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Isolation and Characterization of Lactic Acid Bacteria from 'Nono' Produced in Owo Area, Ondo State, Nigeria

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ABSTRACT

The microbiological analysis of locally fermented Nono produced and hawked by Hausa/Fulani women in Owo was carried out by selecting three different locations in Owo metropolis. Three samples were purchased from these locations and convey to the laboratory in an ice-packed flask. Serial dilution and pour plate method was used and incubated for 24-48hrs for the organisms to grow. The range of bacterial growth is found to be between $4.2x10^5$CFU/ml-$20x10^5$CFU/ml for De-man Rogossa Agar, and $3.0x10^5$CFU/ml-$10x10^5$CFU/ml forMI7 Agar after 48hrs. The pH also of the Nono was found to be between 4.5-4.8 .While the lactic acid bacteria isolated belongs to the genera, *Lactobacillus, Lactococcus and Streptococcus*. While Lactobacillus had the highest percentage of frequency of occurrence because of its ability to convert lactose to lactic acid faster and better than other lactic acid bacteria.

Keywords: 'Nono', Lactic acid bacteria, lactose, lactic acid.

1. Introduction

"Nono", 'Nunu', in English language and Hausa Language is a spontaneously fermented yoghurt – like product, is 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 a 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).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 turning the product sour within few days.

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).

Milk is an excellent culture medium for many kinds of microorganisms, being high in moisture, nearly neutral in pH and rich in microbial foods (Wouters *et al.*, 2002). When milk becomes sour, it is usually considered spoiled, especially if it curdles. The evidences of acid formation are first, a sour flavour and then coagulation of the milk to give a solid jelly-like curd or a weaker curdles that releases clean whey.

In raw milk at temperature from 10^{o}-37^{o} C, *Streptococcus lactis* is most likely to cause the souring, with possibly some growth of Coliform bacteria, Enterococci, Lactococci and Micrococci. At higher temperature, 37^{o}C to 50^{o} C, *S. thermophilus* and *S. feacalis* may produce about one percent acid and be followed by *Lactobacillus bulgaricus* which will produce more acid (Frazier and Westhoff, 2004). The coliform bacteria produce some lactic acid and considerable amount of volatile products such as hydrogen, carbon (IV) oxide, acetic acid, formic acid and alcohol. Species of Micrococcus, Microbacterium and Bacillus can produce acid in milk, mostly lactic acid but ordinarily cannot compete with the lactics (Pradeep and Leena, 2007). Butyric acid may be produced in milk by the action of *Clostridium sp* under the condition that prevent or inhibit the normal lactic acid formation. Thus, after a heat treatment which destroy all vegetative cells of bacteria but allows the survival of spores of Clostridium, may undergo butyric acid fermentation with the production of hydrogen and carbon(iv)oxide gas (Pradeep and Leena,2006).

The hydrolysis of milk proteins by microorganisms usually is accompanied by the production of a bitter flavour caused by some of the peptide released. Proteolysis is favoured by storage at a low temperature, by the destruction of lactic and other acid formers by heat and by the destruction of formed acid in the milk by moulds and film yeasts or the neutralization of acids by products of other organisms (Frazier and Westhoff, 2004). Proteolysis may be acid proteolysis in which acid production and proteolysis occurring together or proteolysis with little acidity, even alkalinity; sweet curdling which is caused by rennin-like enzymes of the bacteria at an early stage of proteolysis. 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).

Starter strain in industrial terms can be defined as isolates which produce sufficient acid to reduce the pH of milk to (5.3 in 6 hours at 30 – 37oC (Beresford *et al*., 2001)

In dairy industry, starter cultures can be divided into three groups:

1. Mesophilic starter cultures
2. Thermophilic starter cultures
3. Artisanal starter culture

Because they produce acid rapidly, withstands the cooking temperature and has high tolerance to salt. The main disadvantages are; they are feacal in origin and some strains are considered to be pathogenic. However, it was concluded that Enterococci represent the dominant microflora of raw milk traditional cheese and should be used as starter in order to produce the typical characteristics (Lopez – Diaz *et al.,* 2000). It has been found that they have beneficial effects on the growth of other LAB species because of their intense proteolytic activity (Lopez – Diaz *et al.,* 2000). 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. Homofermentative lactic acid bacteria which produce mainly lactic acid.
2. Heterofermentative lactic acid bacteria produce lactic acid, carbon-dioxide, ethanol and others.

This classification originated from metabolic routes that organisms used and 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).

2. Materials and Methods

2.1 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 these locations in an ice-packed flask which was taken to the laboratory for microbiological analysis.

2.2 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 and Malt extract Agar (MEA) for fungi isolation. The culture media were prepared according to manufacturer's instructions and sterilized in the autoclave at 121^{o}C and 15psi for 15 minutes.

2.3 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.

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Biochemical tests such as catalase, oxidase, coagulase, indole, urease, citrate utilization and sugar fermentation tests were carried out according to Cheesbrough (2006) for further identification of bacterial isolates.

3. Results

The samples of "Nono" collected from three different locations in Owo, Ondo States were taken to the laboratory in an Ice packed cooler for microbiological analysis to isolate the organisms responsible for fermentation of the milk. The total bacterial growth on MRS Agar ranges between $4.2x10^5$CFU/ml to $20x10^5$CFU/ml while total bacterial growths on M17 Agar range between $3.0x10^5$CFU/ml to 10x105CFU/ml as shown in Table 1.

The pH of the Nono Obtained from the three locations were also determined and found to be within the range of 4.5- 4.8 as shown in Table 2.

The morphological characterization of the isolates were found to be milky, whitish milky while the shape ranges between Round or oval, circular and the elevation were raised, serrated and flat as shown in Table 3.

Table 1: Total bacterial counts in nono samples from three locations

SN	Samples Location	Media Used	
		MRS AGAR	**MI7**
1a	GRA(Housing,Owo)	$20x10^5$CFU/ml	$10x10^5$CFU/ml
2b	Ikare Junction.	$4.2x10^5$CFU/ml	$3.0x10^5$CFU/ml
3c	Iyere (Owo)	$4.5x10^5$CFU/ml	$3.5x10^5$CFU/ml

Table 2: pH of Nono from the three locations:

S/N	Sample Location	pH
A	GRA(Housing,Owo)	4.50
B	Ikare junction.	4.60
C	Iyere (Owo)	4.80

Table 3: Morphological characteristics of the bacteria isolates

	SID	Shape	Colour	Elevation	Appearance
1A	A1	Round Oval	Milky	Raised	Soft
	A2	Circular	Milky	Flat	Smooth
	A3	Round	Whitish Milky	Flat	Smooth
	A4	Oval	Milky	Raised	Smooth
	B1	Round Oval	Milky	Flat	Soft
2B	B2	Circular	Milky	Flat	Smooth
	B3	Circular	Whitish Milky	Raised	Smooth Staff
	C1	Circular	Milky	Raised	Soft
3C	C2	Round Oval	Whitish Milky	Flat	Smooth
	C3	Round	Round Butter Colour	Flat	Soft
	C4	Circular	Whitish	Flat	Smooth
	C5	Circular	Milky	Flat	Soft Smooth

SID: Sample Identity

The results of the biochemical characteristics of the Isolates in the samples from the three different locations showed that they are catalase positive to catalase –ve while the Gram reaction showed that they are Gram +ve Cocci, or +ve/ -ve rods while the sugar fermentation reveals that acid and gas are produced from the fermentative activities of the Isolates as shown in Table 4.

Table 4: Biochemical characteristics of the isolates

Isolates		Gram Reaction	Citrate	Coagulase	Indole	Catalase	Urease	SUGAR FERMENTATION Lactose	Glu	Fructose	Suc	Mal	Probable Identity
A	A1	+ve cocci (in chains)	-	-	-	-	-	-	A	A	-	-	*Streptococcus sp*
	A2	+ve Rods	+	-	+	-	-	+	A	AG	AG	AG	*Lacto bacillus sp*
	A3	-ve Rods	+	-	-	-	-	-	-	-	-	-	*Pseudomonas sp*
	A4	+ve Rods	+	-	+	-	+	+	A	AG	AG	AG	*Lactobacillus sp*
B	B1	Gram +ve Rods	-	+	-	-	+	AG	AG	AG	AG	AG	*Lactobacillus sp*
	B2	Gram +ve Cocci (chain)	-	-	-	-	-	-	A	A	-	-	*Lactobacillus sp*
	B3	Gram –ve Rods	+	-	-	+	-	-	A	-	-	AG	*Pseudomonas sp*
C	C1	Gram +ve Rods	+	-	-	-	+	-	A	AG	AG	AG	*Lactobacillus sp*
	C2	Gram +ve Cocci	+	-	-	-	-	-	A	AG	AG	AG	*Lactococcus sp*
	C3	Gram +ve Rods	+	-	-	-	+	-	A	AG	AG	AG	*Lactobacillus sp*
	C4	Gram +ve Cocci	-	-	-	-	-	-	A	A	-	-	*Lactococcus sp*
	C5	Gram +ve Cocci	-	-	-	-	-	-	A	AG	AG	-	*Lactococcus sp*

KEYS: **-** *Negative* **+** *Positive* ***A****: Acid only* ***AG:*** *Acid and Gas*

4. Discussion

The total viable bacteria counts from the three locations were in the range of 4.2x 10^5-20x10^5CFU/ml with de-Man Rogossa (MRS) Agar, while the range of bacterial load for M17 Agar is between 3.0x10^5-10x10^5CFU/ml in agreement with the total viable counts in artisanal cheese obtained by De angelise *et al*.,2001; Ojo *et al*.,2017.

The rep-PCR and 16srDNA sequence analysis of lactic acid bacteria isolated from 'Wara', a local cheese in Owo showed high frequency of *Lactobacillus plantarum,* while the second most occurring is *Enterococcus faecalis* (Ojo *et al*., 2017) which is in agreement the results of biochemical characterization which showed that the genus *Lactobacillus, Lactococcus* and *Streptococcus* were dominant with the highest frequency of 60% for *Lactobacillus sp* which is in agreement with the work of Alberno *et al*., 2001. The fermentative activities of *Lactococcus* and *Lactobacillus* especially in milk and milk products has been well studied by Centeno *et al*.,1999.This is majorly due to the ability of the organisms to metabolize the milk (Lactose) present in milk and convert it to lactic acid (Pradeep and Leena, 2007). The ability of *Lactobacilli* to lower the pH of the milk within few hours of the fermentation make it suitable to be used as starter culture in most of the fermented milk products such as hard cheese (Pradeep and Leena,2007). The lactic acid produced also helps to preserve the food (Marino *et al*.; 2003). *Lactococcus sp* also produces lactic acid from lactose, but at a much slower rate than *Lactobacillus sp*, which makes the latter a better starter culture, although other metabolic products produced by *Lactococcus sp* contributes to flavour and aroma of the 'Nono' (Marino *et al.*; 2003).

The range of the pH of the 'Nono' from the three locations is between 4.5- 4.8 which is majorly due to the high presence of *Lactobacillus sp* which rapidly converts the lactose to lactic acid and eventually lowers the pH of the' Nono' (Macedo *et al*., 2000).The acid produced by converting the lactose sugar to lactic acid also helps to preserve the food (Marino *et al.,* 2003).

5. Conclusion

The results of the biochemical characterization was in agreement with the previous work done and therefore it can be concluded that this isolates should also be characterized using molecular methods in order identify the organisms to species level. The lactic acid bacteria present in the fermented milk, 'nono' can also be used as starter culture to produce the products in the laboratory and compare it with the locally fermented 'nono' produce by the 'Hausa/Fulani' women. However further should be

done to standardize the isolates before it can be used as starter culture.

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