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RESEARCH ARTICLE

MOLECULAR CHARACTERIZATION AND ANTIBIOTIC SUSCEPTIBILITY PATTERNS OF BACTERIA ISOLATED FROM *WARA* (WEST AFRICAN CHEESE) SOLD IN OSUN STATE, NIGERIA

Monsuru Adebayo ADELEKE[1], Janet Olubukola OLAITAN[1], Oluseye ABIONA[2], Juliet CANICE[1], Sukurah OLAJIDE[1], Waheed OLUOGUN[3], Muinah FOWORA[4], Adekunle Bashir OKESINA[3]

[1]Department of Biological Sciences, Osun State University, P.M.B 4494, Osogbo, Nigeria

[2]Department of Chemical Sciences, Osun State University, P.M.B 4494 Osogbo, Nigeria

[3]College of Health Sciences, Osun State University, Osogbo, Nigeria

[4]Molecular Biology and Biotechnology Division, Nigerian Institute of Medical Research, P.M.B 2013, Yaba, Lagos, Nigeria.

*Corresponding author: healthbayom@yahoo.com

Abstract: This study was carried out to determine the species distribution and antibiotic susceptibility patterns of bacteria isolated from thirty samples of *Wara* (West African Cheese) sold in Osogbo, Ede and Ikire, all located in Osun State, Southwestern Nigeria. The bacteria were isolated from the samples using standard protocol and subjected to RAPD-PCR to determine the probable variants in each species. Antibiotic sensitivity test and plasmid profiling were also carried out on each isolated bacterium. Five bacteria species namely, *Serratia marcesens, Lactobacillus delbrueckii, Lactobacillus casei, Corynebacterium* species and *Staphylococcus aureus* were isolated from the samples. Results of RAPD-PCR showed two band patterns of genes for *S. aureus* and four band patterns *L. casei* and C*orynebacterium* species. Most of the isolates had multiple resistances to antibiotics but did not harbour plasmids. The contamination of the cheese with human body flora which has been implicated as causative agents of gastro-intestinal, urinary tract, and food poisoning indicates poor hygienic conditions in the processing and handling of the cheese. Therefore, there is need for periodic public health enlightenment for the people involved in the processing and handling of the cheese to reduce the incidence of microbial contamination.

Keywords: West African cheese, microbial contaminants, RAPD-PCR, antibiotic susceptibility

Introduction

Wara (West African cheese) is one of the processed dairy products obtained from cattle's milk (Oladipo and Jadesimi, 2012). It is an unripened, soft and moist curd. The cheese is prepared by heat treatment of the milk followed by addition of coagulant and removal from whey (which contains water, lactose, and vitamins) (Adetunji and Babalobi, 2011). The coagulation of the milk is traditionally done by the Fulani pastoralists through the addition of leaf extract of Sodium apple (*Calotropis procera*), until recently, when the use of *Carica papaya* (pawpaw leaf) and lemon juice was demonstrated (Adetunji *et al.*, 2008).

Wara is a staple product and widely consumed in Southwestern Nigeria. The nutritional and proximate analysis of the cheese revealed that it contains vital components of balanced diet such as protein, fat, minerals, vitamins and essential amino acids (Adetunji *et al.*, 2008; Oladipo and Jadesimi, 2012). However, the reduction in shelf-life and public health risk associated with microbial contaminants of West African cheese has been reported in literature (Adegoke *et al.*, 1992; Adetunji *et al.*, 2008; Oladipo and Jadesimi, 2012). The microbial contaminants are introduced into the cheese through the use of unsterilized containers, handling and contamination of the milk by the plant extracts (Adetunji and Babalobi, 2011)

In order to address this challenge, researchers have proposed several methods of treatment and preservation of the cheese. Notable among these methods are the use of antibiotics and chemical preservatives such as propanoic acid and sorbic acid (Joseph and Akinyosoye, 1997; Sanni and Onilude, 1999), and the use of lemon juice (Adetunji *et al.*, 2008).

Reduction in microbial load and relative prolong in shelf-life of the West African cheese was achieved using these methods; nevertheless, none of the methods was able to inhibit the growth of microbial flora.

Though, previous authors had documented the microbial contaminants of West African cheese in Southwestern Nigeria (Adegoke *et al.*, 1992; Joseph and Akinyosoye, 1997; Sanni and Onilude, 1999; Adetunji *et al.*, 2008; Oladipo and Jadesimi, 2012), the thrust of their reports was skewed to the effects of these microflora on shelf-life of the cheese with a neglect on the public health risks associated with consumption of microbial contaminated cheese.

This study was therefore designed as part of longitudinal epidemiological studies on microbial contamination of West African Cheese in Southwestern Nigeria. Here in, we report the molecular studies and antibiotic susceptibility pattern of bacteria isolated from vended West African cheese in different parts of Osun State, Southwestern Nigeria.

Materials and Methods

Study area and sample collection

The study was carried out in Osun State (7^o 30N, 4^o 30E and 5^o 50N, 4^o 50E), Southwestern Nigeria. A total of 30 samples of *Wara* were collected from different hubs of Fulani Pastoralists in three towns; Ede, Ikire and Osogbo, all in Osun State, Southwestern Nigeria. The samples were collected in sterile universal containers and transported to the laboratory for analysis.

Isolation and identification of the bacteria

In the laboratory, 10 ml of sterile water was added to each container containing each of the thirty samples of cheese. Each sample was crushed with sterile mortar and pestle. About 0.1 ml of the samples were cultured in MacConkey and Chocolate agar (Sigma, Germany) plates overnight at 37^oC and the bacteria isolates were subjected to series of morphological, gram staining and biochemical analysis as earlier described by Cowan and Steel (1975), Baron and Finegold (1990) and Adeleke *et al.*, (2012). Briefly, catalase test was done to differentiate the bacteria that produce the enzyme catalase, such as staphylococci from the non-catalase producing bacteria such as streptococci. Citrate utilization test was done to identify enterobacteria; which was based on the ability to use citrate as its only source of carbon. A bright blue colour in the medium indicates a positive citrate test (e.g *Klebsiella pneumoniae*), while a non-change in appearance of the medium gives a negative citrate test (e.g *E.coli*). Coagulase test was done to identify *S. aureus* which produces the enzyme coagulase. A drop of distilled water was placed at the end of a slide; a colony of the test organism is emulsified on it to make a suspension. A loopful of plasma was added to it and mixed gently; presence of clumping within 10 seconds indicates the presence of *S. aureus*, while absence indicates presence of *E. coli* or *S. epidemidis.* Oxidase test was used to identify *Pseudomonas, Neisseria, Proteus, Brucella* and *Pasteurella* species. A piece of filter paper was placed in a clean sterile petri dish, and 2 or 3 drops of freshly prepared oxidase reagent was added to it.

Using a piece of stick, a colony of the test organism was smeared on the filter paper. A blue-purple

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colour within 10 seconds shows an oxidase positive test. Urease test was done to differentiate enterobacteria, e.g *Proteus* strains. The test organism is inoculated in a bijou bottle containing 3 ml sterile Cristensen's modified urea broth and incubated at 35-37^{o}C for 3-12hours. A pink colour in the medium gives a positive test result.

Molecular characterization of the bacteria isolates

DNA extraction was carried out from the bacteria by boiling as follows. Overnight culture of the bacteria in broth was centrifuged at 10,000 rpm for 5 minutes to pellet cells. The cells were washed twice with 1ml sterile water and then homogenized in 200µl of sterile water and vortexed. The samples were then boiled in a water bath at 100^{o}C for 10 minutes. This was followed by vortexing and centrifugation at 12,000 rpm for 15 minutes. The supernatant containing the DNA were transferred to another tube and stored at -20^{o}C. The concentration and purity of the extracted DNA was estimated using a Nanodrop spectrophotometer.

Randomly Amplified Polymorphic DNA - Polymerase Chain Reaction Assay (RAPD-PCR)

RAPD-PCR was used for the characterization of the isolates to determine the clonal relatedness and diversity of the isolates. Three operon primers; OPR-02: CACAGCTGCC, OPC-05: GATGACCGCC and OPC-04: CCGCATCTAC were used.

The PCR was performed in 25µl of a reaction mixture containing DNA (10-200ng), 200 µM of each deoxynucleoside triphosphates (dNTP) (Promega), 2.5 mM $MgCl_2$ 1X PCR Buffer, 20 pMol primer, 2.5 unit of *Taq DNA* polymerase (promega) and sterile distilled water.

Thermal cycling was conducted in an Eppendorf Nexus Thermal cycler for an initial denaturation of 94^{o}C; 1 minute at 28^{o}C and 1 minute at 72^{o}C. This was followed by a final extension step of 10 minutes at 72^{o}C.

The amplification product was separated on 1% agarose gel electrophoresis and visualized by ethidium bromide staining. 1kb DNA ladder (Fermentas, USA) was used as DNA molecular weight standard.

Antibiotics sensitivity test

The standard antibiotic test was carried out on the identified isolates. Briefly, sterile nutrient agar plate was prepared and 0.5 MacFarland equivalent standard of the test organisms was streaked on the surface of the agar and allowed for 15 - 20 min to pre-diffuse.The following antibiotic discs, Ceftazidime (30µg), Cefuroxime (30µg), Gentamicin (10µg), Ceftriaxone (30µg), Erythromycin (10µg), Cloxicillin (10µg), Ofloxacin (10µg), Amoxycillin/Clavulinate (30µg), Ampicillin (10µg), Nitrofurantoin (10µg), Ciprofloxacin (5µg) and Vacomycin (5µg) were placed on the surface of agar plates with a sterile forceps. The plates were incubated at 35°C for 18 - 24 h, after which the inhibition zone diameter (mm) was taken and interpreted in accordance with manufacturer's instruction. Based on the interpretation, thezone of inhibition was then classified as susceptible or resistance. The isolates with resistance to more than one antibiotic were later selected for plasmid profiling.

Plasmid profiling

TENS –Mini prep (Tris 25mM, EDTA 10mM, NaOH 0.1N and SDS 0.5%) was used for the isolation of the plasmid in the bacteria. Details of the procedure were as described by Oleghe *et al.*, (2011).

Each isolate was run on 1% agarose gel electrophoresis at 80V for 1 hour 30 minutes, and HIND III digest of Lambda DNA (Fermentas, USA) was used as molecular weight marker.

Data analysis

The data on frequency of occurrence of the bacteria was analysed with chi-square using Statistics Package for Social Science (SPSS version 15.0). The Multiple antibiotic resistance (MAR) was calculated in accordance with the method proposed by Krumperman *et al*., (1983).

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Results and discussions

Five bacteria species namely *Serratia marcesens, Lactobacillus delbrueckii, Lactobacillus casei, Corynebacterium* species and *Staphylococcus aureus* were isolated from the samples. The samples from Osogboharboured all the five bacteria encountered while Ede and Ikire harboured four and three species respectively (Table 1).

L. casei, Corynebacterium species and *S.aureus* were isolated in all the study locations. There was significant variation in the occurrence of the bacteria species in the sample ($p<0.05$) with preponderance of *Corynebacterium* species over other bacteria encountered.

Table 1. *The occurrence and distribution of bacteria isolated from Wara (West African cheese) in Osun State, Nigeria*

Isolates	**Occurrence in each location**			
	Ede	**Osogbo**	**Ikire**	**Total (%)**
Serratia marcesens	1	3	0	4 (13.3)
Lactobacillus delbrueckii	0	1	0	1 (3.3)
Lactobacillus casei	5	1	1	7 (23.3)
Corynebacterium spp	4	3	5	12 (40.0)
Staphylococcus aureus	0	2	4	6 (20.0)

The results of the molecular characterization of the isolates using RAPD-PCR are presented in Table 2 and Figure 1. All the isolates were subjected to molecular characterization but only three species had successful PCR amplification. Based on the base pair patterns of the genes, *S. aureus* has two band patterns while *L. casei* and *Corynebacterium* species have four band patterns.

The band pattern of each species varies within and between locations.

Table 2. *Probable variants of the bacteria isolated from Wara in Osun State using RAPD-PCR using operon primers*

Isolates	**Variants**	**Base pair of the bands**	**Location**
Lactobacillus casei	1	250,350, 500, 650,1000	Ede, Osogbo
	2	300, 500, 750, 1000	Ede
	3	300, 500, 700, 1000	Ikire
	4	300, 500, 750	Ede
Corynebacterium spp	1	250, 300, 500, 700	Ede
	2	350, 500, 1000, 1100	Ede
	3	250, 300, 500, 650, 900, 1450	Osogbo
	4	300, 400, 500	Ikire
Staphyloccus aureus	1	250	Ikire, Osogbo
	2	250, 400, 550, 900	Ikire

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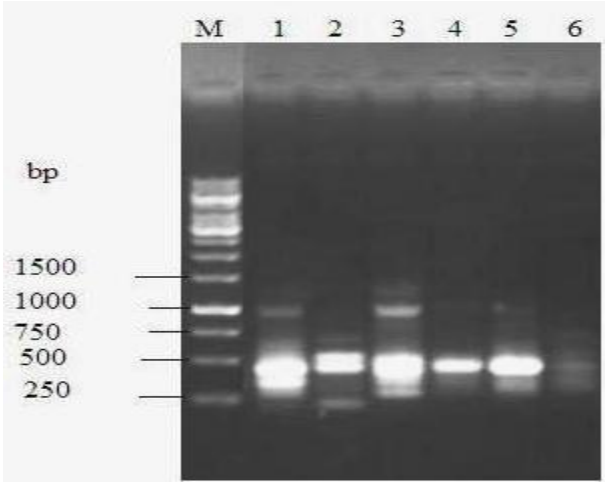

Figure 1. *The gel electrophoresis of RAPD-PCR using operon primers for the characterization of the bacteria isolated from Wara in Ede, Osun State, Nigeria*

Legend Lane M: molecular weight marker; Lane 1: *Lactobacillus casei;* Lane 2: *Corybacterium spp*; Lane 3: *Corybacterium spp;* Lane 4: *Lactobacillus casei*; Lane 5: *Lactobacillus casei*; Lane 6: *Lactobacillus casei*

The results of antibiograms showed that most of the isolates were susceptible to Ofloxacin, Ceftriaxone and Gentamicin (10µg) but had multiple resistance to antibiotics which include Amoxycillin/ Clavulinate, Cloxicillin, Erythromycin and Cefuroxime (10µg) in all the study locations. *L. delbrueckii* as resistant to all antibiotics except Ofloxacin. The MAR index of the isolates was higher than 0.20 in all the studied locations (Tables 3-5).

Table 3*. Antibiotic susceptibility patterns of bacteria isolated from Wara in Osogbo, Nigeria*

S/N	Name of isolate	Sensitivity	Resistance	MAR
1	*Serratia marcesens*	GEN, OFL, CAZ, CPR	AUG, AMP, CRX	0.42
2	*Lactobacillus delbrueckii*	OFL	CRX, GEN, CTR, ERY, CXC, AUG, CAZ	0.88
3	*Corynebacterium spp.*	CRX, GEN, CTR, OFL, CAZ	ERY, CXC, AUG	0.60
4	*Lactobacillus casei*	CRX, GEN, CTR, OFL, CAZ	ERY,CXC, AUG	0.38
5	*Staphylococcus aureus*	GEN, CTR, OFL	CRX, ERY, CXC, AUG, CAZ	0.63

Legend MAR-Multi-antibiotic resistance index, CAZ (Ceftazidime 30µg), CRX (Cefuroxime 30µg), GEN (Gentamicin10µg), CTR (Ceftriaxone 30µg), ERY (Erythromycin 10µg), CXC (Cloxicillin 10µg), OFL (Ofloxacin 10 µg), AUG (Amoxycillin/Clavulinate 30µg), AMP (Ampicillin 10µg), NIT (Nitrofurantoin 10µg), CPR (Ciprofloxacin 5µg), VA (Vacomycin 5µg).

Table 4*. Antibiotic susceptibility patterns of bacteria isolated from Warain Ede, Nigeria*

S/N	Name of isolate	Sensitivity	Resistance	MAR
1	*Serratia marsecens*	CAZ, GEN, CPR, OFL, NIT	AMP, VA, AUG, CRX	0.44
2	*Lactobacillus casei*	CAZ, CRX, GEN, CTR, OFL	ERY, CXC, AUG, VA	0.44
3	*Corynebacterium spp.*	CAZ, CRX, GEN, CTR, OFL	ERY, CXC, AUG, VA	0.44

Legend MAR-Multi-antibiotic resistance index, CAZ (Ceftazidime 30µg), CRX (Cefuroxime 30µg), GEN (Gentamicin10µg), CTR (Ceftriaxone 30µg), ERY (Erythromycin 10µg), CXC (Cloxicillin 10µg), OFL (Ofloxacin 10 µg), AUG (Amoxycillin/Clavulinate 30µg), AMP (Ampicillin 10µg), NIT (Nitrofurantoin 10µg), CPR (Ciprofloxacin 5µg), VA (Vacomycin 5µg).

Table 5. *Antibiotic susceptibility patterns of bacteria isolated from Warain Ikire, Nigeria*

S/N	Name of isolate	Sensitivity	Resistance	MAR
1	*Staphylococcus aureus*	GEN, CTR, OFL	CAZ, CRX, ERY, CXC, AUG	0.63
2	*Lactobacillus casei*	GEN, CTR, OFL	CAZ, CRX, ERY, CXC, AUG	0.63
3	*Corynebacteriumspp*	GEN, CTR, OFL	CAZ, CRX ERY, CXC, AUG,	0.63

Legend MAR-Multi-antibiotic resistance index, CAZ (Ceftazidime 30µg), CRX (Cefuroxime 30µg), GEN (Gentamicin10µg), CTR (Ceftriaxone 30µg), ERY (Erythromycin 10µg), CXC (Cloxicillin 10µg), OFL (Ofloxacin 10 µg), AUG (Amoxycillin/Clavulinate 30µg), AMP (Ampicillin 10µg), NIT (Nitrofurantoin 10µg), CPR (Ciprofloxacin 5µg), VA (Vacomycin 5µg).

The plasmid profiling showed that none of the isolates harboured resistance plasmid but has chromosomal DNA at 23.130 bp (Figure 2).

The isolation of bacteria in *Wara* collected from the study locations suggest the high microbial contamination and public health risks associated with raw consumption of *Wara* sold in major Fulani hubs in Osun State, Southwestern Nigeria. The observation is in consonance with previous reports that *Wara* harbours many microbial contaminants (Adegoke *et al.*, 1992; Adetunji *et al.*, 2008; Oladipo and Jadesimi, 2012). The presence of all the isolates in the samples from Osogbo is expected since the town is a cosmopolitant and the capital of Osun State. The variation in band patterns for each successfully characterized isolate as indicated by RAPD-PCR possibly suggests the existence of strains within the species. *S. aureus*, *Corynobacterium* species and *L. casei* usually exhibit strains, and sympatric existence of the strains is possible within specific geographical location (Takeda and Okumora, 2007).

Therefore, two probable strains of *S. aureus* and four strains of *L. casei*and *Corynebacterium* species were isolated in the present study. Aside *L. casei*, other four bacteria species isolated from the samples have been implicated in causing varying degree of gastro-intestinal, reproductive, urinary infections and food poisoning (Tagoe *et al.*, 2011; Dessi *et al.*, 2009)

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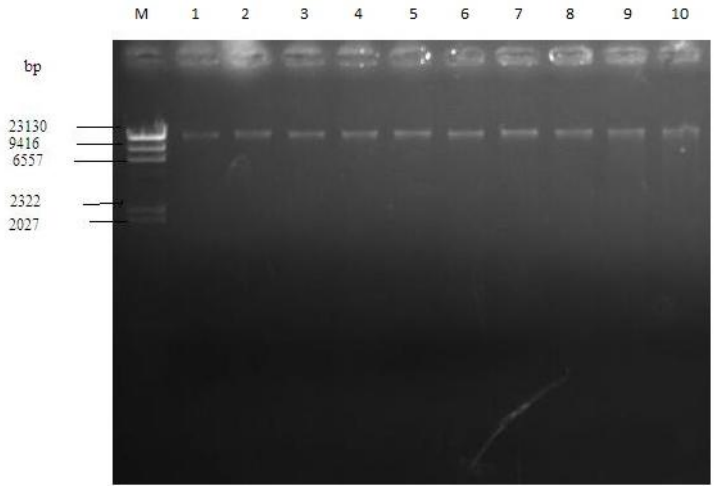

Figure 2. *Plasmid profiling of the gel electrophoresis of the bacteria isolates showing no plasmids*

The preponderance of *Corynebacterium* species isolated from the samples collected in the three locations may suggest poor hygienic conditions of the Fulani pastoralist. *Corynebacterium* species are known as normal body flora of skin and female urino-genital tract, until recently when the pathogenicity of the infection was reported mostly in immune-compromised individuals (Shukla *et al.*, 2003).

The samples could have been contaminated during processing and/ or handling of the raw milk and *Wara* by the Fulanis through the use of unclean containers, sweat, faecal and urine contamination and many other routes. This reason may also be used to explain the appreciable proportion of *S. aureus* (a skin flora which have implicated in food poisoning and urinary tract infection), *L. delbrueckii* (an opportunistic urinary tract flora but causes severe urinary infection) and *S. marcescens* (a human pathogen which causes respiratory and urinary tract infections, conjunctivitis, keratitis and tear duct infections) (Dessi *et al.*, 2009). However, *L. casei* is a beneficial lactic acid bacterium which aids fermentation of the milk.

It has been known to possess immunomodulatory potential and also helps in the prevention of cancer reoccurrence (Takeda and Okumora, 2007). The results of the antibiotic sensitivity test and MAR index revealed multi-drug resistant patterns of the isolates mostly to Amoxycillin/ Clavulinate, Cloxicillin, Erythromycin and Cefuroxime (10µg).

The resistance of pathogenic bacteria to Amoxycilin and Erythromycin (which are among the first-line antibiotics) has also been reported in Osogbo (Adeleke *et al.*, 2012) and elsewhere in Nigeria (Ehinmidu, 2003). Oleghe *et al.*, (2011) earlier posited that antibiotic resistance of pathogenic bacteria is usually mediated by plasmid, thus facilitating rapid transfer of resistance gene to susceptible bacteria in the environment. Conversely, none of the isolates screened in the present study harboured plasmid. MAR index greater than 0.20 usually suggests that the bacteria were isolated from drug pressurized environment (Ehinmidu, 2003).

Despite multi-drug resistant potential of the isolates, they were found to be susceptible to Ofloxacin, Ceftriaxone and Gentamicin (10µg) except *L. delbrueckii* which was susceptible to Ofloxacin alone. Expectedly, there was slight variation in resistance pattern of each species in different study locations. The existence of strains (variants) at different locations as indicated by RAPD-PCR could be the factor responsible for this variation. In conclusion, the present study reported microbial contamination of West African cheese (*Wara*) sold in Osun State, Nigeria. Some of the bacteria isolated exist in strains, and plausibly pose serious public health risks as causative agents of gastro-intestinal, urinary tract infections and food poisoning. The isolated bacteria showed multiple antibiotic resistance but susceptible to Ofloxacin, Gentamicin and Ceftriaxone. The plasmid profiling showed that non of the isolates harboured. Therefore, the consumption of *Wara* in the study area without

proper cookingshould be discouraged. Moreover, periodic public health enlightenment for the Fulani Pastoralists on ways to keep personal hygiene and hygienic processing of *Wara* should be instituted to reduce the incidence of microbial contaminants of the cheese. Public health legislation and enlightenment to reduce abuse of antibiotics in the Nigeria would also go a long way to reduce incidence of antibiotic resistant bacteria in our environment.

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