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Molecular Characterization of Lactic Acid Bacteria Isolated from locally produced 'nono' in owo lga, Ondo State.Nigeria.

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ABSTRACT:
Lactic acid bacteria (LAB) were isolated from locally produced 'nono' samples from three different locations in Owo, Ondo State (South Western Nigeria). Microbiological analysis was carried out using serial dilution and pour plate method for the isolation. The Agar used was De Mann Roggosa Agar (MRS) Oxoid, Nutrient Agar and Nutrient Broth (Oxoid). Four (4) species of (LAB) were mostly present in the "Nono": *Lactococcus lactis, Lactobacillus plantarum, Lactobacillus casei* and *Enterococcus faecalis.* Further identifaction at the species level indicated that all the lactococci isolates were *L. lactis* while three isolates of the *Enterococci* were *Enterococci faecalis* and *lactobacilli* were identified as *Lactobacillus* plantinum (1 Isolate). *Lactobacillus casei* (2 Isolates). PCR – RFLP method which is based on the amplification of 16s rRNA – ITS genes was used for the molecular characterization.

Keyword: *"Nono", Lactic Acid bacteria, molecular characterization.,*

INTRODUCTION:

"Nono", 'Nunu', in English language and Hausa Language is a spontaneously fermented yoghurt – like product, produced and consumed in some parts of West Africa (Akabanda *et al.;* 2014) unlike other fermented milk products where milk of goats, sheep and camels is used. Nono is solely prepared from Cow milk. It is yoghurt like in taste (a sharp, acid taste) and it can be taken alone or with "fura" (Owusu – kwarteng *et al.*, 2012; Akabanda *et al.*, 2013).

turning the product sour within few days. Milk nutritional composition makes it not only suitable for human nutrition but also ideal for microbial life (Nebedum and Obiakor, 2007; Paula, 2014)

MILK COMPOSITION: Milk is a white fluid secreted by female mammals for the purpose of rearing their offspring (O'Connor, 1993).The earliest tribes of ancient Egypt and south-west Asia discovered sometime around 5000BC that cow milk was a nourishing human food. While the ancient Egyptians recognized that cow was a wholesome and sustaining food they had little knowledge of its composition. The earliest evidence of knowledge of the composition of milk

Predominantly, "Nono" is being hawked by the nomadic Hausa or Fulani women, who control over 80% of Nigerian's cattle production (Obi and Ikenebomeh, 2007; Adesokan *et al.*, 2011). Nono contains good quantities of amino acids, calcium, phosphorus and vitamins A, C, E and B complex (Nebedum and Obiakor, 2007).

"Nono" is processed by collecting fresh Cow milk and allowing it to ferment for a day or two (Akabanda *et al.*, 2014). Raw milk has low keeping quality and at room temperature spontaneous microbial spoilage occurs is dated back to 350BC when Aristotle wrote that casein, fats and water are all the known substances of milk. Since the middle of the 19th Century knowledge of the Chemistry of milk has been developed and today there is extensive literature on the chemistry of the major and minor constituents of milk especially cow milk, variations exist in the composition of milk and for the various species of these animals.

The composition of Cow milk varies for a number of reasons e.g. individuality of the cow, the breed, age, stage of lactation, health of the cow, climatic conditions and herd management which includes feeding and general care.(Silanikove and Merin,2016)

Table 1: Proximate composition of milk from various species of mammals

ANIMAL		COW	GOAT	EWE	CAMEL	BUFFALO
Fat		3.75	6.0	9.0	3.0	6.0
Casein		3.0	3.3	4.6	4.5	3.8
Lactose		4.75	4.6	4.7	5.5	4.5
Albumin		0.4	0.7	1.1	1.7	0.7
Ash		0.75	0.84	1.0	1.5	0.75
Water		87.3	94.4	79.6	84.8	85.0

Source: Connor, 1993.

Fermentation is the conversion of sugar into organic acid or an alcohol. Fermentation occurs naturally in many foods and humans have intentionally used it since ancient times to improve both the preservation and organoleptic properties of food. (Zhony, 2011). However, the term "fermentation" is also used in a broader sense for the intentional use of microorganisms such as bacteria, yeast, and fungi to make products useful to humans (biomass, enzymes, primary and secondary metabolites, recombinant products and products of biotransformation) on an industrial scale (Willaert and Neclovic, 2006).

Fermentation is also a term used by microbiologists to describe any process for the production of a product by means of the mass culture of a microorganism (Mitchell *et al.,* 1999). The product can be either the cell itself (biomass production) or microorganisms metabolite or products.

LACTIC ACID BACTERIA: Generally Lactic acid bacteria (LAB) can be defined as Gram positive, non – spore forming, catalase negative devoid of cytochromes, acid tolerant and facultative anaerobe group that produce lactic acid as the major end product during fermentation of carbohydrate (Cisem, 2000). According to carbohydrate metabolism, they can be divided into two main groups:

(1) Homo fermentative lactic acid bacteria produce mainly lactic acid.

(2) Heterofermentative lactic acid bacteria produce lactic acid, carbon-dioxide, ethanol and others.

This classification originated from metabolic routes that these organisms used and the resulting end-products. While homofermentatives use glycolysis (Embeden-Meyerhof pathway) heterofermentatives use the 6 –phosphogluconate or phosphoketolase pathways (Doo Hyun, 2018)

Although LAB comprises of eleven genera, only six of them are dairy associated. These are Lactococcus,Enterococcus,Pediococcus and Lactobacillus (De Martins *et al*,;2011; Erich *et al*.;2018).Dairy microflora is further divided into two groups. Primary group includes starter flora which refer to starter LAB and secondary group includes non starter lactic acid bacteria (NSLAB), propionic acid bacteria (PAB) smear bacteria, moulds and yeast (Beresford *et al*., 2001).

MOLECULAR IDENTIFICATION OF LACTIC ACID BACTERIA: Identification of bacteria isolated from natural microflora involved in milk fermentation has been limited by the complexity of the bacteria association (Garvie, 1984). Additionally, bacterial population involved has similar nutritional and environmental requirements. As a result, the application of molecular methods can be used to resolve identification problems. Nucleic acid probe technology could be an alternative for faster and more reliable differentiation. Several species – specific probes have also been designed.

Furthermore, 16s or 23s rRNA targeted *oligonucleotide* have been used for the specific identification of lactic acid bacteria (LAB). Hence it is now possible to identify various lactic acid bacteria in fermented food without cultivation step at species level within one day (Scheifer and Ludwing, 1995). Additionally, DNA restriction fragment analysis and ribotyping have been used to distinguish Lactic acid bacteria (LAB), especially polymerase chain reaction based methods (PCR – RFLP, REP – PCR, PCR ribotyping and RAPD). Pulse field gel electrophoresis can be used as main molecular tools (Farber, 1996 and olive, 1999).

TABLE 3: Procedural steps of main genotypic methods

RAPD	**PFGE**	**REP – PCR**	**AFLP**	**DNA Sequencing**
PCR Amplification With single Primer ↓ Gel Electrophoresis ↓ Gel Staining ↓ Interpretation	Embeded Organisms in Agarose plug ↓ Protease Digestion ↓ R.E.Digestion ↓ Electro – phoresis ↓ Interpreta – tion	PCR Amplification With REP or ERIC primers ↓ Gel Electrophoresis ↓ Gel Staining ↓ Interpretation	R.E.Disgestion ↓ Linker Ligation ↓ Selective PCR ↓ Gel E. Through an Automated DNA Sequencer ↓ Gel Interpretation	PCR Sequencing Reactions ↓ Gel Electrophoresis ↓ Computer aided Sequence analysis ↓ Interpretation

Source: Cisem, 2003.

LACTIC ACID FERMENTATION

Fermentation is an energy yielding microbial metabolism in which an organic substrate usually carbohydrate is incompletely oxidized and an organic carbohydrate acts as the electron acceptor (Adams, 1990). Fermentation is either because "they lack an electron transport chain (Nester *et al.,* 2004), they lack the ability to respire; they only ferment, regardless of the presence of oxygen (O_2)" (Nester *et al.,* 2004). They can grow in the presence of oxygen but never use it as terminal electron acceptor. This is why they are sometimes called obligate fermentors (Nester *et al.,* 2004).

MATERIALS AND METHODS

SOURCES OF MATERIALS: Freshly prepared Nono were obtained from Fulani hawkers from three different locations; Iyere, Oke- Ogun and Ikare junction in Owo, Ondo State. Three samples were collected from each of these locations in an ice-packed and the samples were taken to the laboratory for microbiological analysis.

PREPARATION OF CULTURE MEDIA

The media used are De Man Rogosa and Sharpe, (MRS), M17 Agar and broth (Oxoid) for isolation of Lactic acid bacteria. The culture media were prepared according to manufacturer's instructions and sterilized in the autoclave at 121^{o}C and 15psi for 15 minutes.

Microbiological analysis: Serial dilution was carried out using the sample and pour plate method was used. The Agar used were De Mann Roggosa Agar (MRS) Oxoid, Nutrient Agar and Nutrient Broth (Oxoid) .Enumeration and identification was carried out by total viable count while morphological characterization using colour, texture shape and size of the isolate while biochemical characterization were carried out using catalase Urease, Coagulase and sugar fermentation on the isolates for identification.

BACTERIAL IDENTIFICATION: The various bacteria colonies were sub – cultured to obtain pure culture. The isolates were coded and maintained on an MRS Agar slants and stored at refrigerated temperature. They were identified based on colonial, morphological, biochemical and genotypic characteristics.

MOLECULAR CHARACTERIZATION:

Extraction of DNA using CTAB method: Bacteria isolates grown overnight was transferred to eppendorf tube and it was spun down at 14,000rpm for 2mins, the supernatant was discarded and 600µl of 2X CTAB buffer was added to the pellet and it was incubated at 65^{0}C for 20mins according to Akinyemi and Oyelakin 2014. The sample was removed from the incubator and allowed to cool to room temperature and chloroform was added, the sample was mixed by gentle inversion of the tube several times. Thereafter, the sample was spun at 14,000rpm for 15mins and the supernatant was transferred into a new eppendorf tube and equal volume of cold Isopropanol was added to precipitate the DNA. The sample was kept in the freezer for 1hr and later spun at 14,000rpm for 10mins and the supernatant was discarded and the pellet was washed with 70% ethanol, later the sample was air dried for 30mins on the bench. The pellet was re-suspended in 100ul of sterile distilled water. DNA concentration of the samples was measured on spectrophotometer at 260nm and 280nm and the genomic purity were determined. The genomic purity was between 1.8 –2.0 for all the DNA samples.

DNA Electrophoresis

Agarose gel electrophoresis was used to determine the quality and integrity of the DNA by size fractionation on 1.0% Agarose gels. Agarose gels were prepared by dissolving and boiling 1.0g Agarose in 100ml 0.5X TBE buffer solutions. The gels were allowed to cool down to about 45^{o}C and 10µl of 5mg/ml ethidium bromide was added, mixed together before pouring it into an electrophoresis chamber set with the combs inserted. After the gel has solidified, 3µl of the DNA with 5µl sterile distilled water and 2µl of 6X loading dye was mixed together and loaded in the well created. Electrophoresis was done at 80V for 2 hours. The integrity of the DNA was visualized and photographed on UV light source.

PCR analysis using I6S primer PCR analysis was run with a universal primer for bacteria called 16S. The PCR mix comprises of 1µl of 10X buffer, 0.4µl of 50mM $MgCl_2$, 0.5µl of 2.5mMdNTPs, 0.5µl 5mM forward primer, 0.5µl of 5mM reverse primer, 0.05µl of 5units/µlTaq with 2µl of template DNA and 5.05µl of distilled water to make-up 10µl reaction mix.

The PCR profile used is initial denaturation temperature of 94^{0}C for 3mins, followed by 30 cycles of 94^{o}C for 60sec, 56^{o}C for 60sec, 72^{o}C for 120sec and the final extension temperature of 72^{o}C for 5mins and the 10^{o}C hold forever.

Purification of PCR products

The amplicon is further purified before the sequencing using 2M Sodium Acetate wash techniques. To about 10µl of the PCR product, add 1µl 2M NaAct pH 5.2, followed by 20µl absolute ethanol, keep at -20^{0}C for 1hr, spin at 10,000rpm for 10 minutes, then wash with 70% ethanol and air dried. Re-suspended in 5µl sterile distilled water and keep at 4^{0}C for sequencing.

PCR for sequencing

The primer used for the reaction was forward I6S. The PCR mix used includes 0.5µl of Big Dye Terminator Mix, 1µl of 5X sequencing buffer, 1µl of M13 forward primer with 6.5µl Distilled water and 1µl of the PCR product making a total of 10µl. The PCR profile for sequencing is a rapid profile. The initial Rapid thermal ramp to 96^{o}C for 1min followed by 25 cycles of rapid thermal ramp to 96^{o}C for 10 seconds, rapid thermal ramp to 50^{o}C for 5 seconds and rapid thermal ramp to 60^{o}C for 4 minutes, then followed by rapid thermal ramp to 4^{o}C and hold forever.

Purification of PCR sequencing products

The PCR sequence product is also purified before the sequencing running using 2M Sodium Acetate wash techniques. To 10µl of the PCR product add 1µl 2M NaAct pH 5.2, then add 20µl absolute ethanol, keep at -20^{o}C for 1hr, spin at 10,000rpm for 10 minutes, then wash with 70% ethanol and air-dried. Re-suspend in 5µl sterile distilled water and keep at 4^{o}C for sequencing running.

RESULTS AND DISCUSSION

The total bacterial growth on MRS Agar ranges between 4.2x 105 and 20x 10^{5} CFU/ml and total bacterial growths on M17 agar ranges between 3.0x10^{5}CFU/ml and 10x10^{5}CFU/ml as shown in Table 1 below:

Table 1: Total Bacterial Counts in Nono Samples from Three Agars Used

SN	SAMPLES LOCATION	MEDIA MRS AGAR	USED MI7
1a	GRA(Housing,Owo)	20x10^{5}CFU/ml	10x10^{5}CFU/ml
2b	Ikare	4.2x10^{5}CFU/ml	3.0x10^{5}CFU/ml
3c	Junction.	4:5x10^{5}CFU/ml	3:5x10^{5}CFU/ml
	Iyere (Owo)		

RESULT OF GENE SEQUENCING 01

GATCCGTCTTAGGTGACATGGCTCAGGTGCGCTTGGT
CACCCCCTTAGAGTTTGAGCGGGCTCGCTTGCGCTGT
GTCTGGCCTCTGCCTGATGGAGGGGGATAACTACTGG
AAACGGTAGCTAATACCGCATAACGTCGCAAGACCA
AAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTG
CCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTC
ACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGA
CCAGCCACACTGGAACTGAGACACGGTCCAGACTCCT
ACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGC
GCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAA

GGCCTTCGGGTTGTAAAGTACTTTCAGCGAGGAGGAA
GGCATTGTGGTTAATAACCACAGTGATTGACGTTACT
CGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGC
CGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATT
ACTGGGCGTAAGCGCACGCAGGCGGTCTGTAGTCAG
ATGTGAAATCCCCGGGCTCACCTGGGAACTGCATTGA
ACTGACAGCTTGAGTCTATGTAAGAGGGGGGGTAGAT
TCAGGTGTAGCGGGTGAATGCTAGAGATCTGGAGATA
CTGGTGGCGGAGGGCCGCCTCCTGACAAAGACTGAC
GCCTCAGGTGCAAAGTCGTGGGGGAGCAACATGATA
AGATACCTGTAGTCACGCCGTAAACGAGTCCACTGGA
GGTGGCCTTGAGGCGTGGCTCGACTTACCGTTAGTCC
ACCGCCTGGGAGATACGCCAAGGTAAAATCAATGATT
GCCGGCTAGA

Streptococcus sp.6151	CP046557.1
Streptococcus sp. J807	CP046446.1

02

GGTTATCTTGTGTCGACATGGCTCAGGTGCGCTGGGA
CCACTCCTTTATAGTTTGACTGGGCTCGGGGGCGTTG
GATCCCCCCCTTAAAATTTTTAGCTGGCCCCAGGTCC
GGGGGACCGCCCCTAATAAAGAATAATCTGTTTCACC
TCATGGTGAAATATTGAAAGACGGTTTCGGCTGTCGC
TATAGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGA
GGTAACGGCTCACCAAGGCGACGATGCGTAGCCGAC
CTGAGAGGGTGATCGGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATC
TTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCG
CGTGAGTGAAGAAGGATTTCGGTTCGTAAAACTCTGT
TGTAGGGAAGAACAAGTACAGTAGTAACTGGCTGTA
CCTTGACGGTACCTTATTAGAAAGCCACGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGTGGCAAGCGT
TGTCCGGGAATTATTGGCGTAAAGCGCGCGCAGGTGG
TTTCTTAAGTCTGATGTGAAGCCCCACGGCTCACCGT
GTAGGTCATTGCAACTGGGAGACTTCAGTGCAGAGAG
GATAGTGATTCCAGTGTAGCGGTGAATGCGTAGAGAT
TGGAGACACAGTGCGAGCGACTATTCTGGTCTGTACT
GACACTGAGGCGCGAAAGCGTGGGAGCAACAGATAG
ATACCTGTAGTCCACGCGTTACGATGGAGTGCTAGTG
TAGGGCTTCGCTCCTTAAGTGCTGCAGCTTACGCATA
GCATCCGCTGGGGAATACGTTCCTAGACTGAATCCTC
AAGGGAT

Lactobacillus lactis strain HCC	KX908104.1
Lactobacillus casei. strain TC3 1	MK078608.1

03

GGTTTAGTCTTATAACTGACATGGCTCAGGTGCGTTG
GACCACCTCTTTATAGTTTAGCGGCGGACGGGTGAGT
AACACGTGGCTCCCTGCCTGTAGACTGGGATAACTCC
GGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACC
GCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCAC
TTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGA
GGTAACGGCTCACCAAGGCAACGATGCGTAGCCGAC
CTGAGAGGGTGATCGGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGGAGGCAGCAGTAGGGAAT
CTTCCGCAATGGACGAAGTCTGACGGACAACGCCGCG
TGAGTGATGAAGGTTTTCGGATCGTAAGCTCTGGTTG
TTAGGGGAGAACAAGTACCGTTCGAATAGGGGCGGT
ACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACT
ACGTGCAGCAGCCGCGGTAATAACGTAGTGGCAAGC
GTGTCGGAATTATTGGGCGTAAGGGCTCGCAGCGTTC
TAAGTCTGATGTGAAGCCCCGGCTCACGGGGAGGTCA
TGGAACTGGGAACTGGATGCGGAGAGGGAGGGGAAT
TCCACTGTACGTGAATGCTAAAGATGGAGAACCATGG
CAAGCACTCTGTCTGAACTGACTAGCAACGTGGAGCC
TGATAGATCTGGATCCAGCTAACGATGCCTAGTGAGG
ATCCGCTTAGTCGCGAAGCCTAGGCCTCTCCGGAGAT
CGTCAAAAAA

Lactobacillus lactis	AY971358.1

Lactobacillus sp.

04

GGATCAATTCTAGGATTGTCATGGCTCAGGTGCGCTT
GGTCCCCCTTTAGAGTTTGACTTGGCTCGGGGGGGGT
GGGTCCCCCCTTAAAAGTTTGACTGGCCCCAGGGCCG
GTGGAGCCCCCTCTTTAAATTGGTTGTTTGAACCCGTG
GTTCAAAAAAAAAAAAAGATTCGGCTACCACTTACA
GATGGGCCCGCGGCGCATTATCTAGTTGTGGAGGTAA
TGGCTCACCAAGGCAACAATGCGTAGCCGACCTGAG
AGGGTGATCGCCCACACTGGGACTGAGACACGGCCC
ACACTCCTATGGGGGGGGGCAGTAGGGAATCTTCTCC
AATGGGCGAAAGTCTGTCGGAGCAACGCCCCGTGAG
TGATGAAGGTTTTTCTGATCTTAAAGCTCTGTTGTTAG
GGGAAAAACACATACCGTTCAAATAGGGGGGGACTT
TGAGGGTACCCTATCCAAAAAGCCCCGGCTAAATACG
TGCCACCACCCCCGGTAATACGTAGGGGGCAAGCGTT
GTCCGGAATTATTTGGGCGTAAAGGGCTCGCAGGCGG
TTTTCTTTAGTCTGATGTGAAAGCCCCGGGCCTCACCC
GCGGAGGGTCTTTGGAAACTGGGGAACTTTAGTGCGG
AGAAGAGAAGGGATTTCCCCTGTGTACGTGAAAGGC
GTAAGATTTGAGAGAACACCCGTGGCAAGGGCATCTT
TTGTCTGTAATCACGCTGAGGAGCGAAAACCTGTGGT
AGCGACAGGATATATACCCCTGTAATCCACAGCCGCT
AACGAAGAAGTGCTAACTGTTAGGAGGTTTCCGCCCC
TTAGTGCTGCAAGATACGCGATTAATATGACATCGCT
CTTGGGAGAGTAATCGTCGTTCTGTCTAGAGACTCGA
TTGAACACTCATTCAAGAGAATGT

Lactobacillus lactis strain IARI-CS-49	JF343143.1
Lactobacillus sp. VP 18	HM150753.1
Lactobacillus sp strain SRR21 e	KY401500.1

05

GGTTTAGTCCAGGTCGACATGGCTCAGGGGCGGTGGG
ACACCCCCTTAGAGTTTGACTGGGCCCGGGGGGGGTGG
ACTCCCCCTTTAGAGTTTAACCCGGGCCCAGGGCCGG
GGGACCACCCCTTATAAAGGATAACATTTTGAACCGC
ATGGTTCAAAATTGAAAGGAGGCTTCGGCTGTCACTT
ATGGATGGACCCGCGTCGAATTAGCTAGTTGGTGAGG
TAACGGCTCACCAAGGCAACGATGCGTAGCCCGACCT
GAGAGGGTGATCGGCCACACTGGGACTGAGACACGG
CCCCAGACTCCCTACGGAGGGAGCAGTAGGGAATCTT
TCCGCAATGGACGAAAAGTTCTGACGGAGCAACCCCC
CCCGTGAGGTGATGAAGGCTTTTCGGGGTCGAAAAAC
TTCCGGTTGGTTAGGGGAAAAAACAAATTGCTAGTTT
GGAATAAGGCTGGCCACCTTGGGACGGGTACCCTAAC

CCGGAAAAGCCGCGGCTTAACTAACGTGCCCACCAGC
CCCCCGGTAATTACGTTAGGTGGCAAGGCGTTTTTCC
GGAAATTTATTGGGGCCATAAAAGCCCCGCCCCAGGT
GGTTTTCCTTTAGGTCTGGATGGGGAAAACCCCCCGG
GCCTCAACCCGGGGGAGGGGGTCCATAGGGAAAACG
GGGGGAAAACTTGGAGGGGCAGAAAAAGAGTA

	Lactobacillus sp. BBT91	FJ981911.1
☑	*Lactobacillus sp.* strain H10WTRM5	MH985183.1

06

GGGTTCAGTCATAGATTGACTGGCTCAGGTGCGGTGG
GACCCCCCTAAGAGTTTGACTGGGCTCGGGGCGGTGG
GTCACCCCCTTTAAATTTTGACCCGGCCCCCGGTGCG
GGGGAAACCCTCCATTAAAGGTTAACCTTTTGAACCG
CATGGTTCAAAATTGAAAAAGGGCTTCGGCTGTCACT
TATGGATGGACCCGCGTCGCATAAGCTAGTTGGTGAG
GTAACGGCTCACCAAGGCAACGATGCGTAGCCCGAC
CTGAGAGGGTGATCGGCCACTCTGGGACTGAGACAC
GGCCCAGACTCCTACGGGGGGCAGCGTAGGGGAATC
TTCCGCATTGGAGGAAAGTCTGACGGAACAACGCCGC
GTGGAGTGAGGAAGGCTTTCGGGTCGTAAAGGGAGG
GGTGGTAGGGAAAACAATTCTAGTTGAATAGCTGGCA
CCTTGGGGGGAACCTAACCGGAAAGCCACGGTTAATT
CATCCCACCCACCCCCCGGGAAATACTAAGGTGCCCA
CCTTTATGGGGGAAATGATTGCGCCTTAAAGCCGGCG
CGAGTGGGATTTTTTTAAGTCCGGTTGGGAAAGCCCC
ACGGCCTCAACCCGTGGAGGGGTCATTGGAAACTTGC
GAGAACTTTGAGGTGCAAAGGGACAA

Lactobacillus casei strain NIOER227		MG205948.1
Lactobacillus lactis strain OU13		KJ626301.1

07

GGTTCCTCTATAACTTGACATGGCTCAGGTGCGCTTG
GACCACCCCTTTATAGTTTGACCTTGGCCCGGGTGCG
TTGGACCTCCCCCTTATAACTTATAGTTTGGGATAACT
CCGGGAAACCGGGGCTAATACCGAATAATCTGTTTCA
CCTCATGGTGAAATATTGAAAGACGGTTTCGGCTGTC
GCTATAGGATGGGCCCGCGGCGCATTAGCTAGTTGGT
GAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCG
ACCTGAGAGGGTGATCGGCCACACTGGGACTGAGAC
ACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAA
TCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCC
GCGTGAGTGAAGAAGGATTTCGGTTCGTAAAACTCTG
TTGTAGGGAAGACAAGTACAGTAGTAACTGGCTGTAC
CTTGACGGTACCTTATTAGAAAGCCACGGCTAACTAC
GTGCCAGCAGCCGCGTAATACGTAGGGTGGCAAGCG
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GGAGGGTCATTGCAACTGGGAGACTTGAGTGCAGAA
GAGGATAGTGCCTTCCAAGTGTAGCCGGTGAATGCGT
AAAGATTGCAGAACACCAGTTGCCGATGCGACTATCT
GATCTGTACTGACACTGAGGCGCGAAGCCTGGGGAG
CAAACAGGATAGAATACCATGGTAGGTTCCACGCCCG
TAACCGATGAGTGCTACGGTCTATGGTATCCGCCCTA
TTAA

Lactobacillus lactis. hb27		
Lactobacillus casei strain		KF011266.1

08

GGGTTCCCTCTATAATCGACATGGCTCAGGTGCGGTG
GGCCACCCCTTTAGAGTTTGTCCTGGGCCCGGGGGGG
GTGGGTCCCCCCCTTTAAATTTTACCTGGGCCCGGGG
GGGGGGGTCCCCCCCCTTTAAAGTTACCCTCGCCCAG
GGGGGGGTTTAAATAATAAAAAAAAAATTCGCCTGC
CCTTATGGCTGGACCCACGATGCATTAGAAAGTTGGC
GAGGTCACGGCTCACCATGGAAAATAATAAGTGGCC
GACCTGCCAGGGTGATCGGCCACACTGGGAACTGAG
ACACGGCCCCAGACTCCTACGGGGGGGCCCCCGTGG
GTTATCTTCCGCGTGGACGAAGTCTGACGAGCTCCCC
CGCGTGGGGTGGGGAGGTTTTCGGGTCGAAAACCCCG
GTTGTTAGGGAAGAACAGTGCCTGTTGAAAAACTGGC
CCCTTGTGGGGCCTAACCCGGAGGCTCGGCGGGGGG
GGGGGCCACCCGCCGCGTAATAGTAGGTGGGGAGCT
TTTTCCCGGATTTTTGGGTCGTTAAGGCGG

Lactobacillus lactis strain A7c		KC936159.1
Lactobacillus fermenti strain A7b		KC936158.1

09

CGTCATCCACGGTAGACATGGCTCAGGTGCGCTTGGC
CACACCCTAATAGTTTGACTGGGCTCGGGGCGTTGGA
ACCCCCCTTAAAAGTTTAACCCGGCCCCAGGTCCGGG
AAACCCCCCCTAATAAAGGATAACATTTTGAACCGCA
TGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTA
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CCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCC
GCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTG
AGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTT
AGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACC
TTGACGGTACCTAACCAGAAAGCCACGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTT
ATCCGGGAATTATTGGCGTAAAGCGCGCGCAGGTGGT
TTCTTAAGTCTGATGTGAAGCCCACGCTCATCGTGGA
GGGTCATTGTAACTGGGAGACTGAGTGCAGAGAGGT
AAGTGTAATTCATGTGTAGCGATGAATTGCGTAGAGA
TATGAGACACAGTGCGAGGCGACTTCTGACTGTAACT
GACACTGAGGCGCGAAAGCGTGGTGAGCAACCAGAA
TAGATACCATGCTAGTCCACGCGTTACGATGAGTGCT
ACTATACAGGGATCGCCTTAGTGCTGAGTAACGCATA
GGACCTCGCTGGATTACGCGCGTAAGTTCTGCAC

Lactobacillus sp. strain 90.2.13		KX649151.1
Lactobacillus casei FN31		DQ462190.1

10

GGACCCTCCTCCGGTTGACTGGCTCAGGTGCGGTGGG
ACCCCCCTAAGAGTTTGACTGGCCCGGGGGGGTTTGT
TCCCCCCCTTAAAAGTTTAAGACTGGGCCAGGTCCGG
GGAACCGACACTAATAAAGGATAACATTTTGAACCGC
ATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCCCTT

ATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGG
TAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTG
AGAGGGTGATCGGCCACACTGGGACTGAGACACGGC
CCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCC
GCAATGGACGAAAGTCCTGACGGAGCAACGCCGCGT
GAGTGATGAAGGCTTTTCGGGTCGTAAAACTCTGTTG
TTAGGGAAGAACAAGTGCTAGTTGAAATAGGCTGGC
CCCTTGACGGGTACCCTAACCAGAAAAGCCACGGCTA
ACTACGTTGCCACCAGCCCCGCGGTAATACGTAGGGG
GCAAAGCGTTTATCCGGGAATTTTTTGGGGCGTAAAG
CCGCGCCGCAGGTGGTTTTCTTAAGTTCTGAATGTGG
AAGCCCCACGGGCTTCAACCCTTGGGAGGGGTTCATT
TGGAAAAACTGGGGAAAATTTGGAGTGGCAGAAAGA
GGGGAAAAGGTGGAAATTTTCCTTTGTGGAAGCGGGT
TGAAAAATGCCACAAAGAAGTATTTTGGAGAGGAAA
CACTCAGGTGGTCAAAAGGGCGAATTTTTTCGGGGCC
TGGTTAACTCTAGACACCTTGGAGGCGCGCCGAACAC
GCGTGGGGTGAGGGCACACCCGGAGGAGTATGAGAT
ACCCCGCTGAGTATATGCTCACACACGCGCGCTGTTA
CACACGAGAGTGAGAGGTGCGCTTCAACAGGTCGTTA
TAGAGAGCGATATTTTCCAGGCCTCATCTATGTCACG
AGCTGC

[*Lactobacillus sp*. strain H10WTRM5](#) [MH985183.1](#)

11

AGGGTTTAGTCTATAATTGCCGTGGCTCAGTTGCGCTT
GGTTCGCCTTTATATGTCGAGTGGCGGACGGGTGAGT
AATGTCTGGGAAACTGCCTGATGGAGGGGGATAACT
ACTGGAAACGGTAGCTAATACCGCATAACGTCGCAA
GACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAG
ATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAAC
GGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAG
GATGACCAGCCACACTGGAACTGAGACACGGTCCAG
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACA
ATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATG
AAGAAGGCCTTCGGGTTGTAAATACTTTCAGCGGGGA
GGAAGGCGTAAGGTTAATAACCCTTGTCGATTGACGT
AACCCGCGAAGAGCCCGGGTAACTCGTGCCGGCAGC
CGCGGTAATCCGGAGGGTGCAGCGTTAATCGAATTAC
TGGCGTAAGCCCGCAGGCGTCTTTCAAGCCGATGTAA
ATCCCGGGGCAACCTGGGACTGATTCGAACTGGCGGC
TAGGTCTGTAAGGGTACATTCCGTTACGTGAATGCCA
AAACTGGAGATCCGTGGAGGCTCCCTGAAAACTGGCT
AATCCAAGGTGGGCACATAGATTATCTGGATCCCGGA
ACGTCCATAAGAGTGCCTAGTGGATCAGATACCGGTA
TCACCCTGTAATCGCCGGGGTAAAAACC
[*Streptococcus sp* Z21](#) [KF835734.1](#)

12

CGTCAAATTAAGCAAACGTTCATGGCTCAGGTGCGTT
TGGATCTCGCCCTAATAGTTTTAGCGGCGGACGGTTG
AGTAACACGTGCTTCTCCTGCCCATAAGACTGGGATA
ACTCCGGGAAACCGGGGCTAATACCGGATAACATTTT
GAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCT
GTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTT
GGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAG
CCGACCTGAGAGGGTGATCGGCCACACTGGGACTGA
GACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGG
GAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAA
CGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAAC
TCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAG
CTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGC
TAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG
CAAGCGTTATCCGGAAGTTATTGGGCGTAAAGCGCGC
GCAGGTGGCTTTCTTAGTCTGATGTGAAGGCCCACGG
CTCATCCGTGGAGGGTCATTGTACTGGGAGACTTGAG
TGCAGAGAGGAAGTGCATTCCATGTGTAGCGGTGAAT
GGCGTACAGATATGGAGGACACCAGTGGGCCGAGGG
CGACTTCTGACTGTAACTGAACACTGAGGTCGCCGAA
CGCGTGGGGAGCAACAGATAAGTACCTTGCAAGATC
CACGCCGTTAAACGATGAGGTGCTAACGTGTAAAGA
GCGCTTCCGCCCTTACGTGCAAGTACGCATAGGCATT
C
[Lactobacillus sp. C02](#) [MN595199.1](#)

[*Lactobacillus sp* strain B12](#) [MG950219.1](#)

13

AGGGTCACTCCTTCGCGTGTCTGGCTCAGGTGCGGTT
GAATCACTTCCTTATAGTTTACGCGCGGCTCACGGGT
GATTAATGTCCGCCTTTATGCCCGATAGAGGGGGATA
ACTACTGGAAACGGTGGCTAATACCGCATGACGTCTA
CGGACCAAAGCAGGGGCTCTTCGGACCTTGCGCTATC
GGATGAACCCATATGGGATTAGCTAGTAGGTGAGGTA
AAGGCTCACCTAGGCGACGATCTCTAGCTGGTCTGAG
AGGATGATCAGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA
CAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTA
TGAAGAAGGCCTTAGGGTTGTAAAGTACTTTCAGCGG
GGAGGAAGGTGATAAAGTTAATACCTTTATCATTGAC
GTTACCCGCAGAAGAGCACCGGCTAACTCCGTGCCAG
CGCCGCGGTTATACGGAGGGTGCAGCGTTAATCGAAT
TACTGGCGTAAGCGCACGCAGGCGGTCATTAAGTCAG
ATTGAAGCCCGAGCTAACTGGGAATGCATCTGAAACT
GGTTGCTAAGCCTTGAGAGGGGTAGAATTCATGTTTA
GCGCGAAATGCTAAATTGGCAGAATTCGGTGGCGAG
GCGGCCCCTGGACAAGACTGGCCTCGGTCCAATCGTG
CGGGATAGGATAGACCCTGGTATTCACCGGAACATTC
ATTAAGGTTGGCCTGACGGGCTTCGGGCTACGGTAAT
CACCCCCTGGAGACGGCCCAAGT
[Proteus sp. strain P211 l](#)
[Proteus sp. strain LMRE72](#)

[Proteus penneri strain 2-63](#)

15

GGCATCTCTCTATAGGTGACTGGCTCAGGTGCGCTGG
GCCCCCCTTTATATTTTGACCTGGGCCCGGGGGGGTT
GGTTCTCCCCCTTAAAATTTTTACCTGGGCCCAGGTCC
GGGGAACCCCCCCCTAAAAAAGAATAATCTGTTTCACC
TCATGGTGAAATATTGAAAGACGGTTTCGGCTGTCGC
TATAGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGA
GGTAACGGCTCACCAAGGCGACGATGCGTAGCCGAC
CTGAGAGGGTGATCGGCCACACTGGGACTGAAACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATC
TTCCACAATGGGGCGAAAGCCTGATGGAGCACCGCCC
GCGTGAGTGAAGAAGGATTTCGGTTCGAAAACTCTGG
TTGTAAGGGAAGAACAAGTTCAGTAGTAACTGGCTGT
ACCTTGACGGTACCTTATTAGAAAGGCCACGGCTAAC
TACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAA
GCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAG
GTGGTTTCTTAAGTCTGATGGTGAAAGCCCACGCTCA
ACCGTGAGGGTCATTTGGAAACGGGAGACTTGAGTGC

AGAAGAGGATGTGGAATTCCAGTGTACCGATGAATTG
CGTACAGATTGGAAGAAACACAGTGGCGAAGCGACT
ATCTGGTCTTGTACTGACACTGAGCTGGAAAGCGTGG
GAGCAACAGGAATGAGATTCCCTGGAGTTTCACGCCG
TTAACGAATGAGGGCTAACCGGTGAGAGTGGTACGTC
CGTAGTTTGTTATGGCCACGGTTAAGTGCATATAATG
AACACT

Lactococcus sp strain	KF261018.1
Lactococcus sp strain SB2.124	JQ744631.1

16

CGTCCACTCTTAAGATTGACATGGCTCAGGTGCGCTG
GACCCCCCTTAAGAGTTTGACTGGGCCCGGGGGGGGT
GGGTCCCCCCCTTTAAATTTTAACCTGGGCCCGGGGG
GGGGGGACCCCCCCTTAAAAAGTTTAACCTTTTGCAC
CGCATGGTTCGAAATTGATAGGCGGCTTCGGCTGTCA
CTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTG
AGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGA
CCTGAGAGGGTGATCGGCCACACTGGGACTGAGACA
CGGCCCAGACTCCTACGGGGAGGCAGCAGTAGGGAA
TCTTCCGCAATGGACGAAAGTCCTGACGGAGCAACGC
CGCGTGAGGTGATGAAGGGTTTTCGGGTCGTAAAAAC
TCTGTTGTTAGGGGAAGAACAAGTGCTAGTTTGAATA
AGCTGGCACCTTGACGGGTACCTAAACCAGAAAGCC
ACGGGCTAACTACGGTGCCAGCCAGCCGCGGTAAAA
ACGTAGGTGGCAAGCGTTTATCCGGAATTATTTGGGG
CGTAAAAGCGCGCGCAGGTGGGTTTCTTTAAGTTCTG
AATGTGGAAGGCCCCCCGGCTTCACCCGTGGAGGGGT
CTTTGGAAAACTTGGGAAACTTGAGTGGCGGAGAGA
GGGAAAGTGGGATTTTCCATGGTGTTACCGGTGGAAA
TTGCGTAGAGATATTGGAGGAAACCACCCAGTGGGC
AGAAGGGCGAATTTTTTCCGGGTCGTGTAACTTGAAC
TCTTGAGGCCCCGAAACGCCGTGGTGTGAGCCAAACA
GGATTAAATATACCCCCTGGTAGTTCCACCGCCCCGT
TACACGAGATGTGAGTGTGTCTAATCTGTTTAGAGGA
GATCTTCTCCGCTCTCCTCTATTATAGTGGCGTGGAGG
AGATTTAAGCACGCCATTATTTAGGGACCTTCTATCC
GCGCTGTGTGGGAGAAGTGATTCGCAATCTGCGC

☐	*Lactobacillus lactis* strain SSe2 1	KF741359.1
☐	*Lactobacillus sp strain NIOER5 1*	MG205787.1

17

AGGGTTCAGTCTAAGGATTGACATGGCTCAGGTGCGC
TTGGATCACCCCTTTAGAGTTTGACCGGGCTCGGGGG
CGTTGGGTCCCCTCCTTTATAGTTTACCTGGCCCCAGG
GCTGGAAACGATGGCTCTTAAAACATACTCTCTCAGG
AGCAAAGCGGGGGACTTCGGTCCTTGCGCTATCGGAT
GAACCCATATGGGGATTAGCTAGTAGGGGGGGTAAT
GGCTCACCTAGGCGACGATCCCTAGCTGGTCTGGGGA
GATGATCAGCCACACTGGGGACTGAGACACGGGCCC
AAACTCCTACGGGGAGGCAGCAGTGGGGGAATATTG
CACAATGGGCGCAAGCCTGGATGCACCCCTGCCGCGG
TGTATTGAAAGAAGGCCCTAGGGTTGAAAAGTACTTT
TCAGTCGGGAGGAAAGGCGTTTGATGCTTATTATCTT
CCAAGGATTGGCGTTACCCAAGGAAAAAGCACCCGG
TTACCTCCTTGGCCAGGGGGCCGCGGGAAATCCGGAG
GGTGCTAGCGTTTATTGGAATTTCTTGGGGCGTAAAC
CCCCCCCCGGGGGTTAATTAATTAAATTTGAAACCCC
CCGGGGTGAAGCCGGGGAATGGAATTTGA

Proteus sp strain JVSP1	KR108920.1

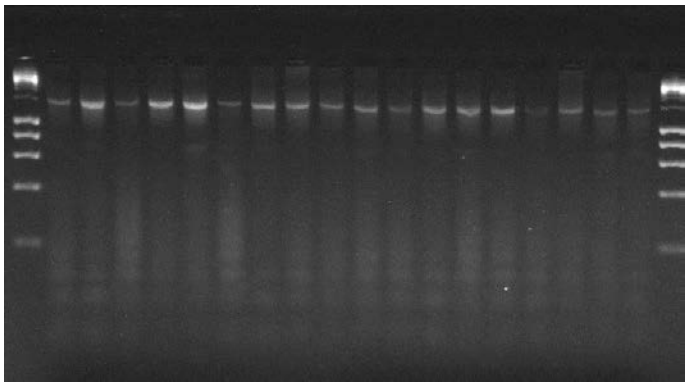
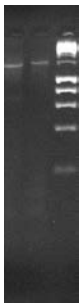


PLATE 1: Showing the bacterial PCR gel.
19-20



Lactic acid bacteria isolated from locally made 'nono' includes *Lactobacillus lactis, Lactococcus sp, Streptococcus sp* which is in agreement with Akabanda *et al.*, 2014.The molecular characterization shows that *Lactobacillus, Lactococcus Streptococcus spp.* dominate the natural microbial flora in the locally produced nono by the Hausa/Fulani women that hawked this product Adesokan *et al*., 2011;Cisem, 2003. *Lactobacillus sp* is widely known for their ability to utilize lactose in milk to produce lactic acid which eventually lowers the pH. The ability to ferment Lactose makes *Lactobacillus spp* an important organisms which can serve as starter culture for the production of "Nono". The lactic acid produced also helps to preserve the food (Marino *et al*.; 2003). *Lactococcus spp* also produces lactic acid from lactose, but at a much slower rate than *Lactobacillus spp*, which makes the latter a better starter culture. Though other metabolic products produced by *Lactococcus spp* contribute to flavour and aroma of the milk products (Marino *et al.*; 2003

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