Evaluation of Some Indigenous Lactic Acid Bacteria Isolated From *Nono* for Starter Culture Production

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Abstract

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 thermophilus. 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 more 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

***Key words**: 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 some good quantities of amino acid, calcium, phosphorus and vitamins A, C, E and B complex (Nebedum and Obiakor, 2007).

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 (Owusu-Kwarteng *et al.*, 2010).

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 (Moayyedi *et al.*, 2010). Probiotics are defined as "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" (Fuller R. 1989). 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 *Bifidobacterium,* considerations are always given for conditions that mimic the 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. 1ml of each sample was taken and homogenized in 9ml of peptone water. Serial dilutions up to 10^{-6} was prepared and 1ml aliquots from 10^{-2}, 10^{-4} and 10^{-6}dilutions respectively were plated on M17 and MRS agar (De Man Rogosa and Sharpe, 1960). Cycloheximide at a concentration of 1% (v/v) was added into the agar plate, prior to pouring so as to prevent fungal growth (Wenjun *et al.*, 2012). 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 *et al.*, (2009).

Assays for probiotic qualities

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, Esherichia coli*, and *Salmonela typhi* was used.

The Antibiotic susceptibility status of the isolates was tested using the disc technique as described by Guerin-Faublee *et al.*, (1996) 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 6h. Viability of LAB was enumerated during the fermentation process using the Total Plate Count (TPC) method, as described by Dave and Shah (1997).

Flow Chart for Yoghurt Production

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

Reconstitution/Adjustment of Milk Composition
⇩
Pasteurization of Milk
⇩
Homogenization of Milk
⇩
Cooling of Milk to 42^{0}C
⇩
Inoculation with Starter Cultures
⇩
Incubation/Fermentation
⇩
Harvesting/Cooling of Yoghurt
⇩
Packaging

Flow chart for yoghurt production, Source: Tamime and Robinson (2008)

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 Chandan, (1988). Samples of yoghurt for sensory evaluation were presented in glass vessels of a volume of 33ml. The overall acceptability, odour, body/texture, flavor, mouth feel and overall appearance were

evaluated (Routray and Mishra, 2011). 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.

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 Schillinger *et al* (2005) 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 600nm

	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 6h 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(30ug); CPX =Ciprofloxacin (10ug) SP = Sparfloxacin (10ug); AU =Augmentin (30ug)
AM =Amoxicillin (30ug); PEF =Pefloxacin (10ug) CN = Gentamycin (10ug); S= Streptomycin(30ug)
OFX =Tarivid (10ug); SXT=Septrin(30ug)
+ Zone<10mm; ++ Zone=10-20mm; +++ Zone> 20mm; - 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 10mm to 22mm 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 highest 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 X 10^6 cfu/ml followed by *L. acidophilus* with 16.83 x 10^6 cfu/ml while the lowest growth was displayed by *L.casei* which had 1.80 x 10^6 cfu/ml.

Table 6 Isolates' viability using TPC

Isolates	**Total Plate Count (Cfu/ml)**
L. bulgaricus	19.42 x 10^6
L. acidophilus	16.83 x 10^6
L. casei	1.80 x 10^6
S. thermophilus	7.25 x 10^6
L .plantarum	3.25 x 10^6

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±0.00^{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±0.00^{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±0.05^{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 19.42 x 10^{6} Cfu/ml which was followed by *L. acidophilus* having 16.83 x 10^{6}. This observation confirmed the data by Antara *et al* (2002) who reported that the number of LAB during fermentation process was rapidly increased from initial count of 1.72x105 to 8.4x10^{8} cfu/ml. Organisms to be used as probiotics must be present in sufficient concentration in order to yield therapeutic effects of yoghurt.

Conclusions

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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Recommendations

It is recommended that a higher concentration of probiotic bacteria greater than the usual 10^6 cfu/g should be present in probiotic food products in order to confer the claimed benefit to consumers. This is very important considering the fact that a reasonable amount of the population of these bacteria would be eliminated during their passage through the gastrointestinal tract.

Microencapsulation is a current technology that creates a barrier between bioactive compounds and protects them against harsh environmental conditions. It is recommended for use in order to achieve this required concentration considering the hurdle being encountered by these organisms in the GI tract, which will ensure that they reach the targeted site in controlled amounts and as at when needed.

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