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Isolation and Identification of Lactic Acid Bacteria with Probiotic Potential from Fermented Cow Milk (Nono) in Unguwar Rimi Kaduna State Nigeria

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Abstract

Lactic acid bacteria (LAB) strains from fermented cow milk (nono) sold in-Unguwar Rimi, Kaduna markets were isolated on lactic bacteria specific medium (De Man Rogosa Sharpe MRS media). Isolated strains were identified and characterized using morphological, biochemical test and carbohydrate fermentation system (API-50 CHL). Six (6) pure colonies were distinctly obtained and identified as *Lactobacillus* strains. Out of the 6 isolated Lactobacilli, 5 were further identified as *Lactobacillus delbrueckii* ssp. lactis 2, *Lactobacillus bulgaricus*, *Lactobacillus salivarius*, *Lactobacillus acidophilus* 2 and *Lactobacillus rhamnosus*. They were further screened for antimicrobial activity and antibiotic sensitivity. *Lactobacillus acidophilus* had higher resistance to all but one of the antibiotics used (Chloramphenicol 30 µg) with no zone of inhibition to Ampicillin, Amoxicillin, Gentamycin, Penicillin, Streptomycin and Tetracycline. The antimicrobial activity against *Escherichia coli* exhibited varying degree of inhibitory activity. It can be concluded that the presence of these microorganisms in fermented cow milk (nono) is of great benefits to humans and animals either as supplements or food production processes.

Keywords

Lactobacillus, Morphological, Antimicrobial, Supplements

1. Introduction

Lactic acid bacteria (LAB) are common inhabitants of fermented products (milk, meat and vegetables) and gastrointestinal tracts, most of which are responsible for maintaining a balance of the micro-biota of healthy host. This group of bac-

teria has the ability to colonize the gastrointestinal tract and hence ferment carbohydrate which produces lactic acid as the major metabolic end product that aids digestion. Also as part of its host benefits, it prevents the action of pathogenic microorganism through the production of inhibitory substances (metabolites) and a formation of a bio-film to protect the intestinal mucosal membrane [1] [2].

Consumption of milk and its products as food substances dates back to thousands of years. Although it was lacking any scientific basis until when it was observed that Bulgarian peasants who often consumed large quantities of soured milk as part of their daily diet had a longer survival age [3]. He believed that consumption of fermented milk would reverse the adverse effect of the lower gut micro flora on the host animal. Fermented milk has a beneficial effect on the host's health [4]. Milk products containing viable LAB have been used as prophylactics for treatment of intestinal infections in adults infected with *Salmonella* and lactose intolerance [5] [6].

Probiotics are live microorganisms that confer health benefits to the hosts by improving intestinal microbial balance [7] [8]. Some LAB species (*Lactobacillus*, *Streptococcus, Enterococcus* and *Pediococcus*) have been reported as active candidates for probiotic use in humans and animals by several researchers [4].

Selection of lactobacilli as potential health-promoting probiotic in food and pharmaceutical preparations entails *in vitro* screening for certain criteria, which include antibiotic tolerance, bile tolerance, inhibiting the growth of other microorganisms and gastric juice which allow them to be established in the intestinal tract. Therefore the present study was undertaken with the objective of isolating and identifying *Lactobacilli* from fermented cow milk (nono) and *in vitro* determination of tolerance to antibiotic, bile and microbial inhibition.

2. Materials and Methods

A total of five (5) nono samples were collected in sterile bags from different locations around Unguwan Rimi Market, Kaduna State. Samples were transported to the laboratory in a cold box and stored in a refrigerator at −4˚C for isolation of LAB.

MRS (De Mans, Rogosa and Sharpe) agar and broth medium in powdered form were weighed and reconstituted using sterile water according to manufacturer's instruction (TITAN Biotech Ltd., India) and sterilized at 121˚C and 15PSI for 15 minutes before cooling.

25 ml each of the collected samples were homogenized in sterile normal saline. Serial dilution up to 10^{-6} was made using a sterile pipette by transferring 1ml from 10 ml of the normal saline culture into 9 ml of diluent in sterile test tubes. Enumeration of LAB was done by pipetting and plating out appropriate dilutions using MRS agar. Plates were incubated in an inverted manner at 37˚C for 48hours. After incubation, colonies were counted and recorded as colony forming units (CFU). Morphologically distinct colonies were sub-cultured and purified by streaking on agar plates repeatedly. LAB cultures were maintained on

MRS agar slant at 4˚C and sub-cultured every 4 weeks.

Isolates were characterized after 48 hours of incubation using: macroscopic examination for shape, elevation, size and pigmentation; microscopic examination by gram staining; growth at 15˚C and 45˚C and; biochemical methods [9] [10]. Further identification of LAB strains by sugar fermentation using API 50 CHL system (Biomerieux® France).

Smear fixation was carried out by spreading loopful of isolate on a glass slide and passing it over low flame 3 times. Smear was covered with 1% crystal violet, Lugol's iodine solution and washed with 95% ethanol and stained with 2% safarin before being observed under light microscope.

A drop of 3% hydrogen peroxide (H_2O_2) was added to a loopfulLAB culture.

The LAB was inoculated in 5ml of tryptone broth and incubated at 37˚C for 24hrs. Five (5) drops of 0.5% Kovac's reagent was added after incubation and mixed by gently shaking.

Test culture was inoculated on slants of Simmon's citrate agar then incubated at 37˚C for 24 hours.

24 hours LAB cultures were inoculated in 5 ml glucose phosphate peptone water and incubated at 37˚C for 24 hours. Following incubation, drops of 0.02% methyl red solution were added.

The rapid identification of different strains using the API 50 CH kit (Biomerieux) which is a standardized system was used to differentiate LAB isolates at strains level. Wells in the incubation trays were filled with sterile distilled water to create a humid atmosphere, strips were placed on the trays accordingly. Pure culture incubated for 24 hours were harvested into ampoules containing sterile peptone water. Bacterial suspension in the ampoule (2.0 McFarland) was dispensed into the mediathen into the strip's microtubules using pipette avoiding bubbles formation. Wells were covered with sterile mineral oil to achieve anaerobiosis and incubated at 37˚C for 48 hours. Reaction based on changes in color of each well was studied and interpreted as negative, positive or doubtful. Identification was obtained after result patterns were analyzed with the numerical profile using api*web*™ (Version 5.1).

The antibiotic sensitivity test was performed as described by [11]. MRS agar plates were prepared with 0.1 ml for each of the identified *Lactobacilli* strains. Standard antimicrobial susceptibility test discs (Sigma-aldrich) were prepared and applied to the surface of the plates and incubated for 18 hour at 37˚C. Following incubation, zones of inhibition surrounding the discs were measured.

The antimicrobial effect of all isolated *Lactobacilli* species against *Escherichia coli* (indicator bacteria) was determined by the disc diffusion method.

The effects of bile on the growth of the probiotic strains were determined using methods from [12]. Bile salt solutions (0.3% and 1.0% conc.) were prepared by dissolving 0.3 g and 1.0 g of sodium desoxycholate in 100 ml distilled water each. Turbidity from cell-lysis was examined after incubating for 4 hours and Gram-staining.

3. Results

Five (5) species of genus *Lactobacillus* were successfully isolated from samples of fermented cow milk (nono) using MRS media. The isolates were identified using conventional bio-chemical methods as presented in Table 1. All the isolates were found to be Gram positive and lack the ability to utilize citrate (negative reaction) and catalase test indicated that all isolates were non-catalase producing bacteria. Further identification was carried out using standard API-50 CHL system Table 2. The first micro tube lacks any active carbohydrate substrate and served as negative control. Entire isolated microorganisms fermented glucose, fructose and lactose which were indicated by the change of color from purple to pale yellow. However, there was variation in fermentation pattern of other substrates. LAB 1 was identified as *Lactobacillus acidophilus* after fermenting Melibiose and raffinose. LAB 2 hydrolyzed most of the carbohydrate substrates and was profiled as *Lactobacillus rhamnosus*. LAB 3 was identified to be *Lactobacillus lactis 2.* Esculin hydrolysis revealed by a change to a darker colour or black was represented by all isolates except LAB 4 which was identified to be *Lactobacillus bulgaricus*. Identified isolates were maintained at 4˚C on MRS agar slants.

The result of the antibiotic sensitivity of selected *Lactobacilli* to commonly used antibiotics is presented in Table 3. They were expressed as sensitive (S) or resistant (R) [13]. *Lactobacillus acidophilus* has higher resistance to all but one of the antibiotics used (Chloramphenicol 30 µg). This is because there was no

Table 1. Morphological and Biochemical Characteristics of Isolated Microorganisms.

Isolate	Characteristics on Agar Plates	Microscopic Characteristics	Growth @ 15˚C	Growth @ 45˚C	Methyl Red Test	Citrate Test	Catalase Test	Indole Test
Lab1	Small, flat, smooth, fuzzy	Gram positive, singly and tapering end	-	+	-	-	-	-
Lab2	Rough, convex, off-white, colonies	Gram positive, rods, straight, singly, non-spore	-	+	-	-	-	-
Lab3	Small, flat, crenated, creamy colour	Gram positive, rods, singly and short chains	-	+	-	-	-	-
Lab4	Circular, irregular, off-white	Gram positive, rods, chained, non-spore	-	+	-	-	-	-
Lab 5	White, smooth, convex	Gram positive, rods, round ends, singly and chains	-	+	-	-	-	-

KEY: (+): Positive Reaction (−):Negative Reaction.

Table 2. Identification of isolated microorganisms using apiweb (v5.1) system.

Isolate	Specie Identified	Identification (%)
LAB 1	*Lactobacillus acidophilus*	93.7
LAB 2	*Lactobacillus rhamnosus*	99.9
LAB 3	*Lactobacillus delbrueckii* ssp. Lactis 2	86
LAB 4	*Lactobacillus delbrueckii* ssp. *bulgaricus*	99.7
LAB 5	*Lactobacillus salivarius*	99.9

zone of inhibition to Ampicillin, Amoxicillin, Gentamycin, Penicillin, Streptomycin and Tetracycline. *Lactobacillus rhamnosus and L. bulgaricus* showed resistance to Amoxicillin and Streptomycin both administered at 25 μg and 10 µg respectively.

The result of the antimicrobial activity of *Lactobacilli* against *Escherichia coli* exhibited varying degree of inhibitory activity against enteric *Escherichia coli* as shown in Table 4.

The result of the bile tolerance test of the isolate is presented in Table 5. Observed values show that all of the 4 isolates are resistant to 0.3% bile salt with gradual decrease of viable cells in 1.0% bile salt. *Lactobacillus bulgaricus* and *L. acidophilus* were more tolerant.

Table 3. Diameter of inhibition zone of Lactobacilli sensitivity to antibiotic.

Isolates	Antibiotics/ Zone of inhibition (mm)						
	AMP 10 µg	AMO 25 µg	GEN 25 µg	PEN 25 µg	STR 25 µg	TET 25 µg	CHL 30 µg
L. rhamnosus	2.5 (R)	5.6 (R)	-	-	4.3 (R)	-	-
L. acidophilus	-	-	-	-	-	-	2.5 (R)
L. bulgaricus	-	5.5 (R)	-	-	3.5 (R)	-	-
***L. lactis* 2**	12.3 (S)	-	-	-	1.5 (R)	-	-
L. salivarius	3.2 (R)	-	13.0 (S)	1.5 (R)	5.0 (R)	-	-

AMP: Ampicillin, AMO: Amoxicillin, GEN: Gentamycin, PEN: Penicillin, STR: Streptomycin, TET: Tetracycline, CHL: Chloramphenicol, R: Resistant, I: Intermediate, S: Susceptible, (−): No inhibition

Table 4. Mean inhibition zone for antimicrobial activity against Escherichia coli.

Isolate	*E. coli* Inhibition Diameter (mm)
L. rhamnosus	9
L. acidophilus	17.5
L. bulgaricus	3.5
***L. lactis* 2**	9.4
L. salivarius	10.5

Table 5. Bile Tolerance Test of Isolated Lactobacilli.

ISOLATE	Concentration/Result	
	0.30%	1.00%
L. rhamnosus	++	+
L. acidophilus	+++	+
L. bulgaricus	+++	+
***L. lactis* 2**	++	+
L. salivarius	++	+

Key: +++ maximum resistance, ++ Moderate resistance, + Minimum resistance.

4. Discussion

Lactobacilli known to be affiliated to lactic acid bacteria (LAB) commonly found in the gastrointestinal tract of animals and humans can also be found in fermented food such as milk and milk product. The creamy or whitish appearance of the isolated *Lactobacillus* species on MRS agar confirms that Lactobacilli have dominance in fermented milk products when compared to other lactic bacteria [14] [15]. One of the characteristics of an ideal probiotics is resistance against antibiotics mostly after antibiotic administration. The results of antibiotic susceptibility of isolated Lactobacilli which showed that almost all the species were found resistant to commonly used antibiotics except *Lactobacillus lactis* 2 and *L. salivarius* that were only susceptible to ampicillin and gentamycin agree with the report of [16]. Other authors reported multidrug resistance [17] [18] [19]. It is unreasonable to use susceptible probiotics in combination with antibiotics in case of bacterial infections [20]. Resistant probiotic bacteria do not have the genes which can be transferred to other bacterial population by conjugation [16].

Antimicrobial activity against entero-pathogens is another important character of probiotic bacteria. All isolated species were tested for antimicrobial activity against *E. coli*. Zone of inhibition (mm) was used as basis to measure antimicrobial activity in this study. Maximum activity (17.5 mm) was shown by *L. acidophilus* while minimum activity (3.5 mm) was shown by *L. bulgaricus*. The average diameter of inhibition zone from the duplicate test clearly showed that all *Lactobacilli* have antimicrobial effect inhibiting the growth of *E. coli*. However, *L. bulgaricus* showed lowest antimicrobial effect against both indicator strains. The strongest antimicrobial effect was shown by *L. acidophilus*. This activity may have been attributed to bacteriocins produced by the antagonistic activity of lactic acid bacteria as observed by [21] [22]. Many investigations have confirmed the antagonistic activities of lactic acid bacteria in humans and animals.

Bacteria must tolerate bile salts concentration for their colonization and metabolic activity in the gastrointestinal tract [23]. However, there was no consensus about the precise concentration *Lactobacilli* strain should tolerate. It is necessary to evaluate the resistance ability of isolated *Lactobacilli* to bile acids before using them as probiotics [24]. This study shows that isolated *Lactobacilli* were tolerant to high concentration (1.0%) of bile salt (Sodium desoxycholate). *L. lactis* 2, *L. rhamnosus* and *L. salivarius* showed minimum resistance at 0.3% and 1.0% concentration of bile salt. These results of resistance against bile salt are supported by other authors who reported that *Lactobacilli* which were isolated from milk products showed resistance to bile salt [25] [26]. Although, research has shown that intestinal strains have more resistance [27]. Resistance ability varies among *Lactobacillus* species as well as among different strains [28]. Because resistance to bile salt is due to the presence of bile salt hydrolase (BSH), an enzyme that reduces toxic effects by conjugating bile. BSH activity is mostly found in the species of *Lactobacillus* which are isolated from feces or intestines of animals [29].

In conclusion, lactic acid bacteria were successfully isolated from fermented cow milk (nono). The characterization of isolates on the basis of microscopic analysis and biochemical properties (phenotypic characterization) is very useful being the most widely recognized and accepted method. Molecular approach based on 16S rDNA restriction analysis should be used for specificity of species because it is believed that identification using carbohydrate is ambiguous and unreliable. *Lactobacillus* species from cow milk are all excellent candidates for further *in vitro*/*in vivo* characterization for application as probiotics in animals to increase production, degrade gluten and cholesterol lowering due to presence of bile salt hydrolase (BSH); bio-preservation of food using bacteriocin produced and gastrointestinal tract delivery vector in both animals and humans.

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