Studies on some indigenous lactic acid bacteria isolated from nono for starter culture production

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Nono is an indigenous yoghurt drink obtained from the mixed fermentation of raw cow milk, basically consumed as a staple food commodity in some parts of West Africa sub region. This study was focused on isolation of some lactic acid bacteria lab from nono identification of the isolates was conducted using standard physiological and biochemical methods. The isolates were identified as *Lactobacillus acidophilus, L. bulgaricus, L casei, L plantarum*, and *streptococcus thermophiles*. In all, *Lactobacillus acidophilus* was the most acid tolerant as it grew better than the *rest of the isolates at a pH of 3 while L.bulgaricus was able to tolerate 0.3% bile acid for 6 hours mo re than the rest of the isolates. L. bulgaricus and L. acidophilus demonstrated high antagonistic activity against helicobacter pylori, Salmonella typhi and Escherichia coli. The lactic acid content showed that there was no significant difference p>5 amongst the isolates. All the isolates were susceptible to all the antibiotics, hence they are considered safe for use as probiotics. The isolates had the ability to ferment milk which was indicated by the increase in bacterial viability with a decreased pH value of 3.9 at the final stage of fermentation. These findings suggest that isolated lab from nono can be used as starter culture for yoghurt production*

Keywords: Nono; Lactic acid bacteria; Probiotics; Starter culture

INTRODUCTION

Nono is an opaque, white to milky coloured, yoghurt-like liquid product that is spontaneously fermented and consumed as a staple food commodity amongst the saharan tribes of the west African Sub region (Nigeria inclusive), extending to the inhabitants of the Mediterranean region and also the middle east. In the Middle East it is called 'dahi' or 'lassi'. Nono contains so me good quantities of amino acid, calcium, phosphorus and vitamins A, C, E and B complex [1]. Its production and consumption derives much food security and economic benefits to the rural people in the region. However, the process characteristics result in products which are not appealing to many people, have very short shelf-life and could have food safety concerns. The traditional production of fermented nono is a spontaneous one involving the action of different types of both pathogenic and beneficial bacteria resulting in a product of questionable quality and reduced consumer acceptance [2].

The enormous benefits of using probiotics for the prevention and treatment of various gastrointestinal disorders are now in the public domain coupled with large experimental and therapeutic evidence [3]. Probiotics are defined as "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host [4]. They play a crucial role in stabilizing the intestinal *Microflora* by competing against pathogens, reducing the incidence of lactose intolerance, prevention of antibiotic-induced *Diarrhoea* and stimulation of the immune system, to mention a few. When selecting lactic acid bacteria as probiotics, especially *Lactobacillus* and *Bifid bacterium*, considerations are always given for conditions that mimic th e gastrointestinal tract. To provide health benefits, probiotics must overcome physical and chemical barriers such as acid and bile in the small intestine; therefore it becomes necessary to test the bile and acid tolerance of potential lactic acid bacteria to be used as probiotics.

The most important criteria for yoghurt production is the selection of starter culture since each culture affects the end product quality differently. Our natural flora and indigenous flavor have consistently been altered due to the introduction of imported commercial starter cultures. Because of the necessity to preserve our natural flora for use as starter culture and increase their availability for industrial use, these cultures must be isolated from artisanal source.

The aim of this study therefore was to isolate and characterize some indigenous lab strains from nono with potential for use as starter culture for yoghurt production.

MATERIALS AND METHODS

Samples of nono that were used in this study were obtained directly and aseptically from *Fulanis* producing milk from local cows within and outskirts of *Abuja metropolis* in sterile universal bottles. The *Lactic Acid bacteria* lab strains were isolated using the pour plate technique. 1 ml of each sample was taken and homogenized in 9 ml of peptone water. Serial dilutions up to 10-6 were prepared and 1 ml aliquots from 10-2, 10-4 and 10-6 dilutions respectively were plated on M^{17} and MRS agar [5]. *Cycloheximide* at a concentration of 1% (v/v) was added into the agar plate, prior to pouring so as to prevent fungal growth. Each sample was plated in duplicate. All plates were incubated for 3 days in *Microaerophilic* conditions using anaerobic gas jar pack system to reduce oxygen level.

Physiological and biochemical identification of isolates

Cell colony morphology

Identification of each colony which was considered as selected lab was conducted by conventional methods based on morphological, biochemical and physiological characteristics of isolates. Standard biochemical methods were used for the identification of the isolated organisms.

The isolates were further characterized on the basis of their sugar fermentation profiles i.e. their ability to ferment different sugars. In this set up, the colour change of the basal medium from purple to yellow and turbidity increase, were recorded accordingly as described by Mehmood.

Assays for probiotic qualities

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The isolated organisms'ability to exhibit certain probiotic characteristics such as bile and acid tolerance during gastro intestinal tract transit as well as their antibacterial activity towards some intestinal pathogenic bacteria was evaluated. Clinical isolates of *Helicobacter pylori, Escherichia coli*, and *Salmonela typhi* was used.

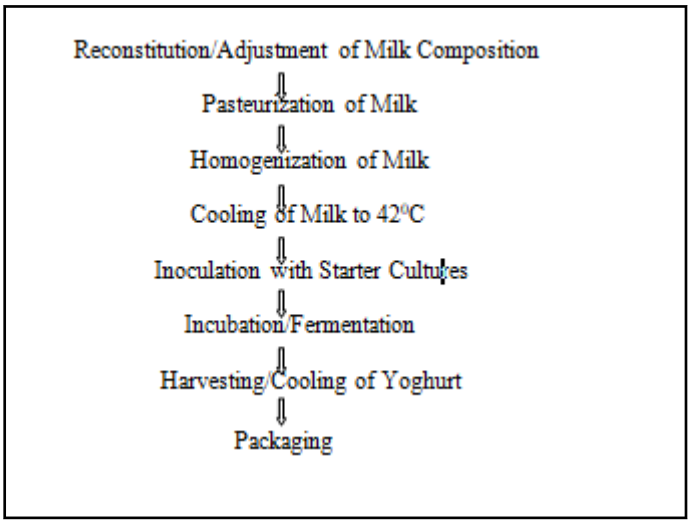
The antibiotic susceptibility status of the isolates was tested using the disc technique as described by [8]. Using freshly grown pure cultures

Fermentation capability of isolates as starter culture for yoghurt production

The ability of the isolates to ferment milk viz-a-viz their use as starter culture for yoghurt production was conducted by inoculating the isolated strains singly and in combination into 8% sterile skim milk and incubated at 42^{o}C for 6 h. Viability of lab was enumerated during the fermentation process using the Total Plate Count (TPC) method, as described by [9].

Flow chart for yoghurt production

The following flow chart outlines the step that was adopted for preparing yogurt.



Flow chart for yoghurt production,

Sensory evaluation of freshly produced yoghurt

The sensory evaluation of produced yoghurt was done on a 5 point hedonic scale (1–worst, 2- bad, 3-good, 4-very good, 5–the best) according to the method of [10]. Samples of yoghurt for sensory evaluation were presented in glass vessels of a volume of 33 ml. The overall acceptability, odour, body/ texture, flavor, mouth feel and overall appearance were evaluated [11]. A trained panel consisting of 10 persons familiar with yoghurt completed the evaluation independently. Their assessments were documented in questionnaires presented to them, analyzed and compared with commercial yoghurt samples [12,13].

RESULTS AND DISCUSSION

Confirmation and characterization of bacterial isolates

The results of the morphologically examined colonies of the isolates with desired characters indicated that all of the isolates have the ability to grow at 4% and 6% concentrations of sodium chloride whilst their growth varied at 2%concentration. These experimental results are in agreement with similar findings of [6]. Who reported that lactobacilli isolated from fermented dairy products were able to grow at 4 and 6.5% NaCl respectively

The capability of the isolates to ferment carbohydrate was also tested during fermentation using different sugars. Almost all the selected isolates were able to utilize hexose sugars like Glucose, Lactose, and Sucrose at different rates. Few isolates were able to ferment Arabinose, while only one isolate was able to ferment the pentose sugar xylose;Consequent upon which the organisms were identified as *Lactobacillus bulgaricus, Lactobacillus acidophilus, Lactobacillus casei, Streptococcus thermophilus* and *Lactobacillus plantarum*. This is as shown in Table 1

Table 1

Confirmatory tests showing the identities of lab isolates

	Growth at different									**Arginine hydrolysis**	**Carbohydrate fermentation**				
	T(0 C)			**pH**	**NaCl**		**Glu**	**Suc**	**Ara**	**Xyl**	**Lac**	**Gal**			
	10	35	40	3	4	6	2	4	6.5						
L. Acidophilus	-	+	+	+	+	+	-	+	+	+	-	-	-	-	+	-
L. Bulgaricus	-	+	+	+	+	+	-	+	+	+	+	+	-	-	+	-
L. Casei	-	+	+	+	+	-	-	+	+	-	+	+	-	-	+	+
S. Thermophilus	-	+	+	-	+	+	-	+	+	+	+	+	-	-	+	+
L. Plantarum	-	+	+	+	-	-	-	+	+	-	+	+	-	-	+	+

Key: - No growth reaction;**+**Growth reaction

Acid tolerance test carried out revealed that all the isolates showed tolerance at varying pH as indicated by cloudiness and change in optical densities. As depicted in Table 2, *L. bulgaricus* and *L. Acidophilus* grew well at pH 4.0 while *S. thermophilus* and *L. plantarum* both grew well at pH 5.0. *L. Casei* had an optimum growth at pH 4.5.

Table 2

Growth of isolates at different pH values (acid concentration) at OD 600 nm

pH values					
Isolates	**3.0**	**3.5**	**4.0**	**4.5**	**5.0**
L. Bulgaricus	0.240 ± 0.0	0.255 ± 0.0	0.354± 0.0	0.248 ± 0.0	0.103 ± 0.0
L. Acidophilus	0.309 ± 0.0	0.356 ± 0.0	0.448± 0.0	0.352 ± 0.0	0.098 ± 0.0
L. Casei	0.182 ± 0.0	0.194 ± 0.0	0.281±0.0	0.272 ± 0.0	0.233 ± 0.0

S. Thermophilus	0.045 ± 0.0	0.201 ± 0.0	0.271± 0.0	0.288 ± 0.0	0.314 ± 0.0
L .Plantarum	0.024 ± 0.0	0.277 ± 0.0	0.298 ± 0.0	0.301± 0.0	0.382 ± 0.0

Values are mean ± standard error of mean duplicate determinations.

Table 3 shows that all lab isolates were able to grow and survive at bile salt condition after six hours which is indicated by the changes in turbidity as well as optical densities of the isolates in the presence of 0.3% bile concentration. This is a prerequisite condition for selection of probiotic organisms

Table 3

Growth of isolates in MRS broth containing 0.3% bile concentration for 6 h at OD 600nm

Time (h)			
Isolates	**2**	**4**	**6**
L.bulgaricus	0.026 ± 0.0 a	0.085 ± 0.0 b	0.141 ± 0.0 c
L. acidophilus	0.012 ± 0.0 a	0.066 ± 0.0 b	0.103 ± 0.0 c
L. casei	0.014 ± 0.0 a	0.074 ± 0.0 b	0.112 ± 0.0 c
S. thermophilus	0.032 ± 0.0 a	0.069 ± 0.0 b	0.135 ± 0.0 c
L. plantarum	0.023 ± 0.0 a	0.037 ± 0.0 b	0.068 ± 0.0 c

Same alphabets in the same column are not significantly different.

Table 4 presents the result of the marked antagonistic activity of the isolates using their cell free supernatant against three intestinal pathogenic bacteria. *L.acidophilus* and *L. bulgaricus* both exhibited the highest antagonistic activity against all the pathogens used. *L. casei* displayed antagonistic activity against H. pylori and E coli but could not suppress the growth of *S. thyphi*. However *S. thermophilus* inhibited the growth of *S. thyphi* but couldn't act against *H. pylori* and *E. coli*.

Table 4

Antagonistic activities of isolates (zone of clearance) against some pathogenic organisms

Indicator organisms			
Isolates	**Helicobacter pylori**	**Escherichia coli**	**Salmonella typhi**
L. Bulgaricus	+	+	+
L. Acidophilus	+	+	+
L. Casei	+	+	-
S. Thermophilus	-	-	+
L. Plantarum	-	+	+

Key: + Presence of zone of inhibition;- Absence of zone of inhibition

Table 5

Antibiotic susceptibility tests of isolates

Antibiotics										
Isolates	**CH**	**SXT**	**SP**	**CPX**	**AM**	**AU**	**CN**	**PEF**	**OFX**	**S**
L. Bulgaricus	+++	++	++	++	++	+	+++	++	+++	++
L. Acidophilus	++	+	+	++	++	+	+++	+++	++	+++
L. Casei	++	+++	++	+	++	++	++	+	+	+
S.Thermophilus	+	+	+	+	++	+	+	++	++	+
L.Plantarum	+	+	++	+	+	++	+++	++	+	+

Key: CH=*Chloramphenicol*(30 ug); CPX =*Ciprofloxacin*(10 ug)SP=*Sparfloxacin*(10 ug); AU=Augmentin(30 ug)AM=*Amoxicillin*(30 ug); PEF=*Pefloxacin*(10 ug)CN=*Gentamycin*(10 ug); S=*Streptomycin* (30 ug)OFX=*Tarivid*(10 ug); SXT=*Septrin*(30 ug)+Zone<10 mm;++Zone=10-20 mm; +++Zone>20 mm; No effect

The susceptibility of the different isolates to various antibiotics was determined and the results are presented in Table 5. The zones of inhibition for susceptible isolates ranged from less than 10 mm to 22 mm and most of the isolates were susceptible to all the antibiotics. The highest zones of inhibition were observed where *L.bulgaricus* and *L. acidophilus* showed highe st sensitivity to *Gentamicin* and less sensitivity to *Augmentin*.

In the same vein, the viability of the lab isolates were determined using total plate count method (TPC) in cfu/ml and the result presented in Table 6. The result as shown in the table suggested that L. bulgaricus was the most viable and had the highest growth of 19.42 x106 cfu/ml followed by L. acidophilus with 16.83 x106 cfu/ml while the lowest growth was displayed by L. casei which had 1.80 x 106 cfu/ml

Table 6:

Isolates' viability using TPC

Isolates	**Total Plate Count (Cfu/ml)**
L. bulgaricus	19.42x106
L. acidophilus	16.83x106
L. casei	1.80x106
S.thermophilus	7.25x106
L .plantarum	3.25x106

Table 7 depicts that there is a general trend of decrease in pH with the passage of time. There was a significantly high concentration of acid produced after six hours (3.98 ± 0.01) by the combination of *Lactobacillus bulgaricus* and *L. acidophilus* making them excellent candidates for use as starter culture while the combination of *Lactobacillus casei* and *Lactobacillus plantarum* had the lowest quantity of acid produced.

Table 7

pH value of milk inoculated with combined isolates during fermentation process

Time (h)						
Isolates	**1**	**2**	**3**	**4**	**5**	**6**
L. b+ L.a	6.1 ± 0.05^{e}	5.60 ± 0.05^{d}	5.15 ± 0.01^{c}	4.59 ± 0.01^{b}	4.01 ± 0.01^{b}	3.98 ± 0.01^{a}
L. b+S.t	5.59 ± 0.01^{f}	4.80 ± 0.00^{e}	4.05 ± 0.01^{d}	4.58± 0.01^{c}	4.49 ± 0.01^{b}	4.22 ± 0.01^{a}

L. a+S.t	6.4 ± 0.0 0^{f}	5.60 ± 0. 01^{d}	5.75 ± 0. 00^{e}	5.45 ± 0. 01^{c}	5.24 ± 0. 01^{b}	4.27 ± 0. 01^{a}
L. a+L.b +S.t	6.3 ± 0.0 0^{f}	5.12 ± 0. 01^{e}	4.20 ± 0. 01^{c}	4.30 ± 0. 00^{d}	4.05 ± 0. 01^{b}	4.01 ± 0. 01^{a}
L. c+L.p	6.1 ± 0.0 5^{e}	5.23 ± 0. 05^{d}	4.85 ± 0. 01^{c}	4.54 ± 0. 01^{b}	4.38 ± 0. 01^{b}	4.56 ± 0. 01^{a}

Values are Mean ± Standard error of mean of duplicate determinations. Same Alphabets in the same column are not significantly different while values with different alphabets are significantly (p 0.05) different along the column.

It could be deduced from the sensorial evaluation on table 8, that the combination of L.a+L.b+S.t was rated highest in appearance followed by combination of L.b+S.t. The combination of L.a+L.b+S.t and the commercial yoghurt were generally accepted followed by combination L.b +S.t.

Table 8

Sensory evaluation of yoghurt produced from combination of isolates

Isolates	Flavor	Texture	Mouth feel	Appearance	Odour	Overall acceptability
L. b+ L.a	3.8	3.7	3.7	3.7	3.6	3.9
L. b +S. t	4.0	4.1	4.0	4.0	3.9	4.3
L. a+ L. p	3.7	3.8	3.9	3.95	3.9	3.8
L. a+L.b +S.t	4.2	4.1	3.8	3.9	4.0	4.1
Cm	3.9	4.0	4.2	4.2	3.9	4.1

Values are mean ± standard error of mean of duplicate determinations

In general, the lab isolates were able to ferment milk for yoghurt production, which was indicated by the increased count of viable lab during fermentation and formation of curd in milk used at the end of fermentation. As shown on Table 8, the viable count of Lactobacillus bulgaricus was 19.42x 106 Cfu/ml which was followed by L. acidophilus having 16.83x106. This observation confirmed the data by [7]. Who reported that the number of lab during fermentation proc ess was rapidly increased from initial count of 1.72x105 to 8.4x108 cfu/ ml. Organisms to be used as probiotic s must be present in sufficient concentration in order to yield therapeutic effects of yoghurt.

CONCLUSION

This study has established that wide varieties of lab are present in nono and lactobacilli are considered to be one of the most important potential starter cultures. Beyond their technological function, demand is currently increasing for new lab strains as starter cultures. Nono, which is naturally endowed with several health benefits, could be a source for obtaining novel strains to be used as starter culture

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