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Original Research Article

Characterization of wild lactic acid bacteria for industrial applications

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Abstract

For some decades now, Lactic acid bacteria (LAB) have been an important raw resource in the food and pharmaceutical industries to produce variety of dairy based products including but not limited to cheese, yoghurt, lactic acid. drugs and cosmetics. The LAB strains include the genera of *Lactobacillus* and *Streptococcus*. The importation of raw materials such as lactic acid, which can be obtained from indigenous sources, however, affects the foreign exchange of Nigeria's economy greatly. This study was designed to screen, characterize and identify LAB for industrial applications. A total of eighty (80) Lactic Acid Bacteria (LAB) strains were isolated from samples of *nunu* (a locally fermented milk product) using basic microbiological procedures such as morphological/phenotypic, gram staining, and other biochemical tests. All the isolates were tested for their acidification, curd formation and aroma production abilities. After forty-eight (48) hours of incubation at 42^0C, twenty-three (23) isolates which satisfied established phenotypic and biochemical criteria were selected for further functional tests. Out of the twenty-three (23) isolates obtained, two (2) strains L53 and L12 had additionally high exopolysaccharide production ability and correspondingly produced yoghurt with high viscosity and very low residual content. Based on the remarkable characteristics obtained from these two species, they are therefore recommended for optimization and proposed for large scale utilization as starter cultures in the dairy industries.

Keywords: *LAB, nunu, indigenous, strain, industrial application*

INTRODUCTION

In many parts of the world, African continent inclusive, the traditionally fermented dairy products have remained the most abundant nutrient-sourced popular foods [1]. They serve as a means of preservation and a way of coping with lack of access to cold storage facilities that are common in such areas. This phenomenon is more popular among cattle rearers and pastoralists in some parts of West Africa [2]. In such areas, the milk obtained from the dairy animals are either drunk fresh or converted to fermented products as a form of value addition and extension of shelf-life [3]. Today, the organoleptic and nutritional characteristics of many food products are frequently being improved due to the activity of known strains of microorganisms used in the fermentation process [4]. The major group of bacteria involved in the fermentation of milk are Lactic acid bacteria

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(LAB). In several literatures, LAB has been described as an heterogeneous group of bacteria which plays a significant role in a variety of fermentation processes both in history and in modern terms. They are gram positive, catalase negative and non-motile organisms that ferment carbohydrates to produce lactic acid as the main product of fermentation. In addition, degradation of proteins and lipids and production of various alcohols, aldehydes, acids, esters and sulphur compounds contribute to the specific flavor development in different fermented products [5].

LAB usually occurs as mixed cultures in the traditional spontaneously fermented milk product like 'Nunu'. However, strains of known characteristics are required in the production of fermented products of certain desirable and predictable qualities in which case, selection of LAB strains with certain known features are sought for [6]. In the search for the identification of cocci and bacilli, classical microbiological techniques employing basic cultural characteristics, morphology, Gram reaction, motility, presence of catalase enzyme, and the spectrum of fermented carbohydrates are usually used in addition to the modern molecular techniques which utilize the analysis of the 16S rRNA gene sequences [7]. These are followed with screening for criteria that enables the particular strain to stand out in terms of the genotypic ability to produce product of consumers' overall acceptance.

In an attempt to discover novel LAB strains that possess excellent functional properties and the potential for application in industries, scientists are in constant search for unique qualities from a variety of LAB- traditionally fermented products [7]. The determination of the performance of newly isolated strains for yogurt manufacturing consequently needs to be further evaluated using advanced classical skills in order to obtain high-performing strains that could enrich the flavor of the product as well as satisfaction of consumers' demands on a sustainable basis [8].

From historical perspectives, traditionally fermented products have low acceptability outside the native folks that consume them due primarily to their short shelf-life, perceived low hygienic quality and generally unpredictable characteristics [9]. Several reports have however suggested the use of controlled fermentations using selected, well-characterized strains to overcome this challenge. However, the selection of pure-culture strains with desirable quality requires skills and expertise that are not easy to come by [10]. The continuous use of back-slopping, non-utilization of starter cultures and small-scale household production of dairy products limits their availability to the local consumers which affects gross earnings and profitability [11]. Also, the reliance on spontaneous fermentation of milk leads to variability in the microbial consortium present in the fermented product due largely to the poor hygienic and handling processes, which invariably affects the quality of the final product culminating in the incidence of foodborne diseases [12].

One important step towards improving and standardizing the fermentation process for dairy traditional fermented products is the development of functional starter cultures. which will enable controlled fermentations with well-characterized strains which will in turn support broader range marketability of the product to the urban consumers without fear of health threats [12]. To achieve this, an accurate understanding of the lactic acid bacteria involved during the fermentation process is required. Unfavorable alterations in the composition of the microbial community of several human population that are often at one time or the other caused by ecological, pharmacological, and other stress factors may be ameliorated by enrichment of the gastro-intestinal tract microbiota with some quantities of beneficial microorganisms such as lactic acid bacteria as probiotic that could help suppress the complex undesirable imbalance of the intestinal microbiomes [13].

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The development of starter cultures that can withstand the environmental conditions by being compatible and efficient will help overcome the challenge of importation of exotic strains thereby conserving foreign exchange. Locally available handy starter cultures with long shelf-life will also pave way for the existence of more indigenous dairy industries to leverage on and expand which will consequently reduce unemployment and crime rates in the respective environments.

Lastly, the consumption of probiotics in adequate amounts has some beneficial effects on human and animal health. The active development of starter cultures for functional foodstuffs containing probiotic microflora with desired beneficial properties will advance both the health sector because the biologically active compounds produced by several LAB can positively affect human health and may contribute to the prevention of certain diseases aside encouraging local dairy industries' sustainability [14]. Therefore, the aim of this research was to isolate, identify and screen the predominant lactic acid bacteria (LAB) involved in the fermentation of *nunu* to produce standard starter cultures for application in modern dairy industries in Nigeria through the investigation of the potential technological and functional characteristics including tolerance to low pH and bile salt, exopolysaccharide production and antimicrobial activity of the LAB isolates against common foodborne pathogens.

METHODOLOGY

Collection of samples

Using the method of Ismail *et al*. [15], traditionally fermented nunu were collected from five farm settlements namely: Karshi, Kurudu, Idu, Gwagwa, and Karmo, producing the dairy product in the Federal capital territory, Abuja, Nigeria. All samples were collected in labeled sterile containers and transported to the Food and Industrial Biotechnology laboratory in ice packs for analysis.

Experimental set-up

Isolation of presumptive lactic acid bacteria

A total of eighty (80) strains of LAB were isolated from *nunu* using basic microbiological procedures including morphological/phenotypic, gram staining, and other biochemical tests. The method of Maqsood and Masud [16] was used for the isolation of LAB with de-Man Rogosa and Sharpe (MRS) agar and M17 agar for rods and cocci respectively. One milliliter (1mL) aliquot of each sample was introduced into 9ml sterile peptone water and serially diluted up to 10^{-6}. An aliquot of 0.1ml from 10^{-2}, 10^{-4} and 10^{-6} dilutions were pour-plated and incubated anaerobically in an anaerobic jar pack for 24-48 hours at 37^0C and 42^0C for the rods and cocci respectively. After the incubation period, the respective morphological characteristics of the developed discrete colonies from each agar were examined, and representative colonies were selected from appropriate dilutions and sub-cultured by series of streaking in order to obtain pure cultures which were maintained on sterile media for subsequent analysis.

Characterization of the isolated LAB

The pure culture obtained were morphologically characterized using features such as the size, shape and color under the microscope as well as other biochemical assays such as Gram staining, catalase enzyme reaction using 3% (v/v) hydrogen peroxide, oxidase reaction using an oxidase reagent and sugar fermentation tests using different sugars.

Screening of the LAB for technological properties

All the isolates were tested for acidification, curd formation and aroma production abilities in line

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with the method of Tamime *et al*. [17].

Acid tolerance tests

The pure, single colony from each culture was sub-cultured from the stock culture and suspended in 1 ml sterile MRS and M17 broth in order to get the inoculum with a final cell concentration of 10^7–10^8 CFU/ml. From the suspension, one ml was added to broth media previously adjusted to pH 3 and 7 using 2 M HCl and 2 M NaOH respectively and subsequently incubated following the procedure of Tamime *et al*. [17]. Afterwards, the survival of the bacteria was determined by plating on MRS and M17 media and incubating anaerobically while the viable cells were counted as CFU/ml.

Tolerance to bile salt

The bile salt tolerance of the isolates was determined by introducing 1 ml of 10^7–10^8 CFU/ml into sterile broth containing 0.6% (w/v) bile salts and incubated for 6 hours after which the survival and viability of the bacteria were obtained by plating on respective media and incubated for 24 hours according to the method of Tamime *et al*. [17].

Acidification activity test

Pasteurized Skimmed Milk (PSM) 10% (w/v) was prepared and overnight grown cultures were introduced, incubated for 8 hours and the pH determined at 2-hour interval following the procedure of Elise *et al*. [18].

Exopolysaccharide (EPS) production potentials

Pasteurized Skimmed milk base comprising of 10% (w/v) skimmed milk, 1% (w/v) sucrose, yeast extract and 1.5% (w/v) agar were prepared. Overnight grown cultures were streaked on separate plates and incubated anaerobically at 37 ^{0}C/42 ^{0}C for 48 hours. Isolates unable to produce exopolysaccharide (EPS) were observed to be similar in appearance with the control being non-ropy, while EPS producers exhibit a ropy texture when picked with sterile inoculating loop which is according to the method of Elise *et al*. [18].

Antimicrobial activity of isolates

The inhibition potential of cell-free supernatants (CFS) of the respective LAB cultures against some indicator food pathogens were determined. Antimicrobial activity resulting from direct antagonism between the CFS of LAB isolates and indicator bacteria in liquid media was tested using the method described by Sharma *et al.* [19]. Cell-free supernatants of the LAB were prepared by growing the LAB in the broth for 24 hours with agitation at 125 revolutions per minute (rpm). The broth was centrifuged and filtered aseptically. The separated liquid was used as crude supernatants. Afterwards, 50 µL of the supernatant was loaded in the wells made on previously cultured *Salmonella typhimurium* and *Escherichia coli* on Mueller Hinton Agar (MHA) agar and incubated at 37^{0}C for 24 hours. Streptomycin antibiotic was used as positive control while MRS broth was used as negative control. After the incubation, the diameters of the inhibitor zones on the agar dish were measured and recorded.

RESULTS

Bacterial identification

The results of the 48-hour incubation of samples obtained from Karshi, Kurudu, Idu, Gwagwa, and Karmo microscopically revealed the cells to be majorly rods singly and in chains; circular,

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low convex and white to milky color. The bulk of the isolates were gram-positive, catalase and oxidase-negative and non-motile, clearly exhibiting the primary features of LAB as depicted in Figure 1.

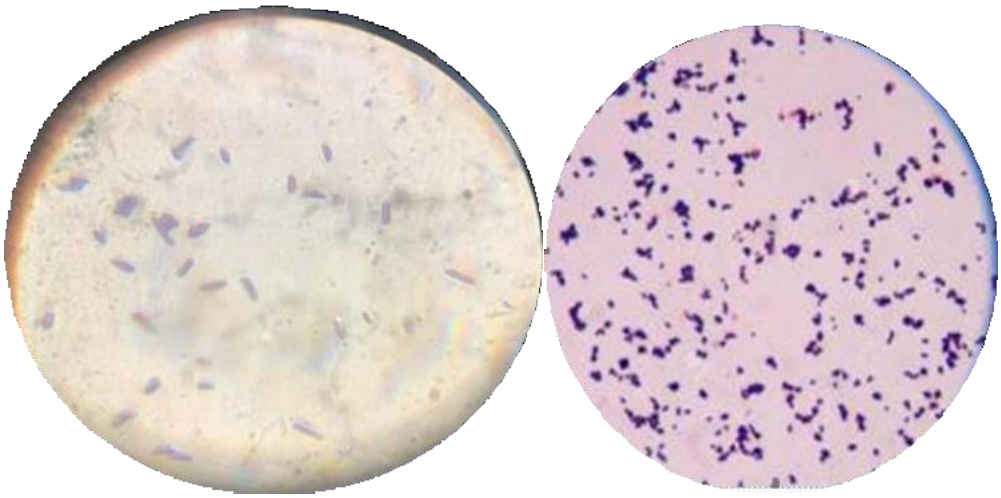

Figure 1: Colony morphology and Gram stain reaction under x100 immersion microscope ascertaining gram positive isolate

A total of eighty (80) LAB strains were isolated from *nunu* samples using the microbiological procedures highlighted. These numbers were screened to four that satisfied the potential criteria of Lactic acid starter cultures. Table I shows the occurrence of the LAB from the different locations.

Table I: The occurrence of lactic acid bacteria in the studied areas

Code	Location	Occurrence (%)
L09	Karshi	34.2
L21	Kurudu	17.8
L53	Idu	29.4
L12	Gwagwa	18.6

Effects of pH and bile on the viability of the test bacteria

The effect of acidity in terms of pH on the viability of the test isolates indicated that all the LAB isolates were tolerant to the acidic conditions being subjected to (Table II).

Table II: The survival of LAB under acid and bile stress conditions

ITEM	Viable Bacteria Count (CFU/ml)			
	pH 7	pH 3	0% Bile salt	0.6% Bile salt
L09	3.2x10^6	2.4x10^6	3.1x10^6	3.0x10^6
L21	5.3x10^6	5.5x10^6	4.1x10^6	4.4x10^6
L53	1.8x10^6	2.3x10^6	3.3x10^6	3.7x10^6
L12	7.2x10^6	6.5x10^6	1.9x10^6	2.5x10^6

The antimicrobial (Plate 1) and exopolysaccharide (Plate 2) production ability of isolates also varied according to the results obtained as shown on Table III. Strain L09 exhibited the highest antimicrobial potential against *E. coli* while L53 recorded the lowest against *E. coli*. In the same vein, all the LAB isolates except L53 displayed long, ropy strands signifying exopolysaccharide production.

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Plate I: Photograph showing antibacterial activity of LAB isolate against E. coli

Plate 2: Photograph showing syneresis (exopolysaccharide) analysis of LAB

Table III: The antimicrobial and exopolysaccharide production ability of the isolates

Item	Antimicrobial Activity Zone of inhibition (mm)		
	S. typhimurium	***E. coli***	
L09	21.5	25.9	++
L21	15.3	19.4	+++
L53	18.3	17.6	-
L12	23.1	14.2	++

+ = Low; ++ = Moderate; +++ = High; and – = None,
Zone of inhibitions measured in mm.

DISCUSSION

Anaerobic cultivation for the isolation of pure bacterial cultures was carried out under anoxic conditions at 37^0C for 24 h on MRS/M17 agar. Under such conditions, bacteria colonies were formed after the period of incubation at a particular temperature. The morphological characteristics of the respective colonies resulted in the production of pure cultures (Figure 1) which was used for subsequent analysis and tests. The research by Bassyouni *et al.* [20] also affirmed the isolation of lactic acid bacteria from dairy products in Egypt using morphological and biochemical analysis.

It is worthy of note that most lactic acid bacteria grow slowly at low pH and bile salt concentration, but acid damage and loss of cell viability may also occur in cells held at extremely low pH since acidic cellular microenvironment modifies carcinogen-induced DNA damage and repair [21]. It was noted that in fermented dairy products, such as yogurt or cultured buttermilk, whether the lactic acid bacteria are viable or injured by the lactic acid and low pH environment, once the desired pH is reached, the product usually becomes stable [21]. In this research, the isolated LAB might possess stable acid tolerant genes as the cultures were able to maintain their technological relevance throughout the stressed time; a feat that was corroborated by similar research conducted by Iqbal *et al.* (2014) [22] who reported that *Lactobacillus. plantarum* MNC 21, *Weissella confusa* MNC 20, and *L. lactis* MNC 24 are fast and high lactic acid producers.

At pH 3, there was a constant viable cell count of around 10^6 CFU/ml of L09atthe 3 h test period, when compared to their numbers in the control at pH 7. There was high viability of L12 and L21cultures after 3 h of incubation, while the viability of others did not reduce. In general perspective, all isolates showed good tolerance to bile, with stable tolerance over the 3 h test period. Exposure to bile salts did not affect their viability. It was however reported that low concentrations of bile salts induced stress responses and reduced motility in *Bacillus cereus* ATCC 14570 [22]. Our isolates have therefore satisfied the criteria to be referred to as probiotic.

Generally, one of the global goals of probiotic bacterial selection involves meeting established safety criteria and being uniquely endowed to produce desirable benefits such as wading off other

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pathogenic and spoilage organisms in their environment. The selection criteria of LAB probiotic are underscored by such criteria including antimicrobial production ability as reported by Adejumo, [23].

The LAB strains isolated in this work exhibited diverse levels of inhibition against the test food bacterial pathogens. L12 was very active against the pathogenic food borne *S. typhimurium* with a 23.1mm zone of inhibition. Similar work was done by Kaewchomphunuch *et al.* [24] who reported that the cell-free culture supernatants of *Lactobacillus* and *Pediococcus* species inhibited the growth of pathogenic *Escherichia coli* isolated from pigs. One noted mechanism of the activity of cell-free supernatants against bacteria pathogens is through secretion of antimicrobial substances, such as bacteriocin and bacteriocin-like substances [25]. All the isolates screened showed antagonistic activities against the test organisms and therefore could be selected for use as yoghurt starter culture when other criteria are met.

Several bacteria are well known for their ability to produce a wide variety of polysaccharides, such as exopolysaccharides (EPS) which are high molecular weight biodegradable polymers formed by monosaccharide residues of sugar and sugar derivatives [26]. Different bacteria groups mostly the lactic acid bacteria (LAB) and bifidobacteria can produce a wide range of carbohydrate polymers during fermentation with several reports of some beneficial roles in the overall functions of the microorganisms producing them as well as their use in yoghurt and other dairy fermented products as stabilizers [27]. Strain L21 isolated from this work was seen to have the highest EPS-production ability (Table 3). The results showed that only L53 does not have the EPS-production ability.

CONCLUSIONS

The subject of this research was the characterization of lactic acid bacteria for multifunctional industrial purposes. Local sources were targeted while four strains were subsequently identified to have suitable criteria such as excellent bile and acid tolerant ability required for further improvement and development as starter cultures namely, L09, L21, L53 and L12. The *Lactobacillus* strains subsequently possessed inherently good antibacterial activity against intestinal bacterial pathogens. The production of EPS by the isolates as well as the cumulative functional potentials highlighted above is requirements met for their selection as yoghurt starter culture for industrial production of safe products.

From the biological and physiological perspectives of LAB starter cultures production from indigenous fermented milk with enhanced functional and technological features, this project results have demonstrated the possibility of the strains in contributing to the development of healthier and consumer-friendly dairy food products. Furthermore, due to dairy product diversifications, highly efficient and viable LAB starter cultures that could exceed the expectations of consumers and achieve the objectives of dairy processors and yoghurt manufacturers are required which further investigation could achieve. Further research is equally recommended to be carried out to develop appropriate conditions for upgrading these indigenously sourced strains for optimal performance.

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