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Molecular Characterization of Lactic Acid Bacteria present in "Wara" in Owo L.G. Ondo State, Nigeria

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ABSTRACT

Lactic acid bacteria (LAB) were Isolated from local cheese samples from three different location in Owo, Ondo State (South Western part of Nigeria). All the "wara" are of the group of semi hard cheese and produced from Cows milk without the addition of starter culture. 378 (Three hundred and seventy eight) isolates, only fifteen (15) were Gram positive and catalase negative through the biolchemical characterization. Four (4) species of (LAB) were mostly Present in the "wara": *LactococcusLactis, Lactobacillus Plantarum, Lactobacillus casei*and *Enterococcus faecalis.* Further identifaction at the species level indicated that all the lactococci isolates were *L.lactis.*three of the *Enterococci* were *Enterococcifaecalis*and *lactobacilli* were identified as *Lactobacillus*plantinum (1 Isolate). *Lactobacillus casei*(2 Isolates). PCR – RFLP method which is based on the amplification of 16srRNA – ITS genes was used for the molecular characterization.

Keywords: "Wara", Starter culture, isolates molecular characterization and polymerase chain reaction

INTRODUCTION

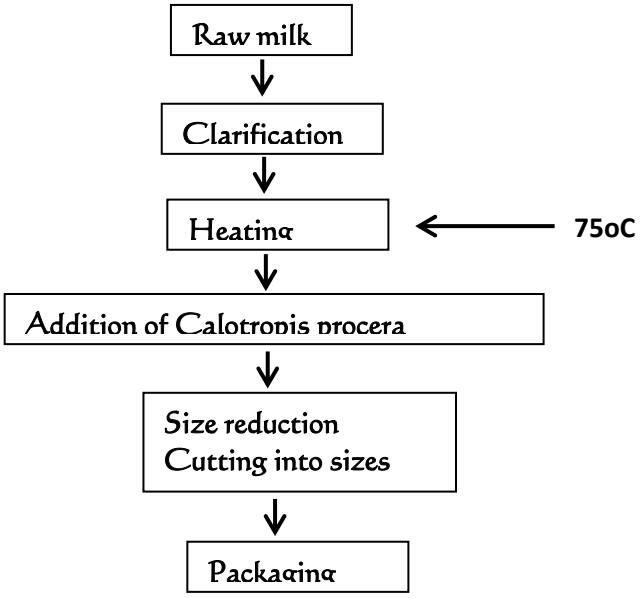
"Wara" is a semi – hard traditional Nigerian cheese manufactured from raw milk and the unique flavour of this cheese variety is the consequence of a complex combination of proteolytic, glycolytic and lipolytic activities which takes place mainly during cheese ripening and which are often brought about by lactic acid bacteria (LAB). Cheese is a concentrated dairy food defined as the fresh or matured product obtained by draining the whey (the moisture or serum of the original milk) after coagulation of casein, the major milk protein (Maron and Barbara, 2000).

Production of cheese is essentially achieved by bringing four ingredients together, milk, rennet, microorganisms and salt. The process includes the following steps: a gel formation, acid production, whey expulsion, salt addition and finally ripening period. The main biochemical changes that occur in cheese manufacture is the production of lactic acid from lactose.

This is achieved by different species of lactic acid bacteria (LAB). The responsible flora that form acid development during cheese production are starter culture that cause decrease in pH, formation of curd, expulsion of whey (Beresford *et al.,* 2001)

However, "Wara" is a type of traditional cheese that is very common in Nigeria. It has been produced from raw Cow milk or sheep milk for many centuries. Although production recipes change from one village to another and even among personal application generally, "wara", production processes include the steps shown in figure 1 below:

Figure 1: Flow sheet of "wara" making process



Therefore the lactic acid bacteria flora of traditional cheese making can be taken as the basis of starter strains with unique characteristics. In order to prevent the loss of microbial diversity and loss of wide range of cheese variety, it is a very important task to build up Lactic acid bacteria collection. Traditional lactic acid bacteria flora in Nigeria still waits for scientific attention and because of uncontrolled industrialization, the folkloric knowledge in "wara" making could be lost in the near future. Hence the need to characterize the biodiversity of lactic acid bacteria (LAB) and preserve the isolates for long term use.

Lactic Acid Bacteria

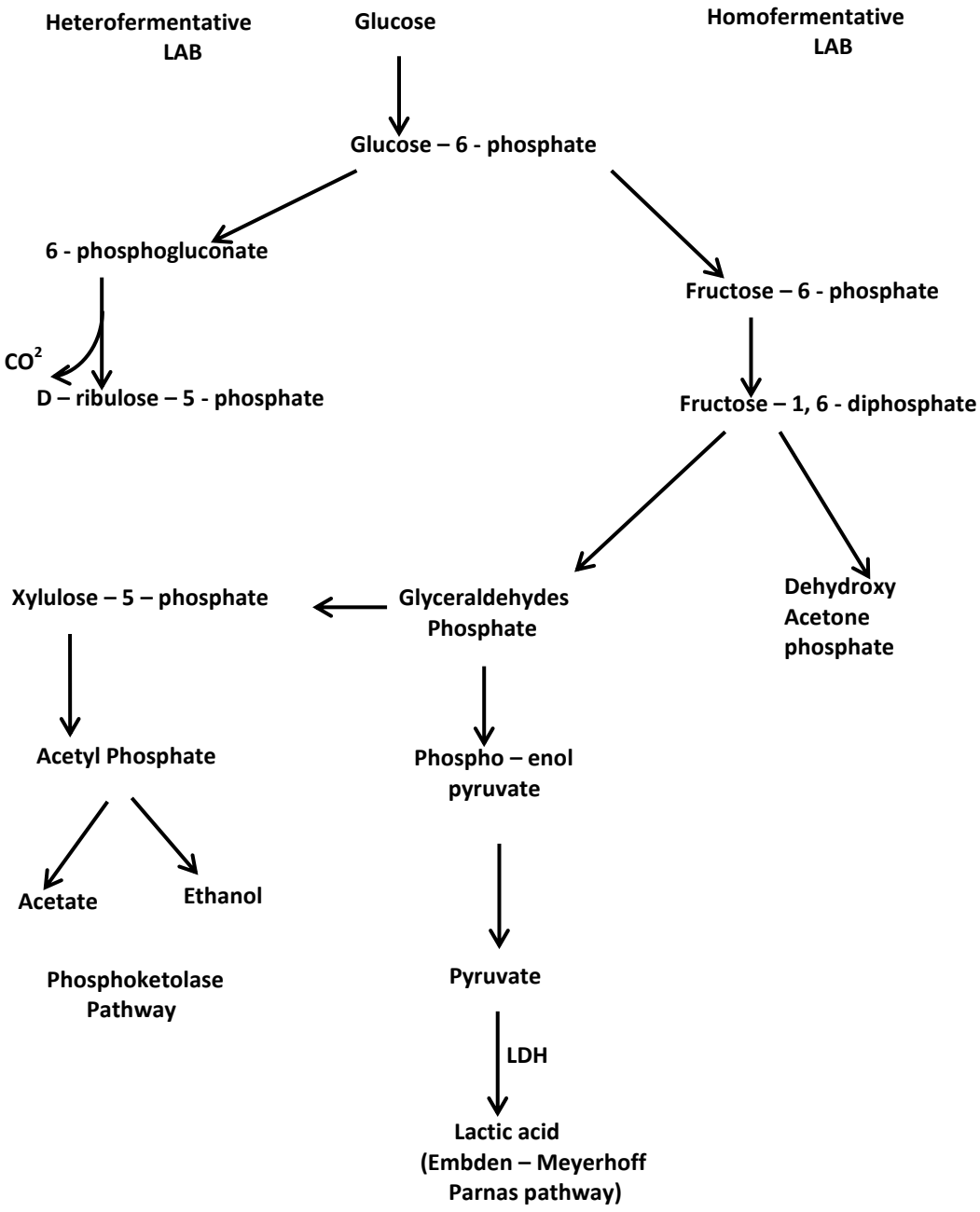
These are Gram positive, non-spore forming, catalase negative, devoid of cytochromes, acid tolerant and falcultative anaerobe group that produce lactic acid as the major end product s during fermentation of carbohydrate (cisem, 2000). According to carbohydrate metabolism, they can be divided into two main groups:

a. Homofermentative lactic acid Bacteria which produce mainly lactic acid.

b. Heterofermentative lactic acid bacteria which produce lactic acid, carbon dioxide, ethanol and or acetic acid.

This classification originated from metabolic routes that organisms used and resulting end-products. While homofermentatives use glycolysis (Embden – Meyerhoffparnas, EMP); heterofermentative use 6 – phosphogluconate or phospho – ketolase pathway (Garvie, 1984). Although LAB comprises ofeleven genera, only six of them are dairy associated. These are Lactococcus, Enterococcus, Pediococcus and Lactobacillus Garvie, 1984; Axelsson, 1998).

Figure 2: Glucose Utilization Pathway of LAB



Nester *et al.*, 2004

Cheese microflora is further divided into two groups. Primary groups include starter flora which refer to starter LAB and secondary group includes non-starter Lactic acid bacteria (NSLAB), propionic acid bacteria, (PAB), smear bacteria, mould and yeast (Beresford *et al.,* 2001). Starter strain in industrial terms can be defined as isolates which produce sufficient acid to reduce the pH of milk to <5.3 in 6h at 30 – 37°C (Beresford *et al.,* 2001).

Molecular Identification of Lactic Acid Bacteria

Identification of bacteria isolated from natural microflora involved in cheese 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 23srRNA 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, PC Rribotyping 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

METHODOLOGY

Sources of Sample

Three (3) samples of fresh cheese were purchased from Fulani settlement in Owo, Ondo State. They were collected using sterile ice – packed flask and taken to the Laboratory for analysis.

Media Preparation and Culture Condition

The media used are:

a. De man Rogosa agar (MRS), pH 6.2 – 6.8., for the isolation of Lactobacilli
b. Nutrient agar (pH 7.4) for the isolation and enumeration of streptococci and Lactococci
c. Milk with 0.1% methylene blue (90ml of milk + 10ml of methylene blue)

All media used were prepared according to manufacturer's specification. MRS agar plates were incubated under microaerophilic condition for three (3) days (Cisem, 2003)

Characterization of Isolates

Pure cultures of the isolates were obtained by streaking repeatedly on sterile MRS and Nutrient agar and characterized on the bases of cultural (appearance of colony on the culture media), morphological (size, shape and colour) and biochemical characterization such as catalase, oxidase, Citrate, Urease and sugar fermentation. The Gram status of the isolates was determined according to Olutiola*et al.,* 1991. The pH of the cheese samples were determined using the pH meter ( ), before and after processing.

MOLECULAR CHARACTERIZATION

Genomic DNA Isolation

Genomic DNA was prepared by using the procedure of cardinal *et al.,* (1997). Amplification of 16s rDNA and ITS (Internally transcribed spacer) region by polymerase chain reaction. This method was based on the amplification of internally transcribed spacer region (ITS) situated between 16s and 23s ribosomal RNA (rRNA) genes, plus 16s ribosomal DNA. For the amplification, the following primers were used.

a. Forward Primer: EGEL: 5' – AGAGTTTGATCCTGGCTCAG-3'C-Mora *et al.,* 1998
b. Reverse Primer; Li5'-CAAGGCATCCACCGT-3'(Jensen *et al.,* 1993.

While the forward primer is complimentary to the 5' – end of 16srRNA genes, the reverse is complimentary to the 3' – end of ITS. After completion of PCR reaction, amplified products were separated in an 0.8% agarose gel (Cisem, 2003). PCR amplification products were purified by chloroform extraction (Sambrook*et al.,* 1989).

RESULTS AND DISCUSSION

Nine sample of "wara" from three different locations were used for the analysis. The morphological characteristics as shown in table 2 below revealed that isolates were irregular or circular in shape raised or convex in elevation. A total of 348 isolate (162 from NA plates 131 from isolates sub cultured to nutrient agar from milk with 0.1% methylene blue and 55 from MRS agar plates

Table 4: Morphological Characteristics of Isolates

Sample number	Number of colonies			Shape			Elevation			Colour		
	NAM(MM)	MRS	NA	NA(MM)	NA	MRS	NA(MM)	NA	MRS	NA(MM)	NA	MRS
1	15	4	19	Irregular, circular	Irregular, circular	Irregular, circular	Raised convex	Raised convex	Raised convex	Milky, yellowish	Milky, yellowish	Milky
2	18	7	20	Irregular circular	Irregular circular	Irregular circular	Raised Convex	Raised convex	Raised convex	Milky, yellowish	Milky, yellowish	Milky
3	13	9	20	Irregular circular	Irregular circular	Irregular	Raised Convex	Raised convex	Raised convex	Milky, yellowish	Milky, Yellowish	Milky
4	17	6	17	Irregular circular	Irregular circular	Irregular	Raised Convex	Raised convex	Raised convex	Milky, Yellowish	Milky, Yellowish	Milky
5	18	8	15	Irregular circular	Irregular circular	Irregular	Raised Convex	Raised convex	Raised convex	Milky, Yellowish	Milky, Yellowish	Milky
6	18	4	18	Irregular circular	Irregular Circular	Irregular circular	Raised Convex	Raised convex	Raised convex	Milky, Yellowish	Milky, Yellowish	Milky
7	12	5	19	Irregular circular	Irregular circular	Irregular	Raised Convex	Raised convex	Raised convex	Milky, Yellowish	Milky, Yellowish	Milky
8	10	5	16	Irregular circular	Irregular circular	Irregular circular	Raised Convex	Raised convex	Raised convex	Milky, yellowish	Milky, yellowish	Milky
9	10	7	18	Irregular circular	Irregular circular	Irregular circular	Raised convex	Raised convex	Raised convex	Milky, yellowish	Milky, yellowish	Milky

KEYSNA (MM)- Isolates subcultured from milk with 0.1% methylene blue into nutrient agarMRS – de Man, Rogosa and Sharp agar
NA – Nutrient agar

Among these fifteen (15) isolates were negative to catalase test. These fifteen (15) isolates were subjected to further biochemical test and molecular characterization. Further biochemical test used are oxidase

citrate, Urease and sugar fermentation. The results as shown in Table 5 below

Table 5: Biochemical Characterization of the Isolates

Isolate Code	Gram Reaction	Catalase test	Oxidase test	Urease test	Citrate test	Sugar Fermentation				Probable Identities
						Maltose	Lactose	Mannitol	Glucose	
A1(MRS)	+vecocci	-	-	+	+	+	+	+	+	*Enterococcus spp.*
A2(MRS)	+ve rods	-	-	+	+	+	+	+	+	*Lactobacillus spp.*
A3(MRS)	+vecocci	-	-	-	+	+	+	-	+	*Enterococcus spp.*
A4(MRS)	+vecocci	-	-	+	+	+	+	+	+	*Lactococcus spp.*
A5(MRS)	+vecocci	-	-	-	+	-	+	-	+	*Enterococcus spp.*
A6(NA)	+ve rods	-	-	+	+	+	+	+	+	*Lactobacillus spp.*
A7(NA)	+vecocci	-	-	+	+	+	+	+	+	*Enterococcus spp.*
A8(MRS)	+ve rods	-	-	-	+	+	+	-	+	*Lactobacillus spp.*
A9(MRS)	+vecocci	-	-	+	+	+	+	+	+	*Lactococcus spp.*
A10(NA)	+vecocci	-	-	-	+	+	+	-	+	*Lactococcus spp.*
A11(NA)	+ve rods	-	+	+	+	-	+	-	+	*Lactobacillus spp.*
A12(NA)	+vecocci	-	-	+	+	+	+	-	+	*Enterococcus spp.*
A13(NA)	+vecocci	-	-	+	+	+	+	+	+	*Lactococcus spp.*
A14(NA)	+vecocci	-	+	+	+	+	+	+	+	*Lactococcus spp.*
A15(NA)	+ve rods	-	-	+	+	+	+	-	+	*Lactobacillus spp.*

The pH of the samples of "wara" used showed that, it has low pH which ranges between 6.4 to 6.6 of shown in table 6 below.

Table 6: pH of samples used

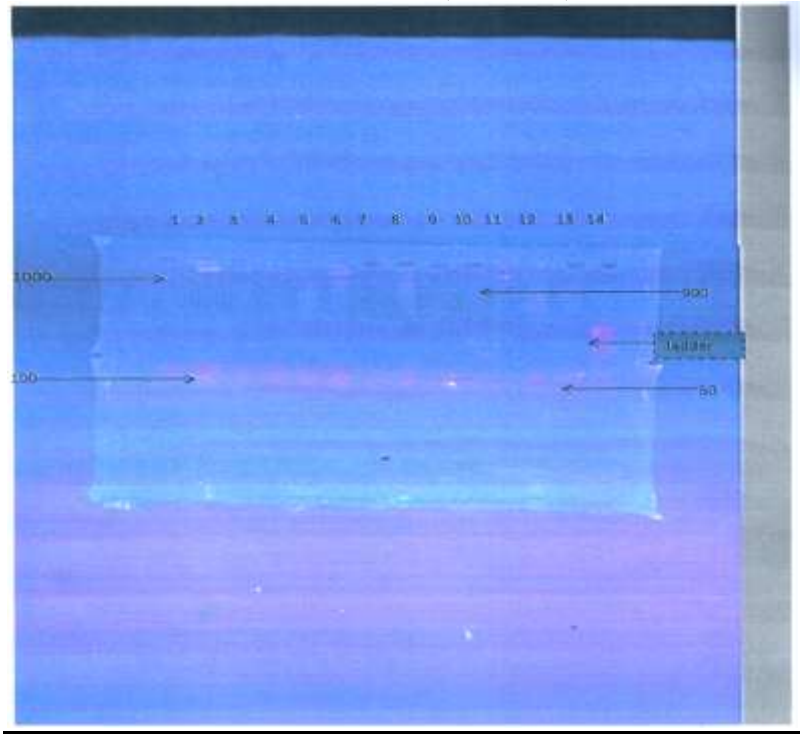
LOCATION/SAMPLE NUMBER	1	2	3

Housing Estate	6.5	6.5	6.5
Emure Ile	6.4	6.4	6.4
Ikare Junction	6.5	6.5	6.6

Molecular Characterization

The isolates determined using morphological and physiological identification methods were further examined using polymerase chain reaction to identify the isolates up to the species level. The result of the analysis is as shown in plate 1 below:

Plate1: Molecular characterization results of isolates found in "wara" using PCR



KEYS

Lane 1……*Enterococcus faecalis*
Lane 2,6,11……..*Lactobacillus casei*(rRNA genes)
Lane 8……*Lactobacillus plantarum*(src gene)
Lane 14……Ladder
Lane 9, 10……*Lactococcuslactis*

DISCUSSION AND CONCLUSIONS

The counts of viable microorganisms in 'wara' cheese ranged between 10^6 – 10^9 depending on the growth media according to Veljovic*et al.,* 2007that the total number of microorganisms ranged between 10^4 to 10^9cfu/g of cheese. Similar data on count of total viable microorganisms in other artisanal cheese are reported according to De angelis*et al.,* 2001. Based on biochemical and morphological characterization, *Lactobacillus,* determined.

However, using rep – PCR and 16srDNA sequencing of these isolates revealed that they are *Lactobacilluscasei, Lactobacillus plantarum, Enterococcus faecalis* and *Lactococcuslactis,* in agreement with the work of Albermo*et al.,* 2001 that *Lactobacillus casei, Lactobacillus plantarum, Enterococcus faecalis and Lactococcuslactis* is often present in raw milk cheeses.

The rep – PCR and 16srDNA sequence analysis also showed that only one of the four lactobacilli isolate from 'wara' was identified as *Lactobacillus*plantarum while the second most occurring LAB in 'wara' is *Enterococci* which are widely distributed in nature and Ayad *et al.,* 2001 showed that *Enterococcus faecalis* is the most frequently occurring species in dairy products which is in agreement with the results because five (5) of the seven (7) cocci isolate) were identified to be *Enterococcus faecalis* while the remaing two (2) belong to *Lactococcuslactis*.

The PCR method also showed that one (1) out of four (4) isolates identified as *Lactobacillus* belong to *Lactobacillus plantarum* while the remaining three (3) belong to *L. casei*. The results clearly indicate that *Lactobacilli, Enterococci* and *Lactococci*are the dominant LAB in the fermentation and producing chain of "wara" cheese (Centeno*et al.,* 1999).

Among the *Lactobacilli,* two (2) species appeared dominant: *Lactobacillus Casei* and *Lactobacillus plantarum;* while *Enterococcus faecalis* and *Lactococcuslactis* predominated among the cocci which is in agreement with the previous studies of which reported high viable counts of *Lactococcus, Lactobacillus* and *Enterococcus spp* in artisanal cheese produced from raw milk (Jurkovic*et al.,* 2006), n this study, homofermentative strains isolated from 'wara' samples used, such as *Lactococcuslactis, Enterococcus spp* and *Lacobacillusplantarum* should be selected as potential starter culture because of their important in fermentation of milk and other beneficial effects such as production of aroma, flavor and other desirable qualities which characterize "wara".

However the most appropriate starter culture combination to give the traditional texture, flavour, aroma and taste to 'wara' should be determined and safe production of the products for consumers in industrial scale with standardized quality should be researched into, also further study or research is needed to evaluate the selected strains or species, single or mixed cultures, on a pilot scale before industrial production level. It would also be beneficial to determine the phage resistance of the selected starter strains (Macedo *et al.,* 2000).

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