antagonistic activity against *E. coli* and all the six *Lactobacillus* species did not show antagonistic activity against *K. pneumoniae* and this result was in line with the result of [15], who reported *K. pneumoniae* ATCC700603 was resistant, but on the contrary [15], also reported as *K. pneumoniae* ATCC 35594 was responsive in different degree to all 5 LAB isolated from fermented flour of finger millet. *S. pneumoniae* was responsive for 3 (50%) isolates with the range of 10.67 ± 1.15 to 16.00, but [8], reported that all 8 of *Lactobacillus* isolates were effective against *S. pneumoniae* with the range of 10 to 22 mm. In this study the effect of cell free supernatant of *Lactobacillus* species were more effective on Gram positive pathogenic bacteria than Gram negative pathogens, it may be because of the antibacterial activity of LAB bacteriocin is more effective against more closely related related bacteria.

Susceptibility of the six isolated *Lactobacillus* species was tested using antibiotics disks with different action site: (i) inhibitors of cell wall synthesis (bacitracin and vancomycin); (ii) inhibitors of protein synthesis (gentamicin, chloramphenicol, and erythromycin) and (iii) inhibitors of nucleic acid synthesis (ciprofloxacine and cotrimoxazole) [33]. All isolates were sensitive to erythromycin, ciprofloxacine, cotrimoxazole and chloramphenicol. Thus, the absence of any strain resistant to these antibiotics was an indication that these isolates didn't possessed or acquired the resistance gene so far. However, susceptibility may be of a disadvantage, if the host takes orally administered antibiotics which may eventually eliminate established probiotic LAB, but report of a joint FAO/WHO stated that “bacteria, which contain transmissible drug resistance genes, should not be used in foods”. This result is consistent with that reported by [7], who stated as all strains of LAB isolated from ergo were sensitive to erythromycin; and also [22] reported that 33 *Lactobacillus* species isolated from fermented milk in various parts of China were shown to be erythromycin and gentamicin susceptible, but all strains were vancomycin resistant. In this research, two isolates (or 33.33%) were gentamicin susceptible; and, only four (66.67%) of the tested *Lactobacillus* species were found to be vancomycin resistant and it may be due to inherent resistance of *Lactobacillus* strains to glycopeptides, such as vancomycin [36]. [12] discussed that resistance to aminoglycosides (streptomycin and gentamycin) has been described as one of the best-characterized intrinsic resistance mechanisms in *Lactobacillus* species. The transferability propensity of resistant genes in probiotic *Lactobacillus* species may pose a threat to food safety. In order to determine if the resistance genes are inherited or acquired, further research must be done.

In this study, all the six *Lactobacillus* species tested were negative for arginine decarboxylase but [29], reported that three out of four probiotics isolates were arginine hydrolysis positive. [13] also reported that a strain of *Lactobacillus rossiae* isolated from sourdough manufactures putrescine from arginine. Amino acid decarboxylation is frequently connected to food fermentations and may lead to the formation of biogenic amines that are toxic to humans [5]. However, it aids the probiotic LAB in producing alkaline compounds (ammonia) to neutralize acid in an acidic environment [37].
None of the tested *Lactobacillus* species had showed hemolytic activity and this result was similar with [27]; [23] also reported that all the four *Lactobacilli* species (*L. animalis* LMEM6, *L. plantarum* LMEM7, *L. acidophilus* LMEM8 and *L. rhamnosus* LMEM9) tested were non-hemolytic.

5. Conclusions

The objective of the current work was to isolate and characterize probiotic bacteria from metata ayib. The results demonstrates that the metata ayib-derived *Lactobacillus* species displayed tolerance to adverse stress conditions, including low pH and the presence of bile salts, as well as inhibition of pathogenic bacteria and adhesion in the gastrointestinal tract. The results of this study have also given important details about the safety of in-vitro characteristics of *Lactobacillus* species, and the isolates were non-hemolytic, all of them displayed varying degrees of antibiotic sensitivity, negative for L-arginine decarboxylase, and non-gelatin solubilizing. This study helps to identify possible probiotic candidates that can be used for more research and development as potential probiotics in foods and complementary and alternative therapies. However, whether the resistance genes are acquired or inherited, the probiotic *Lactobacillus* species' capacity for gene transferability must be defined.

Declarations

**Supplementary Materials:** Not applicable.

**Author Contributions:** Conceptualization, M.A. and B.A.; methodology, M.A.; software, M.A.; validation, M.A. and B.A.; formal analysis, M.A.; investigation, M.A.; resources, M.A.; data curation, M.A.; writing—original draft preparation, M.A.; writing—review and editing, M.A. and B.A.; visualization, M.A.; supervision, B.A.; project administration, M.A.; funding acquisition, M.A.. All authors have read and agreed to the published version of the manuscript.

**Funding:** “This research received no external funding”.

**Data Availability Statement** https://doi.org/10.6084/m9.figshare.21815832

**Acknowledgments:** The authors would like to acknowledge Ethiopian ministry of Education, University of Gondar and Debre Tabor University.

**Conflicts of Interest:** “The authors declare no conflict of interest.”

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