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In-vitro probiotic evaluation of *Lactobacillus* species

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Abstract
Probiotics are health promoting viable microorganisms that exhibit beneficial effects on the health of humans and animals. *Lactobacillus* species were isolated from nono and kunun zaki local drinks and the pH, bile salt and temperature tolerance of all the isolates were tested. Most of the isolates were shown to be able to tolerate a pH range of 2.0 to 7.0 except *L. fermentum* and *L. oris* which were unable to tolerate the pH of 2.0. However, all the *Lactobacillus* species were unable to tolerate the pH of 1.0. The *Lactobacillus* species isolated were able to survive in a range of 0.5 to 2.0 % bile salt concentrations and at a temperature range of 25 to 40°C. The *Lactobacillus* species exhibited remarkable tolerance to stress factors which confers on them useful probiotic characteristics. Thus, they can be used as potential probiotic strains, both by serving as antagonistic agents to prevent harmful bacteria from colonizing the gastrointestinal tracts and other body parts as well as bio-preservatives even in foods with extreme physicochemical conditions such as acidic foods. Local food and beverage manufacturers were advised to exploit commercially prepared cultures of these strains as useful natural additives in their products.

Keywords: Beverage, bile salt, Ph, Probiotics, temperature, tolerance

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Introduction

Probiotics are live microorganisms thought to be beneficial to the host organism. Probiotics are commonly consumed as part of fermented foods with specially added active live cultures; such as in yogurt, soy yogurt, or as dietary supplements. Consumption of probiotics has many health benefits. Some of these benefits include: enhancing bowel function, prevention of colon cancer, cholesterol lowering, lowering of blood pressure, improving immune function and reducing infections, reducing inflammation, improving mineral absorption, preventing growth of harmful bacteria, fighting off diseases like candidiasis and eczema, and many more. Because of all these benefits of probiotics, it was reported that use of probiotics in food products will be helpful on food attribution and microbial quality of food (Goktepe *et al.,* 2006; Gorbach, 2002; Holzapfel and schilling, 2002; Holzafel *et al* 2001; Jay *et al.,* 2005; Prado *et al.,* 2008; Ranadheera *et al.,* 2009).

Probiotics do not colonise the human intestinal tract permanently, but some strains are able to transiently colonise and modulate the indigenous microbiota. Specific probiotic bacteria have been reported to modulate local and systemic immune responses (Isolauri *et al.,* 2002). Although the mechanisms of immune modulation are not fully understood it is known that bacterial components are recognized by the immune system through their interaction with specific Toll-like receptors resulting in the modulation of immune responses (Ning and Walker 2004). The specific receptors implied in some of

these interactions have been reported (Grangette *et al.,* 2005). Probiotic bacteria may also counteract inflammatory processes by stabilizing a healthy microbiota and thus improving the intestine permeability barrier. In addition to influencing gut microbiota and immune system, other mechanisms of probiotic action have been proposed, such as inhibition of pathogens by competition for nutrients and attachment sites or by production of antimicrobial substances, reduction of cholesterol levels through deconjugation of bile salts or binding of toxins and carcinogens and preventing their absorption (Mercenier *et al.,* 2002).

Genomic research has also provided information about the adhesive mechanisms present in probiotic microorganisms which comprise a basis both for populating the gut and for communicating developmental signals to specific areas and sites of the gut mucosa (Kleerebezem *et al.,* 2003). In addition, factors related to the immune-modulatory ability of specific strains have been found and bacteriocin operons have been identified.

This study evaluated the ability of Lactic acid bacteria species to withstand the stress conditions as could be obtained in the gut and other habitats where conditions are tough and competition for survival between different bacterial species fierce. Ability of lactic acid bacteria to withstand such conditions is a good indication of their ability to succeed in outcompeting harmful bacteria in human and animal bodies and to confer other well-known probiotic or health promoting benefits ascribed to them.

Materials and Methods

Sample collection

Samples of Nono and Kunun Zaki, were collected from various locations across Dutse metropolis. The collection process involved placing the samples in plastic containers to ensure proper preservation. Following collection, the samples were immediately transported to the laboratory and stored in refrigeration conditions, until they were processed for further analysis.

.Isolation of Lactic Acid Bacteria from the samples

Lactic acid bacteria were isolated by serial dilution where 10ml of the sample was added to 90 ml of sterile distilled water and mixed thoroughly; 1ml from the mixture was transferred to a tube containing 9 ml of sterile distilled water, this was labeled 10^{-1}. The process was repeated up to a dilution factor of 10^{-5}. Aliquot from 10^{-4} diluents were spread plated on the surface of plates of de-Mann, Rogosa and Sharpe (MRS) agar in duplicates (Awan and Rahman, 2005). The plates were incubated at 32°C in anaerobic jar for 48 hrs. A pure colony was randomly picked and sub cultured onto plates of MRS agar (Baita *et al*., 2021).

Morphological and Biochemical Identification of Lactic Acid Bacteria Isolates

To identify the lactic acid bacteria colonies obtained from MRS agar, several methods were employed including Gram's staining, endospore staining, and catalase tests. These tests were used to determine the species-level identification of the lactic acid bacteria isolates. Additionally, molecular characterization techniques such as DNA extraction, polymerase chain reaction (PCR), gel electrophoresis, and sequencing were utilized to further confirm the identity of the lactic acid bacteria isolates (Reid *et al*., 2013). Pure cultures were maintained in MRS broth for further tests.

Gram's Staining

A smear of the culture was made on a clean glass slide and heat fixed over a flame, the fixed smear was flooded with crystal violet stain for 60 seconds. The crystal violet stain was washed off rapidly with distilled water. The smear was then flooded with Lugol's iodine for 30 to 60 seconds. The iodine was then washed off with distilled water. The stain was decolorized for a few seconds with acetone–alcohol. It was then washed immediately with distilled water. The smear was covered with safranin stain for 2 minutes. The safranin stain was washed off with distilled water. Cotton wool was used to wipe the back of the slide and was placed in a draining rack for the stain to air-dry. It was

later examined microscopically, first with the x40-objective to check the staining and to see the distribution of material, and then with the oil immersion X100 objective (Cheesbrough, 2000).

Endospore staining

A smear of the bacteria was made on a clean glass slide and heat fixed. The slide was placed over the steaming water bath and malachite green (primary stain) was applied for 5 min. The slide was removed from the water bath and rinsed with water. It was then flooded with the counter stain safranin for 20 seconds and rinsed with water after which the slides were blotted, dried and observed under the light microscope. Red colour indicates a positive result while green indicates a negative result (Awan and Rahman, 2005).

Catalase Test

Two millilitres (2 mls) of hydrogen peroxide solution was put into a test tube, using a sterile glass rod several colonies of the test organisms were picked and immersed in the hydrogen peroxide solution on a clean glass slide. Active bubbling indicates a catalase positive result while absence of bubbles is an indication of catalase negative result (Cheesbrough, 2000).

pH Tolerance

The lactic acid bacteria isolates were inoculated into sterile MRS broth tubes with different pH levels, ranging from pH 2 to pH 7. Subsequently, the tubes were incubated at a temperature of 37°C for a duration of 2-3 days. After the incubation period, 0.1ml inocula from each tube were poured onto MRS agar medium using the pour plate method. The plates were then incubated at 37°C for 48 hours. The presence of lactic acid bacteria growth on the MRS agar medium was used as an indicator to classify the isolates as pH-tolerant (Kabore *et al*, 2012).

Bile Salt Tolerance

To determine the bile salt tolerance of the selected lactic acid bacteria cultures, nutrient broth with different concentrations of bile salt (0.5%, 1.0%, 1.5%, and 2.0%) was prepared. Each lactic acid bacteria isolate was inoculated into the respective broth and incubated at 37°C for 48 hours. After the incubation period, 0.1ml of the inoculum was transferred to MRS agar plates using the pour plate method. The plates were then incubated again at 37°C for 48 hours. The growth of lactic acid bacteria cultures on the agar plates was observed and used to classify the isolates as bile salt tolerant (Kabore *et al*, 2012).

Sensitivity to Temperature

The selected bacterial isolates were grown at varying temperatures of 25, 30, 37 and 40°C for 72 hrs. After the incubation period, 0.1ml of the inoculum was transferred to MRS agar plates using the pour plate method. The plates were then incubated again at 37°C for 48 hours. The growth of lactic acid bacteria cultures on the agar plates was observed and used to designate isolates as temperature tolerant (Tambekar and Bhutada, 2010).

Molecular identification of Lactic Acid Bacteria

DNA Extraction

Two hundred microlitres (200 µl), 400 µl lysis buffer and 10 µl proteinase from the culture were vortexed and incubated at 65°C for 1hour, mixing was repeated thrice at intervals of 20 minutes during incubation, then 400µl of phenol chloroform was added to the mixture and vortexed for 15 seconds, this was then spun at 13,000rpm for 10 minutes. The supernatant, was carefully poured into a separate tube, and then 400µl of chloroform was added. The tube was thoroughly mixed. Next, the mixture was subjected to centrifugation at a speed of 13,000 rpm for 5 minutes. Following centrifugation, the supernatant was carefully transferred to a new tube. To this tube, 1000 µl of 100% ethanol and 40 µl of 3M sodium acetate were added. The tube was inverted several times to ensure proper mixing and then stored at a temperature of 20°C overnight. Afterwards, the tube was subjected to centrifugation once again, this time at 4°C for a duration of 10 minutes. The resulting supernatant was discarded, and 400 µl of 70% ethanol was added to the pellet. Finally, the mixture was centrifuged at a speed of 12,000 rpm for 5 minutes. The supernatant was finally discarded and allowed to dry and after drying, 50 µl of deionized water was added (Arief *et al*., 2015).

Amplification of DNA Fragment by Polymerase Chain Reaction (PCR)

To amplify the 16S rRNA gene, universal primers GGACTACAGGGTATCTAAT forward and AGAGTTTGATCCTGG reverse were employed. The expected size of the PCR product was approximately 1.5kb. The PCR amplification was carried out in 25 µL reaction mixtures using a thermal cycler. The PCR reaction mixture consisted of 2.5 µL of PCR buffer. The PCR conditions were set as follows: an initial denaturation step at 94°C for 4 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute each, primer annealing at 55°C for 30 seconds, and primer extension at 72°C for 2 minutes, with a final step of primer extension at 72°C for 5 minutes. The PCR product with expected size of 1.5kb was excised from the gel and purified using MEGA quick-spin PCR and Agarose Gel DNA Extraction System (Arief *et al*., 2015).

Gel Electrophoresis

To each 100mg of agarose gel, 300ml of QG buffer was added. It was heated at 50°C until completely dissolved. It was then mixed at intervals by inversion and observed for change in colour. If orange or violet colour appears, 3ml Sodium acetate was added and mixed. DNA fragments were added at 500bp (base pair) and 4kb (kilobase pair) equal volume of isopropanol. It was mixed well before transfer to a spin column in a collection tube and was centrifuged for 1 minute at 10000 rpm. The flow was discarded and the collection tube was re-used, 0.75ml (750μl) of buffer was added and allowed to stand for 5 minutes and then centrifuged at high speed of 14000 rpm for 1 minute. The flow was then discarded through and the wash step is repeated. The speed was reduced down to 11000 rpm and centrifuged immediately, the empty tube was centrifuged for 3 minutes at high speed 13,000rpm, DEPC water (25μl) was used for elution and was allowed to stand for 5 minutes, it was centrifuged at 13000 rpm for 1minute (Baita *et al.*, 2021).

Sequencing

To 20µl reaction mixture 100µl of phosphate buffer (pb) was added into the sequencing reaction, it was mixed by pipetting in and out. The entire reaction mixture was transferred into the binding column and centrifuged at 10000 rpm for 1 minute. The flow was discarded through and the collection tube is re-used, 650µl of Pf was added and allowed to stay for 3 minutes, it was spun at 13000rpm for 1 minute and the empty column was spun for 2 minutes at 13000rpm. New collection tube was used, 13μl of SLS was added and allowed to stay for 5 minutes, it is spun at 13000 rpm for 1 minute, the elution step is repeated and spun immediately. It was then loaded onto the sequencing machine (Baita *et al.*, 2021).

Results

The Lactic acid bacteria isolates were subjected to different pH and they have shown tolerance to a pH range of 2.0 to 7.0. However, they were unable to survive in a very low pH of 1.0. *L. oris* showed the most remarkable pH tolerance (Table 1). All the five lactic acid bacteria were able to survive at different concentrations of bile salt. They were all able to thrive in bile salt concentration of 0.6% through 2.0% (Table 2). The lactic acid bacteria isolates survived at varying temperatures of 25, 30, 37, and 40°C (Table 3).

Table 1: pH Tolerance of Isolated Lactic Acid Bacteria

Sample	LAB isolates	pH								
		7.0	6.0	5.0	4.0	3.7	3.5	2.5	2.0	1.0
1	*L. plantarum*	+	+	+	+	+	+	+	+	_
2	*L. fermentum*	+	+	+	+	+	+	+	_	_
3	*L. oris*	+	+	+	+	+	+	+	_	_
4	*L. plantarum*	+	+	+	+	+	+	+	+	_
5	*L. pantarum*	+	+	+	+	+	+	+	+	_

Key: += presence of growth _= absence of growth

Table 2: Bile Tolerance of Isolated Lactic Acid Bacteria Cultures

Sample	LAB isolates	Bile salt concentration (in %)			
		2.0	1.5	1.0	0.5
1	*L. plantarum*	+	+	+	+
2	*L. fermentum*	+	+	+	+
3	*L. oris*	+	+	+	+
4	*L. plantarum*	+	+	+	+
5	*L. plantarum*	+	+	+	+

Key: +=- presence of growth

Table 3: Temperature Tolerance of Isolated Lactic Acid Bacteria Cultures

Sample	LAB isolates	Varying temperature (°C)			
		40	32	30	25
1	*L. plantarum*	+	+	+	+
2	*L. fermentum*	+	+	+	+
3	*L. oris*	+	+	+	+
4	*L. plantarum*	+	+	+	+
5	*L. plantarum*	+	+	+	+

Key; += presence of growth

Discussion

Probiotic potentials of LAB may be due to their ability to resist bile salts and acidic pH. In this study five isolated lactic acid bacteria showed an excellent tolerance to acidic pH and bile salt. Before reaching the intestinal tract, probiotic bacteria must first survive transit through the stomach where the pH can be as low as 1.5 to 2.0 (Dunne *et al*, 2001). Tolerance to bile salts is a prerequisite for colonization and metabolic activity of bacteria in small intestine of the host. This will help Lactobacilli to reach the small intestine, colon and contribute in balancing the intestinal microflora (Mathur *et al*., 2020).

Similarly, lactic acid bacteria isolated from baobab (maari) fermented seeds were able to survive at pH 2.5 and tolerate bile salt concentration of 0.3% only (Kabore *et al*., 2012). Tambekar and Bhutada (2010) also reported that the three isolated LAB showed excellent acid tolerance at pH 2.0 and bile salt tolerance at 2.0%. Tolerance to bile salts is a prerequisite for colonization and metabolic activity of bacteria in the small intestine of the host (Havenaar *et al.*, 1992).

This study found that, all the selected LAB isolates were able to survive at temperatures of 25, 30, 37 and 40°C respectively this is in line with the work of (Nicolas *et al.*, 2007). The temperature is an important factor which can dramatically affect the bacterial growth. The reason for choosing this temperature range was to detect whether the isolated cultures are able to grow within the range of normal body temperature or not. And if the isolates were unable to survive within the selected temperature range then they would not have been able to survive in the human gut, which is an essential factor potentially determining the effectiveness of probiotics.

Conclusion

In conclusion The *Lactobacillus* species in this study exhibited a remarkable tolerance to stress factors. Thus, they can be used as potential probiotic strains, both by serving as antagonistic agents to prevent harmful bacteria from colonizing the gastrointestinal tracts and other body parts as well as well as bio-preservatives even in foods with extreme physicochemical conditions such as acidic foods.

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