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Evaluation of Lactic Acid Bacteria for Potential Probiotic: Biochemical characterization, Resistance to Antibiotics, Bile Salts, and Low pH Conditions

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Abstract

This study aimed to evaluate the enzymatic characteristics, acid and bile salt tolerance, and antibiotic resistance profiles of lactic acid bacteria (LAB) to identify potential probiotic candidates and enzyme producers. Several LAB isolates exhibited diverse enzymatic activities, including L-aspartase, βglucosidase, and esculin hydrolase, which are associated with amino acid metabolism, glycoside hydrolysis, and improved nutrient bioavailability. Isolates P1a and P1e demonstrated the highest enzymatic potential. Tolerance testing revealed that isolates P5c, P5a, and P5d exhibited resistance to both 0.3% bile salts (≥70%) and acidic conditions at pH 2.5 (≥50%), indicating their ability to survive gastrointestinal conditions. Antibiotic susceptibility testing revealed variable resistance patterns; notably, isolate P5d exhibited full susceptibility, highlighting differences in safety profiles. The integration of enzymatic, stress tolerance, and resistance data allowed the selection of promising candidates such as P5a and P5d, which combine beneficial enzyme activity, gastrointestinal survivability, and minimal antibiotic resistance. These findings suggest potential applications in the food industry. However, the study is limited by the lack of molecular identification and in vivo validation. Future research should incorporate genetic analysis and functional testing in biological models to confirm safety and efficacy. Overall, this study contributes to the development of safe and functional LAB strains for industrial applications.

Keywords: Lactobacillus; Probiotics; Enzymes; Bile; Anti-Bacterial Agents

Introduction

Lactic acid bacteria (LAB) are a group of Grampositive microorganisms widely known for their role in food fermentation and their probiotic potential. They have beneficial biochemical activities that promote human and animal health. They possess the ability to produce various hydrolytic enzymes, such as proteases, lipases, and amylases, making them promising candidates in the food and pharmaceutical industries (1,2). Some LAB strains also exhibit anti-inflammatory and anti-cancer properties, act as antioxidants, and play a crucial role in maintaining gut microbiota balance

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as concerns about antimicrobial resistance (AMR) grow, it is important to select lactic acid bacteria (LAB) based not only on their biochemical and probiotic properties, but also on their genetic safety. This is particularly important with regard to their potential to carry or transfer antibiotic resistance genes (6,7). Several studies have reported that LAB isolated from various sources, including fermented foods and probiotic supplements, can exhibit resistance to several classes of antibiotics, such as tetracycline, aminoglycosides, and β -lactams (8–10). This indicates the risk of horizontal gene transfer, which could trigger the spread of AMR in food and clinical environments (11,12).

However, lactic acid bacteria (LAB) are known to have antagonistic properties against pathogens through the production of organic acids, hydrogen peroxide, and antimicrobial compounds, such as bacteriocins. This antibacterial activity creates opportunities to use LAB to control zoonotic and foodborne pathogens, such as Listeria monocytogenes and Salmonella spp. (13,14). Therefore, identifying and biochemically characterizing LAB, along with evaluating their antibiotic resistance, are crucial steps in selecting safe and effective strains for use as probiotics or industrial enzyme producers (15-17)

This study aims to characterize the biochemistry of LAB isolates and screen their probiotic potential, focusing on tolerance to low pH and bile salts, enzymatic activity, and antibiotic resistance profiles. This approach is essential to ensure that the selected strains possess both functional value and microbiological safety for food and therapeutic applications.

Methods

Characteristic testing (Enzymatic Activities, Antibiotic Sensitivity Patterns, and Fermentation Potential using the VITEK 2 fluorescent system (ID-BCL card).

The VITEK 2 fluorescent system (ID-BCL card). Testing was performed following the instructions of the manufacturer. Briefly, strains were cultured on de Man Rogosa Sharpe Agar (MRSA) for 18 to 24 h at 37 ° C before the isolate was analysed. A bacterial suspension was adjusted to a McFarland standard of 0.50 to 0.63 in a solution of 0.45% sodium chloride using the VITEK 2 DensiCheck instrument (bioMérieux). The duration between preparation of the solution and filling of the card was always less than 1 h. The analysis was done by using an identification card for lactic acid bacteria (ID-BCL card). Cards are automatically read every 15 min. Vited with VITEK 2 software version VT2-R03.1(18,19).

If the turbidity is still low, then a bacterial colony should be added. If the turbidity is high, then the amount of inoculum volume is extracted and diluted by adding a NaCl solution. An antibiotic sensitivity test is performed by taking 280 μ l gram of positive bacteria from the first inoculum tube to the second tube using a micropipette and a sterile tip(20).

Vitek 2 cards were placed on the second tube to test the antibiotic sensitivity on the cassette, according to the identification sequence and antibiotic sensitivity. Vitek card for sensitivity test with grey hose, in suspension tube, was placed in the cassette, then was inserted into VITEK 2 Compact 11. It was automatically read every 15 minutes. The current NCCLS breakpoints for oxacillin susceptibility used: MICs of _2 mg liter_1 indicated susceptibility and MICs of _4 mg liter_1.mj as Instrumen user manual (21).

Acid Tolerance

The bacterial acid tolerance (2.5) was tested through the cup count method by modifying the pH of the media for the acidity test. 10 ml of lactic acid bacteria culture in 24 hours- MRSB was harvested and then dissolved at 1% into 9 ml MRSB (control) and MRSB pH 2.5 and 3.0 (added with 10% HCl) and subsequently incubated at 37 ° C for 120

minutes. Bacterial cultures were cultured on de Man Rogosa Sharpe Agar (MRSA) media using a pour plate method and incubated at 37 ° C. for 48 hours. The acid tolerance test was performed three times. The resistance of the isolates was presented in resistance percentage and log decline after incubation (19,22,23).

Bile Salts Resistance

The bacterial resistance to gall salts was tested through the cup count method. 10 ml of lactic acid bacteria culture in 24 hour-MRSB were harvested and then diluted 1% respectively into 9 ml MRSB (control) and MRSB added with Oxgall 3%, then incubated at 37 ° C for 24 hours. Bacteria were cultured on de Man Rogosa Sharpe Agar (MRSA) media using a pour plate method and incubated at 37 ° C. for 48 hours. The bile saline resistance test was performed three times. The resistance of the isolates was presented in tolerance percentage and log decline after incubation (23,24).

Results

As illustrated in Table 1, the biochemical characterization of five lactic acid bacteria (LAB) isolates was conducted using the VITEK 2 system. The characterization specifically focused on the fermentation reactions of the isolates to a variety of protein and carbohydrate substrates. The five isolates were coded as follows: P1a, P1c, P1e, P1g, P5a, P5c, and P5d. The isolates P1a, P1e, P5a, P5c, and P5d demonstrated positive activity toward leucine, alanine, and tyrosine, suggesting the presence of proteolytic enzyme activity. Pla and demonstrated the capacity to utilize P1e phenylalanine. Substrates such as L-proline, Lpyroglutamate, glycine, and glycogen were generally not fermented by the tested isolates. The translation was executed using the DeepL.com free version of the translation service.

Several isolates can ferment complex carbohydrate substrates, such as D-glucose, D-ribose,

D-mannitol, D-mannose, and N-acetyl

D-glucosamine. This indicates a wide range of fermentation, particularly by isolates P1a, P5a, P5c, and P5d, which consistently show positive reactions. Additionally, isolate P1c exhibits a limited fermentation spectrum. Some isolates reactions to showed positive D-trehalose, palatinose, and tagatose, indicating the potential to utilize alternative carbohydrate substrates (Table 1). According to the Salt Tolerance Test, most isolates showed tolerance to 6.5% NaCl, except for P1g and P5d, which exhibited sensitivity (negative reaction).

Table 2 shows the results of the enzymatic activity tests of seven lactic acid bacteria (LAB) isolates: P1a, P1c, P1e, P1g, P5a, P5c, and P5d. These tests were performed using the VITEK 2 system. The tested enzymes included 12 important enzymes involved in carbohydrate and protein degradation. Table 3 illustrates the seven isolates' resistance to three major antibiotics: Kanamycin, Oleandomycin, and Polymyxin B. P1a, P1g, and P5c are resistant to all three antibiotics. P1c and P1e were resistant only to Polymyxin B; P5a was resistant to Kanamycin and Oleandomycin but sensitive to Polymyxin B; and P5d was sensitive to all antibiotics tested.

The results of the isolate resistance test on the 0.3% (oxgall) bile concentration (Fig. 1) showed the presence of inhibition of BAL isolate growth by bile salts. The bacteria are resistant to treatment. P1A, P1C, and P1E bacteria had a small percentage. This indicates that the bacteria cannot survive the conditions of exposure to bile salts, while P1G, P5A, P5C, and P5D can survive with a resistance percentage ranging from 60 to 80.

The result presented in Fig. 2 shows that P1E bacteria cannot survive under pH 2.5. P1C, P1A, P1G, and P5A have nearly the same resistance of about 50%. P5C and P5D isolates had the highest resistance (range from 70%). There was an inhibition in the growth of LAB isolates at acidic pH, which was indicated by the decreasing number of colonies.

Table 1. Biochemical characterization of lactid acid bacteria based on protein and carbohydrate interpretation using vitek 2

No	Strain	P1a	P1c	P1e	P1g	P5a	P5c	P5d
1	Leucine	+	-	-	+	+	+	+
2	Phenilalanin	+	-	-	+	+	+	+
3	L-prolin	-	-	-	-	-	-	-
4	L-pyrolidonil	-	-	-	-	-	-	-
5	Alanin	+	+	-	+	+	+	+
6	Tyrosin	+	-	-	+	+	+	-
7	Ala phepro Fermentasi gula	+	-	+	-	-	-	-
8	Cyclodextrin	-	-	-	-	-	-	-
9	d-galactosa	-	-	-	-	-	+	-
10	Glycogen	-	-	-	-	-	-	-
11	Inositol	-	-	-	-	-	-	-
12	Metal-A- Dglukopiranosid	-	-	-	-	-	-	-
13	Ellman	-	-	-	-	-	-	-
14	Metil D xilosid	-	-	-	-	-	-	-
15	Maltotriose	+	+	+	+	+	+	+
16	Glisin	-	-	-	-	-	-	-
17	D-mannitol	+	+	+	+	+	+	+
18	D-mannose	+	+	+	+	+	+	+
19	D-Melezitoze	-	-	-	+	+	+	-
20	N-acetil D-glukosamin	+	+	+	+	+	+	-
21	Palatinose	+	+	+	-	+	-	-
22	L-rhamnose	-	-	-	-	-	-	-
23	Phosphoril choline	-	-	-	-	-	-	-
24	Piruvat	+	+	+	-	-	-	-
25	D-Tagatose	+	-	-	+	-	-	
26	D-trihalose	+	-	-	+	+	+	-
27	Inulin	-	-	-	-	-	-	-
28	D-Glukose	+	+	+	+	+	+	+
29	D-Ribuso	+	-	-	+	+	+	+
30	Putrescine	-	-	-	-	-	-	-
31	NaCl 6,5%	-	+	+	-	-	-	-

Note: (+) and (-) symbols indicate the positive and negative reaction, respectively

In this study, the bacterial resistance to antibiotics was also tested. Antibiotics which were tested, including Kanamycin, Oleandomycin, Polymixin B. Test bacteria P1a, P1G, P5c are resistant to all antibiotics which were tested (Kanamycin, Oleandomycin, Polymixin B.). P1c bacteria test are resistant to Polymyxin B. P1e is resistant only to 1 ie Polymyxin B antibiotic.p5a is resistant to Kanamycin, Oleandomycin, Polymyxin B, whereas p5D is not resistant to all antibiotics.

Table 2. Biochemical characterization of lactid acid bacteria based on enzymes using Vitek 2

No	Strain	P1a	P1c	P1e	P1g	P5a	P5c	P5d
1	Betaxilosidase	-	+	-	-	-	-	_
2	L-Lysine arylamidase	-	-	-	-	-	-	-
3	L-aspartase	-	+	+	-	-	-	-
4	Beta galactosidase	-	-	-	-	-	-	-
5	Beta N-acetil Glucosaminidase	-	-	-	+	+	+	-
6	Alfa galactosidase	-	-	-	+	-	-	-
7	Beta-glucosidase	-	+	-	+	+	+	-
8	Betha mannosidase	-	-	-	-	-	-	-
9	Alpha mannosidase	-	-	-	-	-	-	-
10	Alpha-glucosidase	-	-	-	-	-	-	-
11	Esculin hydrolase	+	+	+	+	+	+	+
_12	Tetrazodium red	+	+	-	+	-	+	-

Table 3. Characterization of lactid acid bacteria based on antibiotic resistances using Vitek 2

No	Strain	P1a	P1c	P1e	P1g	P5a	P5c	P5d
1	Kanamicin resistance	+	-	-	+	+	+	-
2	Oleandomycin resisting	+	-	-	+	+	+	-
3	Polymyxin B resistant	+	+	+	+	+	+	-

Note: (+) and (-) symbols indicate the positive and negative reaction, respectively

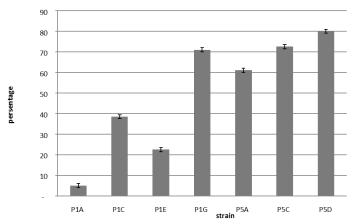


Figure 1. LAB resistances for Bile Salt 0,3% minutes.

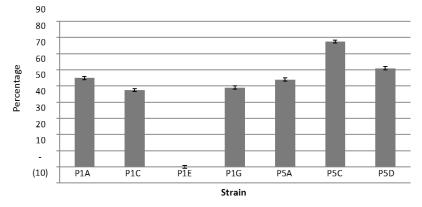


Figure 2. Acid resistance at pH 2,5

Discussion

The results of this study indicate that lactic acid bacteria (LAB) isolates exhibit high variability in biochemical activity and antibiotic resistance. This variability is crucial in the selection process for probiotic candidates and enzyme producers. The positive characterization of various amino acids and carbohydrates suggests that most isolates have good metabolic capabilities, especially in P1a, P5a, and P5c, which have the widest fermentation spectrum. These characteristics suggest their potential as probiotics, particularly given their ability to ferment vital compounds such as glucose, mannitol, and ribose in the intestinal environment. Positive reactions to compounds such as alanine, tyrosine, and palatinose suggest potential enzyme production, including protease and amylase. These activities contribute to the ability to digest complex substrates and enhance nutrient availability in the digestive tract, giving some isolates good enzymatic potential for biotechnology and food industry applications. However, antibiotic resistance raises significant concerns regarding the safety of probiotic use. Isolate P5d, which shows no resistance to antibiotics, is the safest candidate in terms of spreading antibiotic resistance genes. In contrast, isolates P1a and P5c require further investigation before widespread application due to the risk of horizontal gene transfer (HGT) in the gut microbiota, as they are resistant to all antibiotics. The primary criterion for selecting safe and effective probiotic strains is the combination of high biochemical activity and low antibiotic resistance. Therefore, isolates P5a and P5d are potential candidates for further testing before application and should be studied in more detail in terms of molecular aspects and in vivo applications.

The biochemical and antibiotic resistance profiling of lactic acid bacteria (LAB) in this study highlights their potential as both probiotic candidates and enzyme producers. Several isolates, such as P1a and P5c, demonstrated high enzymatic activity, particularly for β -glucosidase and esculin hydrolase, which are enzymes often associated with the

breakdown of plant polysaccharides and bioactive compound release, thus enhancing host digestion and health. These results align with the findings of Gizachew et al, who reported that LAB strains isolated from Ethiopian fermented dairy products exhibited strong antibacterial and enzymatic properties, including β -glucosidase activity, which may contribute to their immunostimulatory effects(14).

Our findings suggest that certain LAB isolates, such as P1a, P1g, and P5c, exhibit resistance to multiple antibiotics, including kanamycin, oleandomycin, and polymyxin B. This pattern reflects growing concerns about the presence of antibiotic resistance genes (ARGs) in probiotics and fermented food microbes. LAB strains resistant to multiple antibiotics from fermented foods and human sources in Nigeria(7), emphasizing the need for strict screening before clinical or food applications. concerning resistance profiles in commercial probiotic LAB, suggesting that even marketed supplements may pose a risk of ARG transfer(8). This phenomenon is influenced by environmental and selective pressures in fermentation or natural environments. Additives in high-moisture sweet corn silage influence ARG abundance in microbial communities(25). This suggests that environmental conditions play a role in shaping resistance profiles. Furthermore, Chu et al revealed that fermentation residues from antibiotic production facilities act as resistance reservoirs for genes(6), thereby increasing the likelihood that LAB will acquire resistance through environmental exposure.

Concerns about the horizontal gene transfer (HGT) of ARGs from probiotics to pathogens and the potential for resistance gene propagation in the gastrointestinal tract have been expressed(11,26). These risks underline the importance of integrating phenotypic screening (e.g., VITEK 2) with molecular approaches (e.g., whole genome sequencing), to ensure the safe application of LAB strains(27,28). Interestingly, some isolates in this study showed minimal resistance (e.g., P5d), which variability may indicate natural less

environmental exposure to antibiotics, as seen in strains isolated from less industrialized or more controlled settings (15,29). This highlights the necessity of careful strain selection depending on the intended application (food, feed, or therapeutic use).

Isolate P5d only showed limited enzyme activity (esculin hydrolase and tetrasodium red). Based on previous literature, LAB strains usually have a broader enzyme spectrum, especially for βglucosidase and α-glucosidase, which play a role in the fermentation of complex carbohydrates (14,30). This low enzyme activity is likely due to differences in the environmental conditions of the isolates, such as pH, substrate availability, or interactions with other microorganisms that do not support optimal expression of certain enzymes. Additionally, isolates such as P1g and P1c exhibit antibiotic resistance that is not commensurate with their enzyme activity. "This contrasts with previous findings, which reported a positive correlation between metabolic diversity increased antibiotic resistance in LAB strains isolated from spontaneously fermented products(15,27). This discrepancy may be due to genetic strain variation or specific selection pressures in the environment that drive resistance without being related to metabolic function or enzymes.

Interestingly, although some isolates showed resistance to antibiotics (kanamycin, polymyxin B), not all had superior enzyme profiles. Resistance can be acquired horizontally through gene transfer, which is not always related to the strain's original metabolic capabilities (11).

Strain P5D and strain PSC stand out in terms of their resistance to bile salts. This indicates that they are well-adapted to the intestinal environment, particularly the duodenum and small intestine, where bile concentrations are relatively high. These results suggest that both strains have great potential as probiotics. These results align with previous studies indicating that LAB strains capable of surviving in bile possess efflux systems or membrane structure modifications that counteract

the detergent effects of bile salts. Strain PSC and P5D demonstrated good performance under acidic conditions again, confirming their potential as probiotics capable of surviving in the stomach. This resistance is important because the extreme pH of the stomach can cause bacterial inactivation or death before they reach the intestines.

These differences indicate that probiotic potential is not always directly proportional to antibiotic resistance or enzymatic strength. Therefore, each isolate has its own advantages, and sometimes, to maximize probiotic potential, a consortium of two or more isolates with complementary advantages is required. For example, in this study, isolate P5a has a broad fermentation spectrum potential, but P5d has the advantage of being non-resistant to antibiotics and and Survival ability in bile and acid conditions is an important indicator in the selection of probiotic candidates, as it illustrates the ability of microbes to pass through the human gastrointestinal tract.

This study provides valuable contributions to food microbiology and probiotic biotechnology through the biochemical characterization of lactic acid bacteria (LAB). The results revealed strain-specific enzymatic profiles, particularly in isolates P1a and P1e, emphasizing the influence of environmental origin on the expression of probiotic traits. Key enzymes such as L-aspartase, β-N-acetylglucosaminidase, and esculin hydrolase, known for their roles in amino acid metabolism, glycoside hydrolysis, and nutrient bioavailability, were identified, supporting previous findings on LAB functional diversity (14,15). Despite these important findings, the study has several limitations that must be acknowledged. First, the diversity of LAB potentially isolates analyzed was limited, overlooking broader enzymatic or resistance characteristics present in LAB from various ecological sources. Second, the study did not incorporate molecular identification or genotyping techniques such as 16S rRNA sequencing or whole genome sequencing (WGS), limiting the taxonomic accuracy and hindering the detection of specific

resistance or virulence genes. Lastly, all functional assessments were conducted in vitro, without in vivo or in situ validation. Further studies using gastrointestinal models or animal trials are necessary to confirm the probiotic functionality and safety of these isolates in real-life biological contexts.

Conclusions

This study successfully characterized several lactic acid bacteria (LAB) isolates for their enzymatic antibiotic resistance activities and highlighting their potential as candidate probiotics and enzyme producers. The results demonstrated that specific LAB strains possess desirable enzymatic functions such as L-aspartase, β-Nacetyl-glucosaminidase, and esculin hydrolase and exhibit varying degrees of antibiotic resistance, suggesting strain-specific adaptations influenced by their environmental origins. These findings contribute valuable insights into the selection of functionally and industrially relevant LAB strains, particularly for applications in fermented food production, probiotic development, and health-related formulations. However, further molecular and functional validations are necessary to ensure safety, efficacy, and regulatory suitability for future application.

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