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Production of Mixed Fruit (Orange, Pawpaw, Pineapple and Water Melon) Wine from Damaged Fruits Using *Saccharomyces cerevisiae*, *Lactobacillus fermentum* and *Lactococcuslactis*Isolated from Palm Wine, Ugba and Yoghurt

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Abstract
Perishable and damaged tropical fruits found in Nigeria which included watermelon, orange, pawpaw and pineapple were used to produce mixed fruit wine, thus reducing the level of postharvest losses while creating novel mixed fruit combination wines. The samples for sourcing the test organisms included yoghurt, ogi, ogiri and ugba. However, the test organisms were isolated from only three sources. Lactobacillus fermentum was sourced from ugba whileLactococcuslactis and Saccharomyces cerevisiae were isolated from yoghurt and palm wine respectively. Genetic analysis was carried out on two of the samples for confirmation.The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolates within the Lactobacillus and Lactococcus, and revealed a close relatedness to Lactobacillus fermentumandLactococcuslactis. The fruit musts were subjected to primary and secondary fermentation for 4 and 21 days respectively. The pH of the wines ranged from 3.80 to 4.90 with the fruit wine fortified with S. cerevisiaehaving the lowest p*H. The temperature of the mixed fruit wines ranged from 27ºC to 31ºCwith the wines inoculated with Lactococcuslactisand S. cerevisiaeboth producing the same highest temperature of 31ºC. The fluctuations could be due to biochemical changes occurring during the metabolism of the substrates.The titratable acid ranged from 0.47g/ml to 0.98g/ml, with the wine inoculated with Lactococcuslactisshowing the highest titratable acid value. According to the sensory evaluations, the wines were generally acceptable but further maturation would lead to more favorable acceptability.*

Keywords: *Tropical fruits, wine production, Lactic Acid Bacteria, genomic characterization.*

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I. Introduction

In Nigeria, massive quantities of well-known tropical fruits such as mango, pineapple, banana, citrus, guava, pawpaw, watermelon and cucumbers are produced (Lawal*et al.,* 2021). Nigeria has lost US$ 10 billion from food loss and waste over the past 20 years (Federal Ministry of Agriculture and Rural Development (FMARD, 2008; Aworh, 2004). Post-harvest losses account for over 30% of all fruits produced in Nigeria, costing farmers, and by extension, the economy an estimate of 9 billion naira yearly. The demand for fruits is on the increase and the production and distribution of bulk fruits requires special care in order to avoid mechanical damage or injury (Onuigbe and Onuoha, 2013). Onyeniran (1988), reported that about 50-70% losses in fruits arecommon in the tropics between the production areas and the points of consumption. Busari *et al.* (2015) reported that postharvest losses in fruits such as citrus, banana and pineapple are enormous and the marketing system of theses fruits places 75% of the burden of these losses on the individuals selling the fruits. The economic implicationsof bruised or damaged fruits is enormous. Reducing the mechanical damaged can increase shelf life of fruits as well as reduce microbial infestation.

Pawpaw, pineapple, watermelon, and oranges are tropical fruits with short shelf-lives under the high temperatures and humid conditions present in Nigeria. Production of wine from these fruits will definitely help reduce postharvest losses. Since the fruits are perishable, large quantities are thrown away yearly because of the lack of storage facilities, poor harvestingtechniques and poor handling by fruit vendors (Akubor *et al.*, 2003; Okoro, 2007; Ugbogu and Ogodo, 2015). However, these fruits can be used to produce novel wines which can earn foreign exchange and also provide employment for our Nigerian youths (Biri*et al.,* 2015). Fruit wines are fermented alcoholic beverages made of fruits other than grapes; they may also have additional flavors taken from other fruits, flowers and herbs.

The main role of Lactic Acid Bacteria (LAB) in wine making is to conduct malolactic fermentation (MLF) which can increase wine aroma, color and mouthfeel, improve microbial stability and reduce acidity of wine (Devi and Ka, 2019; Devi *et al.*, 2019; Virdis*et al.*, 2021). LAB has the ability to contribute to the sensory profile of wine through many different enzymatic pathways. Enzymes from LAB that can exert their activity in wine include glycosidase, esterases and proteases (Liu, 2002; Sumby*et al.*, 2019). The activity of these enzymes can add to the appearance, flavor, texture and aroma of wine and thus define its structure (Swiegers*et al.*, 2005). Before the use of MLF starters became a common winemaking practice, a secondary fermentation in wine was often enabled by the microbial populations that originated in vineyards. LAB starters previously reported include *Lacticaseibacillusparacasei*, *Lentilactobacillusparacasei*and*Lactiplantibacillusplantarum*(Bravo-Ferrada *et al.*, 2013; Lopez-Seijas*et al.*, 2020).

At present*Oenococcusoeni*is one of the three, and the most known, species in the *Oenocuccus* genus (Lorentzen and Lucus, 2019). *O. oeni*is the main LAB of choice in winemaking due to its high tolerance for low pH and high ethanol concentrations (Bartowsky, 2005). However, with increasing temperatures during growth and harvest, and a consequent rising pH trend for many wines, other LAB have the potential to become an alternative to *Oenococcus* (Krieger-Weber *et al.*, 2020; Shao-Yang *et al.*, 2020; Sun *et al.*, 2020). In view of this the current research seeks to investigate other LAB.

II. Materials And Methods

Sample Collection

Physically damaged fruits such as watermelon (*Citrulluslanatus*), orange (*Citrus sinensis*), pineapple (*Ananascomosus*) and pawpaw (*Carica papaya*) were obtained from 3 different markets in Owerri, in Imo State, Nigeria, through random selection of physically damaged fruits from fruit vendors.

Source of Materials

The test samples for sourcing Lactic Acid Bacteria and *Saccharomyces cerevisiae* includefreshpalmwinefrom*Raphiahookeri*, yoghurt, ogi (fermented maize, *Zea mays*), ogiri (fermented melon seed, *Citrullus vulgaris)*, and ugba (fermented African oil beans, *Pentaclethramacrophylla*) which were sourced randomly from the market.

Sample Preparation

The samples were collected into sterile universal bottles and were taken to the laboratory for further studies. The samples were homogenized by adding 1 ml of yoghurt into 9 ml of sterile peptone physiological saline solution, while 1g of ogi, ugba and ogiri were homogenized respectively into 10 ml of sterile peptone solution as described by Ngene *et al.* (2019).

Media Preparation

67.1 g of De MannRogosa Sharpe (MRS) medium was dissolved in 1 litre of distilled water as described by the manufacturer, gently heated, autoclaved at 15 psi for 15 minutes, cooled to room temperature and dispensed into sterile Petri dishes. MRS broth was prepared by dissolving 67.1 g of the medium in 1 lire of distilled water and filtering to remove the agar prior to sterilization.

Isolation of Lactic Acid Bacteria

Serial dilutions of each of the prepared samples were carried out as described by Ngene*et al.* (2019). Dilutions of each of the prepared samples were carried out with 0.1 ml of the third diluent (10^{-3}) inoculated into MRS agar by spread plate method and incubated at 37°C for 48 hours. After incubation, colonies were purified by successive streaking on MRS agar, maintained on MRS agar slants and stored at 4°C in a refrigerator.

Isolationof*S.cerevisiae*fromPalmWine

Culturingofthefreshpalmwinewasdoneonpotatodextroseagar(PDA)andincubatedatroomtemperature for 48 h. The isolates were obtained and sub-cultured on fresh medium to obtain pure cultures. Theyeast cultures were transferred to modified malt extractagar (MEA) containing yeast extract and 2 % glucose and then incubated for 24 h. Isolates were identified as *S. cerevisiae*based on their cultural characteristics,microscopyandtheirpattern of fermentationas describedbyAmoa-Awua*etal.* (2006) and Walker and Stewart (2016).

Identification of Lactic Acid Bacteria Isolates

Gram Staining

Gram staining was carried out on the samples as described by Cheesbrough (2010). A smear of the bacterial isolates was made and fixed by air drying. The smears were covered with crystal violet for 60 seconds and rapidly washed off with waterthereafter. The smears were then covered with Lugol's iodine for 60 seconds and washed off with water. The smears were decolorized with acetone alcohol and washed off after 10 seconds. The smears were finally flooded with safranin for 2 minutes and washed off with clean water. The back of the slides were then wiped and placed in a draining rack for the smear to dry before viewing with 100x oil

immersion objective lens. Gram positive bacteria gave a purple coloration while gram negative bacteria appeared pink.

Biochemical Test

The tests were carried out as described by Cheesbrough (2010). The tests carried out include motility, catalase, indole, oxidase, coagulase, and sugar fermentation.

III. Genomic Characterization

1. DNA Extraction (Boiling Method)

Five milliliters of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) was spun at 14000rpm for 3 min. The cells were re-suspended in 500µl of normal saline and heated at 95^{o}C for 20 min. The heated bacterial suspension was cooled on ice and spun for 3 min at 14000rpm. The supernatant containing the DNA was transferred to a 1.5ml microcentrifuge tube and stored at -20^{o}C for other downstream reactions.

2. DNA quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was launched by double clicking on the Nanodrop icon. The equipment was initialized with 2 µl of sterile distilled water and blanked using normal saline. Two microlitre of the extracted DNA was loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the "measure" button.

3. 16S rRNA Amplification

The 16s rRNA region of the rRNA gene of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.5uM and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 30 minutes and visualized on a blue light transilluminator.

4. Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10µl, the components included 0.25 µlBigDye® terminator v1.1/v3.1, 2.25µl of 5xBigDye sequencing buffer, 10µM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing conditions were as follows: 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min.

5. Phylogenetic Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using MAFFT. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analysed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969).

Identification of Yeasts

The yeast was identified based on its cultural characteristics and microscopy as described by Barnett *et al.* (2000).

Preparation of Must for Mixed Fruit Fermentation

The must was prepared for four-mixed fruit fermentation as described by Okokon and Okokon (2019) and Ogodo*et al.* (2015) in three replicates. The fruits were taken through three levels of treatment because of the physically damaged nature of the fruits; physical, chemical and hot water treatments. The fruits were first washed with sterile distilled water to remove debris from soil and sand then all the fruits soaked in hot water at about 55ºC for 3 minutes and lastly the fruits were dipped in 1% calcium chloride solution.

Fruit Juice Extraction

The fruits were sorted, trimmed, peeled, cut into small pieces and placed in a sterile juice extractor. The extracted juice was filtered using a clean muslin cloth into conical flasks. The blended fruit samples were transferred into a clean new transparent bucket and mixed with distilled water (1:1 w/v). Exactly 0.541kg of sugar was added to the must followed by vigorous stirring. Approximately 4 g of sodium metabisulphate ($Na_2S_2O_5$) was dissolved in 400 ml of water and poured in 100 ml aliquots into each of the three mixtures and stirred properly. The sugar concentrations were measured and the musts were mixed in the combination of orange, pawpaw, pineapple and watermelon (33.4 °Brix).

Preparation of Starter Cultures

The starter cultures were prepared from a known quantity of the must for fermentation, small quantity of sugar and known volume of water. Exactly 3.7 ml representing approximately 10^8cfu/ml (measured using McFarland standard) of *Saccharomyces cerevisiae, Lactobacillus fermentum, and Lactococcuslactis* were added to each of the mixtures separately, stirred properly and allowed to stand for 24 h before use.

Fermentation

The primary fermentation was initiated by the addition of the starter cultures. The must was stirred every 12 hours for a period of 4 days. After the 4 days, the wine was racked into the secondary fermenter which was an air tight container. Secondary fermentation was done for 21 days after which the wines were clarified. The clarification was done using bentonite. Approximately 500 g of bentonite was dissolved in 2 liters of boiling water and stirred to form a gel which was allowed to stand for 24 hour. About 150 g of the gel-like bentonite was transferred into each of the mixed fruit wine and stirred briskly to dissolve. The wine was left for 1 month to clarify after which filtration was carried out with the aid of muslin cloth. The filtrates were then allowed to mature for a period of 5 months.

Screening of LAB for Probiotic Activity

Determination of Optimal Growth at Different pH

The following procedure was carried out as described by Prabhurajeshwar and Chandrakanth (2017). 1% (v/v) fresh overnight culture (a single colony was sub-cultured in MRS broth) of *Lactobacillus* was inoculated into MRS broth with varying pH ranging from 2 to 6. The pH was adjusted with concentrated acid and 5 N NaOH. The inoculated broths were incubated in anaerobic condition for 24h at 37ºC. After 24h of incubation, growth of the bacteria was measured using a spectrophotometer, reading the optical density at 560nm (OD_{560})against the inoculated broth.

NaCl Tolerance Test

For determination of NaCl tolerance, all the isolates were grown in MRS broth supplemented with different concentrations of NaCl (1%-6%). The broths were inoculated with 10µl overnight culture of the isolates and incubated anaerobically at 37ºC for 18 – 24h, bacterial growth was monitored by measuring absorbance at 600nm and NaCl free MRS broth used as control.

SensoryEvaluation of the Various Wines

Thewinesproducedwere compared for color, flavor,taste,clarity,andoverallacceptabilitybyapanelof 10student judges on a seven point hedonic scale wheresevendenotesexcellentandoneverypoor.

IV. Results And Discussion

Experimental Isolates

Table 4.1 represents the cultural and biochemical characteristics of the lactic acid isolates. The isolates were differentiated based on their morphological and biochemical characteristics. *Lactobacillus fermentum* is a gram positive rod while *Lactococcuslactis*is a gram positive cocci. However, both organisms are negative for spore, motility, oxidase and catalase tests but positive for KOH.In addition, optimum growth at various pH and salt concentrations was also examined and confirmed to be positive.

Genetic characterization was carried out for two of the samples. The obtained 16s rRNA sequence from the isolates produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The 16S rRNA of two of the isolates showed a percentage similarity to other species at 99-100%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolates within the *Lactobacillus and Lactococcus,* and revealed a close relatedness to *Lactobacillus fermentum*and*Lactococcuslactis*.

Table 4.2 represents the colonial and morphological characteristics of the yeast sample. The observed characteristics identify the isolate as *Saccharomyces cerevisiae.*

The results demonstrate the diversity of lactic acid bacteria in dairy and nondairy fermented Nigerian foods.The samples for sourcing the test organisms included yoghurt, ogi, ogiri and ugba. However, the test organisms were isolated from only three sources. *Lactobacillus fermentum* was sourced from ugba while*Lactococcuslactis* and *Saccharomyces cerevisiae* were isolated from yoghurt and palm wine respectively. Yeasts from genera such as *Saccharomyces, Pichis, Schizosaccharomyces, Kloekera, Saccharomyeoides*and *Candida* have been reported to be isolated from palm wine(Duarte *et al*., 2010; Adedayo and Ajiboye, 2011) as also evidenced from our findings. However, the major fermentation is undertaken by about twenty indigenous strains of *S. cerevisiae*which are genetically different from the strains used to make wine from grape (Ogode*et al.,* 2015). Sani and Udeme (2013) were not able to isolate *Lactobacillus lactis* from yoghurt unlike the present

work. In addition, *Lactobacillus fermentum*was only isolated from uba samples while Ngene*et al.*(2015) were able to isolate the organism from ogi and ogiri samples.

Table 4.1: Cultural and biochemical characteristics of lactic acid bacteria isolates

Colonial characteristics	Gram	Spore	Motility	KOH	Oxidase	Catalase	NaCl (%)			pH			Possible bacteria
							1.5	2	4	4	6	9	
Small colony	Gram positive rods	-	-	+	-	-	+	+	+	+	+	+	*Lactobacillus fermentum*
Small colony	Gram positive cocci	-	-	+	-	-	+	+	+	+	+	+	*Lactococcuslactis*

Key: - = Negative + = Positive NaCl = Sodium chloride % = Percentage KOH = Potassium hydroxide

Table 4.2: Colonial and morphological characteristics of the yeast sample

Colonial Characteristics	**Morphological Characteristics**	**Fungal Isolates**
Large creamy smooth colonies with dry surface.	Oval to convex, budding yeast cells colonies	*Saccharomyces cerevisiae*

Physicochemical Parameters of Fruit Wine Must during Fermentation

There was a fluctuation in the temperature of the mixed fruit wines during the fermentation process. The temperature ranged from 27 to 31ºC. The fluctuations could be due to biochemical changes occurring during the metabolism of the substrates by the various organisms during fermentation (Ogodo *et al.*, 2015).

The pH of the wines was low and ranged from 3.80 to 4.90 with the fruit wine fortified with *S. cerevisiae*having the lowest pH of 3.80. According to Boulton *et al.* (1996),acidity plays a crucial role in winemaking since it influences taste and mouthfeel perception, color intensity and the solubility of tartrate and proteins. In addition, the lower the pH, the lower the susceptibility of wines to microbial spoilage (Ferreira and Mendes-Faia, 2020; Botezatu*et al*., 2021).

The titratable acid ranged from 0.47 to 0.98 g/ml but Oba *et al.* (2018) observed a range of 0.11 to 1.08g/ml. The mixed fruit wine inoculated with *Lactococcuslactis* had the highest level of titratable acid level of 0.98 g/ml. There was a constant increase in titratable acid throughout the fermentation process as also observed by Oba *et al.* (2018). However, Ogodo*et al*. (2015), observed an up and down variation in titratable acid.

Table 4.3: Physiochemical parameters of fruit wine must during fermentation.

Day	**Must**	**Temperature (°C)**	**pH**	**TA (g/ml)**
0	1	27	4.9	0.47
	2	27	4.9	0.52
	3	27	4.5	0.68
7	1	28	4.5	0.52
	2	28	4.7	0.60
	3	28	4.4	0.72
14	1	29	4.4	0.70
	2	29	4.5	0.81
	3	29	4.2	0.75
21	1	30	4.3	0.82
	2	30	4.4	0.92
	3	30	4.0	0.78
28	1	30	4.2	0.91
	2	31	4.2	0.98
	3	31	3.8	0.92

Key: Must 1 = Fruit wine fortified with *Lactobacillus fermentum*
Must 2 = Fruit wine fortified with *Lactococcuslactis*
Must 3 = Fruit wine fortified with *Saccharomyces cerevisiae*

Sensory Evaluation

According to the sensory evaluations in table 4.4, the wines were generally acceptable as previously reported for other tropical wines (Panda, 2014; Akubor*et al.,* 2003). However, the wines may be more palatable if allowed to mature further (Lawal*et al.,* 2021). The proteases and esterases found in LAB may have accentuated the flavor and aroma of the wines (Swiegers*et al.,* 2005). In addition, some of the enzymes found in LAB may have helped to improve both colour and clarity of the wines (virdis*et al*., 2021).

Table 4.4: Sensory evaluation of fortified fruit wine

Must	Colour	Flavour	Clarity	Taste	General acceptability
1	5.3	5.1	6.2	5.3	5.2
2	5.1	5.2	6.1	5.1	5.2
3	4.2	4.1	5.2	5.2	5.3

Key: Must 1 = Fruit wine fortified with *Lactobacillus fermentum*
Must 2= Fruitwine fortified with *Lactococcuslactis*
Must 3 = Fruit wine fortified with *Saccharomyces cerevisiae*

The colour of must 1 and 2 are dark orange while must 3 is light orange. According to table 4.5, the number of yeast cells declined as fermentation proceeded in the must fortified with *Saccharomyces cerevisiae*. This is the reason yeast is acclimatized to adapt to the hostile environment of base wines (i.e. low pH, high acidity and alcohol) for a second alcoholic fermentation in sparkling wine production (Kemp *et al.,* 2020). Yeast performance during second alcoholic fermentation can further be increased by addition of micronutrients (Borrull *et al.,* 2016). The total variable count which was obtained from nutrient agar plates decreased as fermentation progressed as also observed by Oba *et al.*(2018). Thus, there was a general decrease in microbial load as fermentation progressed as was also observed with both *Lactic Acid Bacteria and Lactococcuslactis*grown on MRS medium.

Table 4.5: Microbiological analysis of the fruit wine must during fermentation.

Days	Must	Growth Medium	Microbial Load (cfu/ml)
0	1	NA	-
		PDA	-
		MRS	-
	2	NA	-
		PDA	-
		MRS	-
	3	NA	-
		PDA	-
		MRS	-
7	1	NA	-
		PDA	5.0×10^3
		MRS	1.80×10^4
	2	NA	-
		PDA	2.50×10^4
		MRS	2.00×10^4
	3	NA	-
		PDA	2.00×10^4
		MRS	3.00×10^4
			1.55×10^4
14	1	NA	-
		PDA	2.0×10^3
		MRS	2.0×10^4
	2	NA	-
		PDA	1.50×10^4
		MRS	-
	3	NA	-
		PDA	8.0×10^2
		MRS	1.50×10^4

Key: cfu/ml = Colony forming unit per milliliter PDA = Potato dextrose agar
MRS = De Mann Rogosa Sharpe agar NA = Nutrient agar
- = No growth

Competing Interests

The authors hereby state that there was no competing interest.

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