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Processing-Water as Source of Gram-negative Foodborne Indicator Bacteria in Traditionally-produced *Iru*

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ABSTRACT

The possibility of introduction of foodborne indicator bacteria into *iru* by processing-water samples used for washing boiled seeds' cotyledons prior to traditional fermentation of *iru* was investigated. Thirty processing water samples (collected from 30 locations in five major cities of southwestern and middle belt Nigeria (Abeokuta, Ibadan, Ijebu-Ode, Lagos and Lokoja) were total coliform positive between 1 month and two years of storage, while 27 (90.0%) and 23 (76.7%) of the water samples were total coliform positive at 28 and 35 months of storage, respectively. Using standard phenotypic taxonomic tools, the viable and easily recoverable Gram-negative bacterial isolates, obtained from the water samples were identified as- *Enterobacter aerogenes* (5.8%), *Escherichia coli* (58.9%), *Klebsiella aerogenes* (11.8%), *Klebsiella pneumoniae* (11.8%), *Proteus mirabilis* (5.8%) and *Pseudomonas aeruginosa* (5.8%). The overall bacterial isolates from various simulated, fermenting *iru* samples were characterised as *Enterobacter aerogenes* (11.2%), *Escherichia coli* (22.3%), *Klebsiella aerogenes* (8.6%), *Klebsiella pneumoniae* (24.3%), *Morganella morganii* (3.3%), *Salmonella Enterica* serovar Paratyphi (5.3%), *Salmonella Enterica* serovar Typhi 4 (2.6%), *Shigella dysentariae* (4.6%), *Proteus mirabilis* (13.8%), *Pseudomonas aeruginosa* 4 (2.6%) and *V. cholerae* (1.3%) but the most recovered bacterial species from the fermenting mesh in the first 5 days of simulated fermentation were *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella aerogenes*, *Klebsiella pneumoniae* and *Proteus*, *mirabilis*, while controlled *iru* fermentations indicated that the fermenting bean cotyledons washed with processing-water samples had higher recovery rates of indicator bacteria than the fermenting cotyledons washed with tap and sterile water samples. The study concluded that water used in the traditional production of *iru* can be source of foodborne indicator bacteria.

Key words: Food protection, foodborne indicator bacteria, Critical Control Points, *iru*, Nigerian indigenous fermented condiments, vegetable fermented foods, water-borne diseases

INTRODUCTION

Food condiments give pleasant aroma to soups and sauces in many countries, especially in Africa and India, where protein calorie malnutrition is a major problem. In Africa, many proteinaceous oily seeds are fermented to produce food condiments, which have great potential as key protein, fatty acids and good sources of gross energy (Umoh and Oke, 1974; Kingsley, 1995; Omafuvbe *et al*., 2004; Achi, 2005). The fermentation process of alkaline-fermented foods has been

reported to be associated with several benefits which include: enhanced digestibility due to degradation of non-digestible oligosaccharides (e.g., stachyose, raffinose), decreased flatulence potential, increased vitamin content in form of thiamine and riboflavin, provision of free glutamate, reduction or elimination of phytic and oxalic acids that could function directly in taste or eventually serve as precursors for aroma active molecules (Odunfa and Oyewole, 1986; Azokpota *et al*., 2006; Anukam and Reid, 2009; Chelule *et al*., 2010).

Industrial processes however, sometimes have difficulty in copying a traditional product without losing some of, its flavour, aroma and other peculiar characteristics. Similarly, many indigenous traditional technologies are not easily adopted by transnational companies without altering the methods of preparation and perhaps ending up with a product of altered aroma, colour and flavour, such, that it is, unacceptable (Onyekwere *et al*., 1989; Ogunshe *et al*., 2008a, b). The conservative food habits of consumers are, also an advantage for small-scale indigenous cottage industries but contaminations always exist in these naturally-fermented traditional foods, some of which may also contain toxin-producing microflora (Ouoba *et al*., 2008). Therefore, since there has been significant increase in the number of reported cases of food-borne illnesses, there must be considerable interest in ways of stopping this upward trend by reduction in the incidence of, microbial food-poisoning (Smith-Palmer *et al*., 1998).

The fermented African locust beans (*Parkia biglobosa*) cotyledons is known as, *iru* (Yorubas of Southern Nigeria), *dadawa*/*dawadawa* (Hausas of the Northern Nigeria), *kpalugu* (Ghana), *khinda* (Sierra Leone), *nététou* (the Gambia/Senegal) and *soumbala*/*soumbara* (many francophone West African countries, including Burkina Faso). It, is a strong-smelling, dark-brownish, food-flavouring condiment and the most important food condiment in the entire savannah region of the West and central Africa. *Iru* is one of the natural sources of plant proteins (Campbell-Platt, 1980), containing about 39-47% protein; 31-40% oil and 11.7-15.4% carbohydrates and it is due to this high protein content that it has a great potential as protein supplement (Oyenuga, 1968). *Iru* is used in the preparation of various delicacies, such as vegetable soups and stews and also as a low cost meat substitute that contributes to, protein and calories intake by several West African families. It is one of the highly cherished Nigerian fermented food condiments among various natives and more recently, the unique organoleptic properties of such indigenous, condiment are appreciated among the elites, compared to the highly advertised industrial seasoning agents in the country (Ogunshe *et al*., 2008a).

Although fermentation plays important roles in the lives of people in the developed and developing worlds, food safety remains a major challenge to producers and consumers of indigenous fermented foods. As an example, fermentation of food condiments like *iru ogiri*, etc., is still a traditional family art carried out in houses, while some of the associated microorganisms are of pathogenic and medical importance (Ogunshe *et al*., 2006; Ogunshe and Olasugba, 2008), even with antibiotic resistance abilities. Due to the emergence of multi-drug, resistant food-borne bacteria posing a challenge, the need to identify the source (s) of such Gram-negative, food-borne indicator bacterial flora present in *iru* is, thus, necessary. This study therefore, is aimed at determining the possibility of possibility of water samples used during the production of *iru* as source of food-borne Gram-negative indicator bacteria in traditionally-produced *iru*, the most popular Nigerian indigenous fermented food condiment.

MATERIALS AND METHODS

Samples' collection: This study which was in various stages, involving field and laboratory works, was concluded in October 2010. Locust bean (*Parkia biglobosa*) seeds were purchased from

Bodija market in Ibadan, Oyo State, Nigeria, while the water samples used for cottage-production of fermented *iru* were collected from 30 sources in five major cities of southwestern (Abeokuta, Ibadan, Ijebu-Ode, Lagos) and middle belt (Lokoja) Nigeria.

Simulated fermentation of *iru* samples: The collected processing water samples from local *iru* producers were used in rinsing the boiled and dehulled cotyledons, while *iru* samples were fermented in the laboratory according to the modified, simulated traditional method (Fig. 1a). The modification in the simulated method was that the *Parkia biglobosa* seeds were boiled under pressure using pressure cooker at lesser time, instead of using fire wood to boil the seeds (Fig. 1b).

pH determination: Ten grams of each fermented condiment samples were homogenised in 90 mL of sterile distilled water. The pH of each homogenate was then determined using a Pye unican pH meter equipped with a glass electrode and determinations were done in triplicates.

Pre-fermentation processing: The fermenting cotyledons were subjected to various pre-fermentation processing such as rinsing the *iru* cotyledons with *iru* processing-water, tap water,

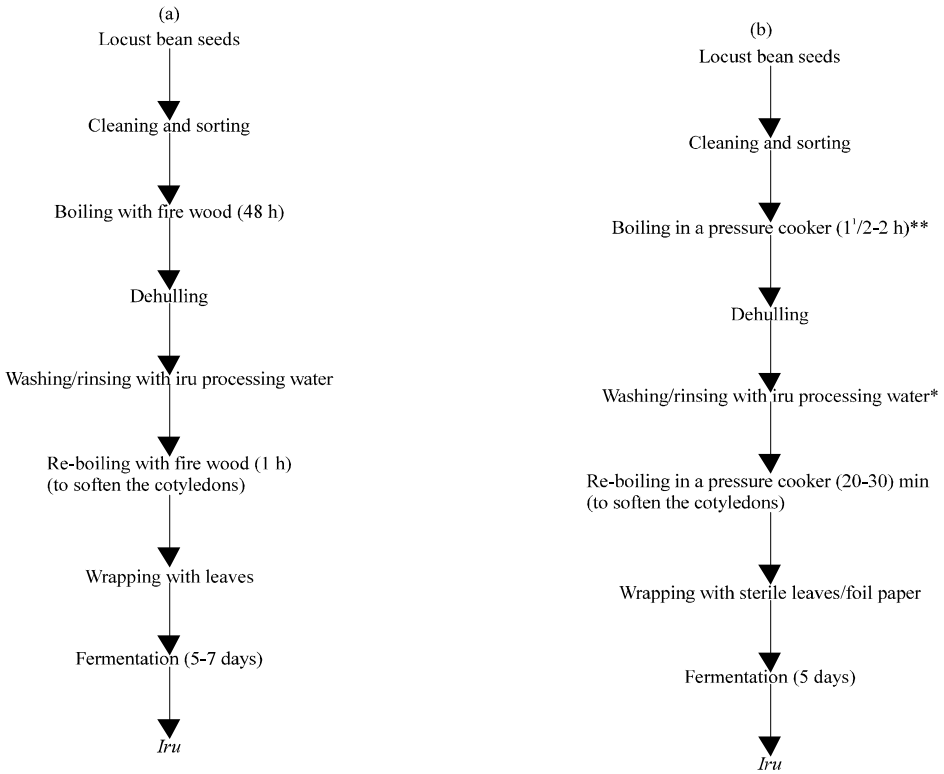

Fig. 1(a-b): (a) Flow chart for traditional (cottage) production of *iru* and (b): Flow chart for the simulated (adapted laboratory) production of *iru*. ** Modified simulated method: the locust bean seeds were boiled under pressure with pressure pot. * Tap and sterile water samples were used in rinsing the boiled cotyledons during the controlled fermentation process

well water and sterile water. Twenty selected processing water samples were used to rinse the already boiled and autoclaved cotyledons, followed by the simulated traditional method of fermentation with cleaned banana leaves. *Iru* processing water samples 7, 9, 11, 24 and 30 were the samples containing the highest faecal coliform counts and were further used as test water samples in the rinsing of the bean cotyledons during pre-preparation treatments for fermentation of the cotyledons for *iru* production.

The banana leaves used for fermentation were cleaned with tap water, sterile water, ethanol-swabbed and sterilised in the pressure pot and the cotyledons were packed while hot and cold into the banana leaves for fermentation. In the first set, different water samples were used to rinse the already boiled and autoclaved cotyledons, followed by the simulated traditional method of fermentation with cleaned banana leaves. Another set of similarly rinsed cotyledons were fermented with ethanol-swabbed banana leaves, while the third set were the similarly rinsed cotyledons but wrapped in clean leaves and re-sterilsed by autoclaving for 15 min. before fermentation.

Bacterial isolation: Isolation of Gram-negative indicator bacteria from *iru* samples were by selective pour-plating culture procedure on MacConkey agar, eosin methylene blue agar, *Salmonella-Shigella* agar, blood agar and cystein-lactose-electrolyte deficient agar (Lab M, England). Pure cultures of the indicator bacterial isolates obtained from the processing-water samples and fermenting *iru* samples were characterised using phenotypic protocols, according to standard bacterial taxonomical methods (Bailey and Scott, 1974; Harrigan and McCance, 1976; Cheesbrough, 1998, 2000).

Determination of presence of coliforms in processing water/fermenting bean cotyledons' samples: One litre of processing water samples were collected from each producer of *iru* and stored in 500 mL quantities under aseptic conditions at ambient temperatures throughout the period of coliform determination. For coliform determination, 5 mL / 5 g of each processing water/fermenting cotyledons' sample were dispensed into each specimen bottle containing 50 mL of sterile MacConkey broth (Lab M, England) with inverted Durham tube. Specimens for the determination of faecal coliform were incubated at 45°C for 24-48 h, while specimens for total coliform determination were incubated at 35°C for 24-48 h. Presence of total or faecal coliform was determined by colour change of the MacConkey broth from red-pink to yellow with the presence of gas in the inverted Durham tubes.

Antibiotic susceptibility test: Antibiotic susceptibility determination on the Gram-negative bacterial isolates using various antibiotic discs: AMX (Amoxycillin; 25 μg), COT (Cotrimoxazole; 25 μg), NIT (Nitrofurantoin; 250 μg), GEN (Gentamicin; 10 μg), NAL- (Nalidixic acid; 30 μg), OFL- (Ofloxacin; 30 μg), AUG (Augmentin; 30 μg) and TET (Tetracycline; 30 μg), was carried out according to the agar disc-diffusion method of NCCLS (2003). The entire agar surfaces of sterile, Mueller-Hinton agar plates were seeded with each test bacterial isolate and the antibiotic discs were later placed on the agar surfaces, followed by incubation of the plates at 35°C for 24-48 h. Zones of inhibition after incubation were measured and recorded in millimeter diameter according to the methods of Bauer *et al.* (1966) and NCCLS (2003), while zones less than 10.0 mm in diameter or absence of inhibition zones were recorded as resistant (negative).

RESULTS

All of the 30 water samples collected from local *iru* producers (used for washing the bean cotyledons for *iru* fermentation) and stored at ambient temperature were faecal coliform positive between 1 month and 2 years of storage, while 27 (90.0%) and 19 (63.3%) of the water samples were total coliform positive at 28 and 35 months, respectively (Table 1). The Gram-negative bacterial isolates recovered from the *iru* processing water samples were phenotypically characterised as *Enterobacter aerogenes* (5.8%), *Escherichia coli* (58.9%), *Klebsiella aerogenes* (11.8%), *Klebsiella pneumoniae* (11.8%), *Proteus mirabilis* (5.8%) and *Pseudomonas aeruginosa* (5.8%). It was observed that all the different fermenting cotyledons that were rinsed with 20 selected processing water samples were total coliform positive between day 1 and day 5 of fermentation and about 60.0% were faecal coliform-positive by day 5 of fermentation (Table 2).

Table 1: Coliform results of processing water samples used for fermenting locust bean seeds' cotyledons

	Periods of coliform determination (months)									
	1	4	8	12	16	20	24	28	32	35
Water sample	F T	F T	F T	F T	FT	F T	F T	F T	F T	F T
1	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- +	- +
2	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- +	- +
3	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- +	- +
4	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- +	- -
5	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- +	- +
6	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -
7	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- +	- +
8	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- +	- +
9	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- +	- +
10	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- -	- -
11	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- +	- +
12	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- +	- +
13	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- +	- +
14	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- +	- +
15	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- +	- +
16	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- +	- -
17	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- -	- -
18	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- -	- -
19	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- +	- +
20	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -
21	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- +	- +
22	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- +	- -
23	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- +	- +
24	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- +	- +
25	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- +	- +
26	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- +	- -
27	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- +	- -
28	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- +	- +
29	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- -	- -	- -
30	+ +	+ +	+ +	+ +	+ +	+ +	+ +	- +	- +	- +

+: Coliform positive, -: Coliform negative, F: Faecal coliform, T: Total coliform

As shown in Fig. 2, an overall total of 152 randomly obtained Gram-negative bacterial isolates from the various fermenting *iru* cotyledons that were rinsed with 20 selected processing water samples obtained in this study were characterised as *Escherichia coli* 34 (22.3%), *Enterobacter aerogenes* 17 (11.2%), *Klebsiella aerogenes* 13 (8.6%), *Klebsiella pneumoniae* 37 (24.3%), *Morganella morganii* 5 (3.3%), *Salmonella Enterica* serovar Paratyphi 8 (5.3%), *Salmonella Enterica* serovar Typhi 4 (2.6%), *Shigella dysentariae* 7 (4.6%), *Proteus mirabilis* 21 (13.8%), *Pseudomonas aeruginosa* 4 (2.6%) and *Vibrio cholerae* 2 (1.3%).

Table 2: Coliform results of fermenting locust bean seeds' cotyledons rinsed with selected processing water samples at 48 h of incubation

Fermenting cotyledons rinsed with water samples	Periods of coliform determination (days)									
	1		2		3		4		5	
	T	F	T	F	T	F	T	F	T	F
FC 2	+	-	+	+	+	+	+	+	+	+
FC 3	+	+	+	+	+	+	+	+	+	+
FC 6	+	-	+	+	+	+	+	-	+	-
FC 7	+	+	+	+	+	+	+	+	+	±
FC 9	+	-	+	+	+	+	+	+	+	+
FC 10	+	+	+	+	+	+	+	+	+	-
FC 11	+	+	+	+	+	+	+	+	+	±
FC 13	+	-	+	-	+	+	+	+	+	+
FC 15	+	+	+	+	+	+	+	+	+	+
FC 17	+	+	+	+	+	+	+	-	+	-
FC 18	+	+	+	+	+	+	+	+	+	-
FC 19	+	-	+	+	+	+	+	+	+	+
FC 20	+	+	+	+	+	+	+	+	+	-
FC 21	+	+	+	+	+	+	+	+	+	+
FC 22	+	+	+	+	+	+	+	+	+	-
FC 24	+	-	+	-	+	+	+	+	+	±
FC 26	+	-	+	+	+	+	+	+	+	-
FC 27	+	+	+	+	+	+	+	-	+	-
FC 28	+	+	+	+	+	+	+	+	+	+
FC 30	+	-	+	+	+	+	+	+	+	±
	20 (100%)	12 (60.0%)	20 (100%)	18 (90.0%)	20 (100%)	20 (100%)	20 (100%)	17 (85.0%)	20 (100%)	12(60.0%)

+: Coliform positive, -: Coliform negative _ : Processing water samples with highest coliform counts, F: Faecal coliform; T: Total coliform

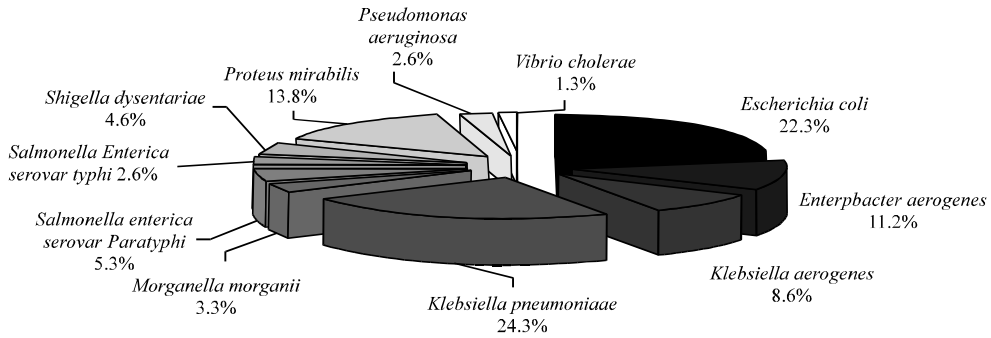

Fig. 2: Percentage recovery rates of indicator foodborne Gram-negative bacteria

Table 3: Recovery rates of isolated Gram-negative bacterial specie from simulated fermenting locust bean seeds' cotyledons

Days	Isolated Gram-negative bacterial species
1	*E. coli* (12.5%), *Enterobacter aerogenes* (6.3%), *Klebsiella aerogenes* (12.5%), *Klebsiella pneumoniae* (43.8%), *Proteus mirabilis* (18.8%), *Shigella dysentariae* (6.3%) [*Kleb. pneumoniae* (43.8%)]
2	*E. coli* (27.3%), *Enterobacter aerogenes* (4.5%), *Klebsiella aerogenes* (4.5%), *Klebsiella pneumoniae* (50.0%), *Pseudomonas aeruginosa* (4.5%), *Proteus mirabilis* (4.5%), *Shigella dysentariae* (4.5%) *Kleb. pneumoniae* (50.0%); *E. coli* (27.3%)]
3	*E. coli* (25.0%), *Enterobacter aerogenes* (15.6%), *Klebsiella aerogenes* (6.3%), *Klebsiella pneumoniae* (25.0%), *Morganella morganii* (6.3%), *Proteus mirabilis* (15.6%), *Shigella dysentariae* (3.1%), *Vibrio cholerae* (3.1%) [*E. coli* (25.0%), *Kleb. pneumoniae* (25.0%)]
4	*E. coli* (32.4%), *Enterobacter aerogenes* (16.2%), *Klebsiella aerogenes* (5.4%), *Klebsiella pneumoniae* (27.0%), *Proteus mirabilis* (16.2%), *Shigella dysentariae* (2.7%) [*E. coli* (32.4%); *Kleb. pneumoniae* (27.8%)]
5	*E. coli* (22.2%), *Enterobacter aerogenes* (13.9%), *Klebsiella aerogenes* (16.7%), *Klebsiella pneumoniae* (27.8%), *Proteus mirabilis* (16.7%), *Shigella dysentariae* (2.8%) [*Kleb. pneumoniae* 27.8%; *E. coli* (22.2%)]

Values in parenthesis are the percentage rates of the most recovered bacterial species. The pH values of the fermenting mesh and fermented condiments were between 7.3 and 8.1

Table 4: Faecal coliform results of the pre-processed laboratory fermenting locust bean seeds' cotyledons

	Coliform results (days of fermentation of cotyledons)									
	Day 1		Day 2		Day 3		Day 4		Day 5	
Processing methods	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Boiled cotyledons IRW 30/ethanol-swabbed leaf	+	+	-	+	+	+	+	+	+	+
Boiled cotyledons IRW 7/ethanol-swabbed leaf	+	+	-	+	+	+	-	+	+	+
Boiled cotyledons IRW 11/ethanol-swabbed leaf	+	+	+	+	+	+	+	+	-	+
Boiled cotyledons IRW 24/ethanol-swabbed leaf	+	+	+	+	-	-	+	+	+	+
Boiled cotyledons Sterile water/ethanol-swabbed leaf	+	+	+	+	+	+	+	+	-	-
Sterile cotyledons (Hot) IRW30/ethanol-swabbed leaf	+	+	+	+	-	+	-	+	-	-
Sterile cotyledons (Cold) IRW30/ ethanol-swabbed leaf	+	+	-	-	+	+	+	+	-	-
*Sterile cotyledons Sterile water/ethanol-swabbed leaf	-	-	-	-	-	-	-	-	-	-
Boiled cotyledons (Cold) Sterile foil paper	-	+	+	+	+	+	+	+	+	+
Boiled cotyledons (Hot) Sterile foil paper	-	-	-	-	-	-	+	+	+	+
Boiled cotyledons (Hot/cold) IRW9 / boiled leaf	+	+	+	+	+	+	+	+	+	+
Sterile cotyledons (Hot/cold) IRW9/ boiled leaf	+	+	+	+	+	+	+	+	+	+
Boiled cotyledons (Hot/cold) IRW9 / sterile leaf	+	+	+	+	+	+	+	+	+	+
Sterile cotyledons (Hot/cold) IRW9 / sterile leaf	+	+	+	+	+	+	+	+	+	+
*Sterile cotyledons/ boiled leaf (Hot/cold) / IRW9	-	-	-	-	-	-	+	+	+	+
*Sterile cotyledons/ sterile leaf (Hot/cold) / IRW9	-	-	-	-	-	-	-	+	+	+

+: Coliform positive, -: Coliform negative, IRW: *Iru* processing water, *: Less fermentation characteristics

The prevailing Gram-negative bacterial species from the simulated fermenting *iru* cotyledons were as shown in Table 3. The most recovered bacterial species from the fermenting mash during the 5- day fermentation period were *Klebsiella pneumoniae* (43.8%) on day 1; *Klebsiella pneumoniae* (50.0%) and *E. coli* (27.3%) on day 2; *E. coli* (25.0%) and *Klebsiella pneumoniae* (25.0%) on day 3; *E. coli* (32.4%) and *Klebsiella pneumoniae* (27.8%) on day 4 and *Klebsiella pneumoniae* (27.8%) and *E. coli* (22.2%) on day 5, respectively (Table 3).

Table 5: Antibiotic resistance pattern of Gram-negative bacterial species from *iru* processing water samples

	Antibiotic discs (μg^{-1})								
Bacterial isolates	AMX	COT	NIT	GEN	NAL	OFL	AUG	TET	MAR (%)
E. coli (58.9%)	66.6	100	33.3	33.3	66.6	33.3	33.3	33.3	25.0-87.5
Ent. aerogenes (5.8%)	100	100	100	0.0	100	0.0	100	100	75.0
Kleb. aerogenes (11.8%)	100	66.6	33.3	0.0	33.3	0.0	100	100	37.5-75.0
Kleb. pneumoniae (11.8%)	100	33.3	33.3	0.0	33.3	33.3	66.6	100	25.0-75.0
Pr. mirabilis (5.8%)	100	0.0	0.0	0.0	100	0.0	100	100	50.0
Ps. aeruginosa (5.8%)	100	100	0.0	100	0.0	100	100	50.0	75.0

AMX : Amoxycillin, COT: Cotrimoxazole, NIT: Nitrofurantoin, GEN: Gentamicin, NAL: Nalidixic acid, OFL: Ofloxacin, AUG: Augmentin, TET: Tetracycline, % MAR: % Multiple antibiotic resistance

Table 6: Antibiotic resistance pattern of Gram-negative bacterial species from fermenting locust bean seeds' cotyledons

	Antibiotic discs (μg^{-1})								
Bacterial isolates	AMX	COT	NIT	GEN	NAL	OFL	AUG	TET	MAR (%)
E. coli (29.0%)	62.5	33.3	50.0	25.0	62.5	0.0	75.0	37.5	25.0-87.5
Ent. aerogenes (10.0%)	75.0	50.0	50.0	25.0	50.0	50.0	75.0	75.0	25.0-87.5
Kleb. aerogenes (10.0%)	80.0	70.0	30.0	30.0	20.0	0.0	60.0	20.0	25.0-62.5
Kleb. pneumoniae (29.0%)	90.9	63.6	54.5	9.1	63.6	18.1	90.9	63.6	37.5-100
Morg. morganii (1.0%)	100	100	100	0.0	100	0.0	100	100	75.0
Pr. mirabilis (10.0%)	66.7	33.3	33.3	0.0	33.3	0.0	66.7	66.7	25.9-75.0
Salm. typhi (2.0%)	50.0	100	50.0	0.0	50.0	0.0	50.0	50.0	75.0
Sh. dysentariae (8.0%)	100	100	100	50.0	50.0	50.0	50.0	100	62.5-87.5
V. cholerae (1.0%)	100	100	100	0.0	100	0.0	100	100	75.0

AMX: Amoxycillin, COT: Cotrimoxazole, NIT: Nitrofurantoin GEN: Gentamicin, NAL: Nalidixic acid, OFL: Ofloxacin, AUG: Augmentin, TET: Tetracycline, % MAR: % Multiple antibiotic resistance

The faecal coliform results of the 5-day fermenting cotyledons that were subjected to various pre-fermentation processing are as shown in Table 4. The presence of coliforms was more, prominent at 48 h of incubation. Although the presence of coliforms was less recorded in the sterilised samples the fermenting characteristics, especially colour, smell and softness of cotyledons were also less pronounced.

The antibiotic resistance patterns of the Gram-negative bacterial species from *iru* processing water samples were as shown in Table 5 - amoxycillin (AMX: 66.6-100%), augmentin/cotrimoxazole/ nalidixic acid/nitrofurantoin/tetracycline (AUG/COT/NAL/NIT/TET: 33.3-100%), were the most resisted antibiotics, while gentamicin and ofloxacin (GEN/OFL; 33.3%) were moderately resisted, except in the only strain of *Ps. aeruginosa*. Multiple Antibiotic Resistance (MAR) patterns of the Gram-negative bacterial isolates from the *iru* processing water samples indicated values between 25.0-87.5%.

Antibiotic resistance patterns of the Gram-negative bacterial species, isolated from fermenting locust bean seeds' cotyledons were amoxycillin / augmentin (AMX /AUG: 50.0-100%); cotrimoxazole (COT: 33.3-100%) while resistance rates of other antibiotics were between 9.1-100%. Ofloxacin was the least resisted (18.1-50.0%) but the overall MAR rates were between 25.0 and 100% (Table 6).

DISCUSSION

Indigenous fermented food condiments are known to be traditional family art practised in households, under primitive and unhygienic conditions, which in most times results in low yield and

poor quality, since microbial flora from the environment freely come in contact with the fermenting cotyledons (Ogbadu and Okagbue, 1988); Thereby, the modes of preparation must have significantly contributed to the high recovery rates of the Gram-negative food-borne indicator bacteria in this most commonly consumed (sometimes eaten even in uncooked state), fermented condiment in the south-western, northern, eastern and middle belt regions of Nigeria. Prior to fermentation the boiled and dehulled cotyledons are cleaned by washing in the river, streams, rain-filled ditches and at times with water from wells etc. The source of water for washing the cotyledons may be responsible for the widely reported observation, in which many housewives nowadays rinse the condiment with clean water, using only the rinsed cotyledons for cooking, due to the sandy/stony mouth-feel effect while eating soups, dishes and stews cooked with *iru* but the portion washed off are the nutritious portion of the condiments.

According to Steinkraus (1991, 1995), the alkaline pH created during fermentation of the proteinous foods and as also observed in the present and other studies (Hesseltine, 1965; Ogbadu and Okagbue, 1988; Ogunshe *et al*., 2007; Parkouda *et al*., 2009) has been reported to make the fermenting substrate unsatisfactory for invasion by microorganisms that might cause spoilage of the product. However; although, there was no correlation between the pH values of the fermenting cotyledons and presence of coliforms, or other food-borne pathogens, this study proved that the food indicator bacterial species were able to withstand the alkaline conditions created by the fermenting process considering the fact that the Gram-negative, foodborne indicator bacteria isolated from *iru* in this study are similar to those previously isolated from fermenting or fermented *iru* (Campbell-Platt, 1984; Ogunshe and Olasugba, 2008; Enujiugha, 2009).

Food microbiologists are usually interested in the determination and studies on microbial flora of industrial importance, especially in selection of starter cultures from fermented foods, including fermented condiments but it is important to note that these groups of pathogens have been implicated in food poisonings and clinical cases, thus becoming a significant public health concern with a worldwide distribution (Baird-Parker, 1994). As an example, the outer membrane of Gram-negative bacteria is rich in a molecule called lipopolysaccharide; therefore, if Gram-negative bacteria enter the bloodstream, lipopolysaccharide can trigger a cascade of events, including high fever and a drop in blood pressure and for this reason, lipopolysaccharide is often referred to as an endotoxin (Kaplan, 2000). Presence of such Gram-negative foodborne bacterial indicators in *iru* as recorded in this study is therefore, of significant pathogenic and medical importance.

In addition, the Gram-negative food indicator bacteria from *iru* samples were mostly multiple-antibiotic resistant, while the global impact of antibiotic resistance is potentially devastating and threatens to set back progress against certain infectious diseases to the pre-antibiotic era (Larson, 2007). Almost all cases of foodborne illnesses in Nigeria are undocumented due to lack of proper health-record keeping, surveillance and government policies. Meanwhile, pathogens, such as those isolated from *iru* samples can cause mild to moderate self-limiting gastroenteritis, while invasive diseases and complications may also occur, resulting in more severe cases (Mead *et al*., 1999; CDC, 2002). It can thus, be suggested that measures that can minimise the risk of foodborne illnesses should be taken during *iru* productions, while the application of hazard analysis critical control points (HACCP), also as advocated by the World Health Organisation (WHO) should be applied to indigenous fermented food condiments like *iru*.

Contamination of foods by disease-producing microorganisms has been known and studied since around 1880 (Jay, 1993) and as far back as the last century, coliform bacteria have been used as indicators of possible faecal contamination in water and food (Chang *et al*., 1989; Jay, 1993).

Current study seems to be the first reported data on the microbial significant effect of processing water on *iru*. Due to high recovery rates of Gram-negative indicator enterobacteria from *iru* processing water samples, as well as the fermenting/fermented *iru* produced with the processing water, it can then be concluded that water used in the preparation of *iru* and other similar condiments is a major source of foodborne indicator bacteria associate with *iru*. In addition, the multiple antibiotic resistance recorded among the bacterial flora from the processing water also indicated the unwholesomeness of the water samples in the fermentation processing of such condiment. This is therefore, likely to be responsible for the multiple antibiotic resistance recorded among the bacterial flora from the fermenting *iru* condiment. A major problem of African researches is that findings are usually limited to documented data; therefore, further studies on HACCP of cottage-production of *iru* and feedback surveillance of the processing methods to the traditional producers are on-going in our laboratories.

CONCLUSION

This study concludes that processing water used in cleaning the boiled and dehulled African locust bean (*Parkia biglobosa*) seed cotyledons during the traditional-production of *iru* is a major source of Gram-negative food-borne indicator bacteria to the fermented condiment.

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