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AEROBIC MESOPHILIC BACTERIA ASSOCIATED WITH IRISH POTATO (*SOLANUM TUBEROSUM* L.) SPOILAGE AND THEIR SUSCEPTIBILITY PATTERN TO LACTIC ACID BACTERIA AND ANTIBIOTICS.

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ABSTRACT

A total of 15 samples of spoiled Irish potatoes (*Solanum tuberosum* L.) were collected from five grocery shops in Ile-Ife, Osun State, Nigeria and were analysed for aerobic mesophilic bacterial load. The isolated aerobic mesophilic bacteria were phenotypically characterized by biochemical tests and their susceptibility to antibiotics was assessed. In addition, lactic acid bacteria (*Lactobacillus casei*, *Enterococcus faecalis* and *Lactobacillus cellobiosus*) previously isolated from yoghurt showing inhibitory activities against indicator organisms were screened for antimicrobial activities against the isolated spoilage bacteria. The population of aerobic mesophilic bacteria ranged from $5.18 - 7.74 \log \text{ c.f.u g}^{-1}$. A total of five strains of bacteria were isolated and identified as *Bacillus* sp. (A₁), *Bacillus* sp. (B₁), *Bacillus* sp. (B₂), *Erwinia chrysanthemi* and *Pseudomonas* sp. The isolated bacterial strains showed multiple antibiotics resistance. Of significant note is the high multiple resistance pattern of *Bacillus* sp. strain B₂, which showed resistance to five out of the eight antibiotics tested. The multiple antibiotic resistance (MAR) index of the isolated bacterial strains ranged from 0.4 to 0.6. Of the lactic acid bacteria screened, only *Enterococcus faecalis* showed inhibitory activity due to the effect of organic acid against *Erwinia* sp. and *Bacillus* sp. (A₁). The results indicated the high prevalence of antibiotic resistant strains associated with the spoilage of Irish potato.

Key words: Irish Potato, Aerobic Mesophilic Bacteria, Antimicrobial Agents, Lactic Acid Bacteria,

INTRODUCTION

Potato (*Solanum tuberosum* L) is a staple crop in 130 countries worldwide, ranking fourth in production after rice, maize, and wheat (Calvo *et al.*, 2010), with Nigeria being the fourth biggest producer in Sub Saharan Africa (FAO, 2008). Potato is a carbohydrate rich food providing a good source of dietary energy and some micro nutrients to consumers. In Nigeria, potatoes are responsible for more than half the total carbohydrate requirements of the populace in localities where potato is cultivated and consumed as a staple food. In comparison with other roots and tubers, the protein content of potato is very high. In many developing countries especially the urban areas, rising levels of income are driving a “nutrition transition” towards more energy dense foods, as part of that transition; demand for potato is increasing (FAO, 2008).

Potato tubers suffer from post harvest losses as a result of physical, physiological or pathological factors or a combination of all three factors (Booth, 1974). The principal factors responsible for losses during storage of potatoes have been reported to include the natural processes of the dormant but living tubers which result in the conversion of starch in the tubers into carbon dioxide and water, evaporation of water from the tuber, and sprouting and infection by microorganisms resulting in tuber decay (Amadioha and Adisa, 1993). Fungi and bacteria causing rots in potato have been reported to produce a wide range of hydrolytic enzymes such as cellulases, pectinases, xylanases, and proteases (Olivieri *et al.*, 2004). These enzymes are responsible for tissue maceration and cell death,

following which the microorganisms have access to the nutritional resources of the dead plant tissues (Aveskemp *et al.*, 2008). Bacterial soft rot is one of the most common potato diseases in the tropics and induces quick and heavy spoilage losses. Its causal bacterium, *Erwinia carotovora*, has been extensively studied (Harrison and Nielson, 1990). Among the fungi reported to be associated with dry rot of potato, *Fusarium solani* has been reported as the most virulent (Mayea *et al.*, 1980; Adisa, 1986). Understandably, preventing spoilage of potato during storage carries a great economic significance. One way to prevent this spoilage is to protect healthy tubers from mechanical and biological injuries. As potato, the world’s largest non-cereal crop, is frequently damaged by *E. carotovora* leading to huge economic losses, a cost effective strategy for spoilage control is therefore critical to potato farmers, retailers, and processors. In line with this, some efforts to inhibit *E. carotovora* development on postharvest potatoes using salts, antimicrobial agents and irradiation and /or disinfectants have been previously described (Afek *et al.*, 1999; Tsai *et al.*, 2001; Cladera Olivera *et al.*, 2006)

In this study, spoilage organisms in potato were isolated, characterized and their sensitivity to antibiotics were investigated. In addition, lactic acid bacteria previously isolated from yoghurt showing inhibitory activities against indicator organisms were screened for antimicrobial activities against the isolated spoilage bacteria, in order to make useful recommendation on possible prevention of spoilage by bacteria.

MATERIALS AND METHODS

Source of Potato Samples

Fifteen samples of spoiled Irish potato tubers were collected from five grocery shops in Ile-Ife, using sterile stomacher bags. The samples were immediately transported to the Food Microbiology laboratory of the Department of Microbiology, Obafemi Awolowo University, Ile-Ife Nigeria for analysis.

Source of Lactic Acid Bacteria Species

The lactic acid bacteria (LAB) strains (*Lactobacillus casei*, *Enterococcus faecalis* and *Lactobacillus cellobiosus*) used in this study were previously isolated from bottled yoghurt and reported to exhibit anti-inhibitory properties (Omafuvbe and Enyioha, 2011). They were obtained from the stock collection in the Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.

Microbial Analysis

The potato tubers from the five different grocery shops were grouped and labeled A to E with three potato tubers in a group. Potatoes in each group were mashed aseptically in a sterile stomacher bag and 10.0g of the mashed potato was suspended in 90 mL of sterile maximum recovery diluent (MRD, Oxiod) and serially diluted in same diluent. One milliliter of appropriately diluted sample was pour plated in nutrient agar and the poured plates were incubated at 30°C for 48 h. The viable aerobic mesophilic bacteria were enumerated and expressed as colony forming units (c.f.u) per gram sample. Representatives of the different colonies were selected according to their morphological characteristics, purified by successive subculturing on nutrient agar and identified phenotypically using standard methods (Harrigan and McCance, 1976; Buchannan and Gibbons, 1985).

Antimicrobial Susceptibility Tests

The bacteria isolates were tested for their susceptibility pattern to antibiotics (Abtek, Scotland) namely; Ampicillin (25 µg), Cotrimoxazole (25 µg), Gentamicin (10 µg), Nalidixic (30 µg), Nitrofurantoin (200 µg), Colistin (25 µg), Streptomycin (25 µg), Tetracycline (25 µg) for the Gram-negative organisms and Ampicillin (10 µg), Chloramphenicol (10 µg), Cloxacillin (5 µg), Erythromycin (5 µg), Gentamicin (10 µg), Penicillin (1 I.u), Streptomycin (10 µg) and Tetracycline (10 µg) for Gram-positive organisms. This testing was performed using the standard disc diffusion method of the National Committee for Clinical Laboratory Standards NCCLS (2008) guidelines. Aqueous suspension of 18 to 24 h old pure culture of the bacterial isolates were made in sterile saline and compared with prepared 0.5 Macfarland standard. The broth cultures (0.1 mL) of the isolates were spread on Mueller Hinton agar plates, the antibiotics disc were placed on it and the plates incubated at 35 °C for 24 h. The antibiotics

susceptibility pattern of the isolates was interpreted using the criteria described by the disc manufacturer and according to NCCLS standards.

Multiple Antibiotics Resistance Indexing of Isolates

Multiple antibiotic resistance (MAR) index was defined as a/b where 'a' represents the number of antibiotics to which the isolate is resistant and 'b' the number of antibiotics to which the isolate is exposed (Adeleke and Omafuvbe, 2011). MAR index values of less than or equal to 0.2 is considered to below risk sources which is an indication that the strain originated from sources where antibiotics are seldom or never used.

Production of Inhibitory Substance and Antagonistic Effect of Lactic Acid Bacteria

The LAB strains were grown in MRS (de Man, Rogosa, and Sharpe) broth for 24h at 30°C under anaerobic condition [in anaerobic jar provided with disposable BBL gas generating pack (CO₂ system envelopes, Oxiod)]. The cell free supernatant of the broth culture was obtained by centrifugation at 15, 000 rpm for 15 min at 4°C (Kivanc, 1990). Half portion of the supernatant was adjusted to pH 6.2 using 2.5 N NaOH to rule out inhibition due to pH reduction caused by organic acids while the other half was used unadjusted. The pH adjusted supernatant was filtered through a syringe filter with a pore size of 0.22µm (Satorius Millipore, UK). Antagonistic activities of both pH adjusted and unadjusted cell free supernatant of the LAB strains were tested on the bacteria isolates from the spoiled potatoes using the agar well diffusion assay technique (Schlinger and Lucke, 1989; Takahiro *et al.*, 1991). Briefly, one milliliter of the bacteria isolate cultured in nutrient broth for 24 h at 30 °C was seeded into 15mL of molten Mueller Hinton agar (Oxoid), maintained at 45 °C. The resulting mixture was poured into Petri dishes and allowed to solidify and wells of 4 mm in diameter were bored into the agar with a sterile cork borer. Aliquots of fifty micro liters (50 µl) of the filtered cell free supernatant of the test organisms (LAB) were transferred into each well. The plates were incubated aerobically for 24 h at 30 °C, after which they were examined for clear zones around the wells. Inhibition occurring with the use of the pH adjusted supernatant was assumed to the presence of inhibitory substance other than pH (organic acid).

RESULTS AND DISCUSSION

The population of aerobic mesophilic bacteria recorded for the spoiled potato samples from the five grocery shops was high and ranged between 5.18 and 7.74 log c.f.u g⁻¹ sample (Table 1). The heavy load of bacteria can be attributed to the rapid colonization of the potato by spoilage organisms. The aerobic mesophilic bacteria population comprised of five bacteria strains of the genera *Erwinia*, *Pseudomonas*, and

Bacillus (Table 2). Our identification was based on phenotypic characteristics only. The isolates could be further characterized using molecular method. All the bacteria strains isolated were found to occur in all the

sampled spoilt potatoes from the five grocery shops (Table 1). *Erwinia* sp. and *Bacillus* sp have been reported to be associated with bacterial soft rot in potatoes (Olivieri et al., 2004; Mahmoud et al., 2008). In Nigeria,

Table 1. Total Aerobic Mesophilic Bacteria Population and their Occurrence in Spoilt Potato Samples

Potato samples*	Bacteria count (log c.f.u.g ⁻¹)**	Occurrence of bacteria isolates***
A	7.74 ± 0.73	<i>Erwinia chrysanthemi</i> , <i>Pseudomonas</i> sp., <i>Bacillus</i> spp. (A ₁ , B ₁ , B ₂)
B	5.18 ± 0.67	<i>Erwinia chrysanthemi</i> , <i>Pseudomonas</i> sp., <i>Bacillus</i> spp. (A ₁ , B ₁ , B ₂)
C	7.60 ± 0.57	<i>Erwinia chrysanthemi</i> , <i>Pseudomonas</i> sp., <i>Bacillus</i> spp. (A ₁ , B ₁ , B ₂)
D	6.86 ± 0.65	<i>Erwinia chrysanthemi</i> , <i>Pseudomonas</i> sp., <i>Bacillus</i> spp. (A ₁ , B ₁ , B ₂)
E	5.23± 0.63	<i>Erwinia chrysanthemi</i> , <i>Pseudomonas</i> sp., <i>Bacillus</i> spp. (A ₁ , B ₁ , B ₂)

* Code represent grocery shops

** Values represent the mean of three determinations ± standard error.

*** A₁, B₁, B₂ represent the code for the three strains of *Bacillus* spp. isolated

Table 2. Characteristics of Bacteria Strains Isolated from Spoilt Potato

Characteristics	Isolate code				
	A ₁	A ₂	A ₃	B ₁	B ₂
Cell morphology	Rod	Rod	Rod	Rod	Rod
Gram reaction	+	-	-	+	+
Catalase	+	+	+	+	+
Spore	+	-	-	+	+
Nitrate reduction	+	-	+	+	+
Gelatin liquefaction	+	-	-	+	-
Citrate utilization	+	+	+	+	-
Indole production	-	-	+	-	-
Starch hydrolysis	+	-	-	-	-
Oxidative-Fermentative test	F	O	F	F	F
VP (acetoin)	-	-	+	+	+
Methyl red test	+	-	+	+	-
Arginine hydrolysis	+	+	+	+	+
Acid from Sugar Fermentation:					
Glucose	-	-	+	+	+
Sucrose	-	-	+	+	+
Lactose	-	-	+	-	-
Maltose	-	-	+	-	-
Mannitol	-	-	+	-	-
Probable identity	<i>Bacillus</i> sp.	<i>Pseudomonas</i> sp.	<i>Erwinia chrysanthemi</i>	<i>Bacillus</i> sp.	<i>Bacillus</i> sp.

+: Positive; -: Negative; O: Oxidative; F: Fermentative; VP: Voges-Proskauer.

Adisa (1986) reported *Erwinia* sp. as one of the most important bacteria causing spoilage of potatoes. Bacteria soft rot is one of the causes of microbial spoilage of potatoes and is the most important disease that can spread extensively during potato storage. Losses due to this disease may range from 0-100% depending upon the method of potato handling.

Although the production of enzymes by the isolated bacterial strains was not investigated in this study, bacteria causing rots in potato have been reported to produce a wide range of hydrolytic enzymes such as cellulases, pectinases, xylanases and proteases (Olivieri et al., 2004). These enzymes are responsible for tissue maceration and cell death, after which the

microorganisms have access to the nutritional resources of the dead plant tissues (Aveskamp *et al.*, 2008). *Erwinia* and *Bacillus* species have been found in soil and they gain entrance through wounds and natural openings such as lenticels. Although *Pseudomonas* sp. has never been reported to be associated with potato spoilage, its isolation from potato in this study was however not surprising, since *Pseudomonas* species commonly occur in soil and are mainly plant pathogens (Collins *et al.*, 2004).

The antibiotic susceptibility pattern of the isolated bacteria strains are shown in Table 3. All the bacteria isolates showed 100% resistance to ampicillin and 100% sensitivity to gentamicin. The *Bacillus* strains were 100% resistant to penicillin and cloxacillin. The isolates showed multiple antibiotics resistance pattern. Of significant note is the high multiple resistance pattern of *Bacillus* sp. strain B₂ which showed resistance

to five out of the eight antibiotics tested (Table 3). The high level of resistance to ampicillin, penicillin, and cloxacillin by the isolates is an indication of the production of b-lactamases. Several workers have reported the production of b-lactamase by bacteria with high resistance to b-lactam antibiotics (Rahman-Khan and Malik, 2001; Lateef *et al.*, 2004). Although to the best of our knowledge there are no reports yet on the antibiotics susceptibility pattern of bacteria associated with potato spoilage, a high level of resistance have been reported among bacteria from other foods (Rahman-Khan and Malik, 2001; Lateef *et al.*, 2004, Islam *et al.*, 2010). The high level of resistance exhibited by the organisms may be a reflection of misuse or abuse of antimicrobial agents in the environment which has created enormous pressure for the selection of resistance among opportunistic bacterial pathogens (Sharma *et al.*, 2005). Multiple

Table 3. Antibiotics Susceptibility Pattern of Bacteria Isolated from Spoilt Potato

Isolate*	Antibiotics												MAR index
	AMP	CHL	COL	COT	CXC	ERY	GEN	NAL	NIT	PEN	STR	TET	
<i>Bacillus</i> sp. (A ₁)	R	S	ND	ND	R	S	S	ND	ND	R	S	I	0.4
<i>Bacillus</i> sp. (B ₁)	R	S	ND	ND	R	R	S	ND	ND	R	I	I	0.4
<i>Bacillus</i> sp. (B ₂)	R	R	ND	ND	R	S	S	ND	ND	R	S	R	0.6
<i>Erwinia chrysanthemi</i>	R	ND	R	R	ND	ND	S	R	S	ND	S	S	0.5
<i>Pseudomonas</i> sp.	R	ND	S	S	ND	ND	S	S	S	ND	R	R	0.4

R- Resistant; I, Intermediate; S, Susceptible; ND, Not determined. MAR, Multiple antibiotic resistance.

* Isolate code in parenthesis

AMP, Ampicillin, CHL, Chloramphenicol, COT, Cotrimoxazole, CXC, Cloxacillin, NAL, Nalidixic, ERY, Erythromycin, NIT, Nitrofurantoin, GEN, Gentamicin, PEN, Penicillin, COL, Colistin, STR, Streptomycin, TET, Tetracycline

antibiotic resistant bacteria are considered a global threat to public health and the transferable nature of the gene clusters encoding high-level multiple antibiotic resistance in the environment have generated great concern in the scientific community (Islam *et al.*, 2010). All the isolates had MAR index > 0.2 (ranges from 0.4 to 0.6) which indicated that they were from high risk sources. Adeleke and Omafuvbe (2011) reported high level of multiple antibiotics resistance in poultry faeces which is in turn used as plant fertilizer. This serves as transfer of antibiotic resistance down the line of food chain towards man (Salehi and Bonab, 2006).

The pH adjusted cell free supernatants of the three strains of lactic acid bacteria tested did not inhibit the growth of the bacteria isolates associated with spoilt Irish potato in this study. It is worthy of note however, that only the pH unadjusted cell free supernatant of

Enterococcus faecalis showed antagonistic activity against *Bacillus* sp. (A1) (Plate 1) and *E. chrysanthemi* (Plate 2). These results indicate that the inhibitory activity showed by *Enterococcus faecalis* was due to organic acids since the pH adjusted cell free supernatant did not inhibit the organisms. Although the production of bacteriocin and bacteriocin-like substances was not tested in this study since the pH adjusted cell free supernatant of the lactic acid bacteria showed no inhibitory activity, bacteriocin-like substance produced by *Bacillus licheniformis* have been reported to inhibit potato soft rot caused by *E. carotovora* (Cladera-Olivera *et al.*, 2006). Bacteriocins produced by lactic acid have been the subject of intense investigation, since they are useful candidates for application in food storage (O'Sullivan *et al.*, 2002; Obadina *et al.*, 2006; Yurdugül and Bozoglu, 2008; Khalil *et al.*, 2009).

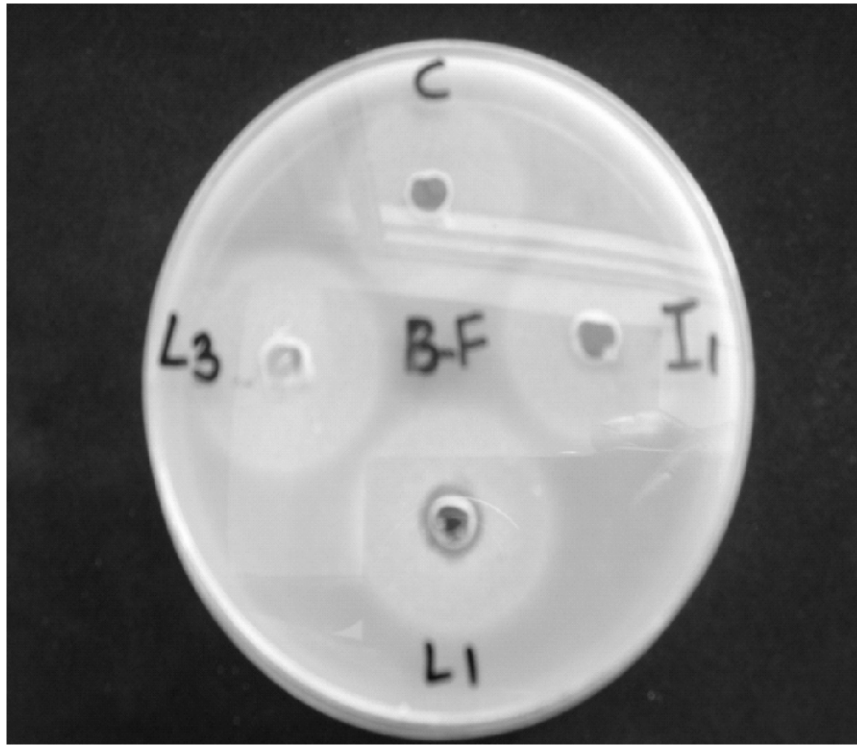


Plate1: Inhibition of *Bacillus* sp. A₁ (B.F) by *Enterococcus faecalis* (L₁) Cell-free Supernatant by Agar Well Diffusion Assay.

B.F, *Bacillus* sp. (A₁); L₁, *Enterococcus faecalis*; I₁, (sterile MRS broth). Wells contain 50 µl aliquot of the *Lactobacillus casei*; L₃, *Lactobacillus cellobiosus*, C, Control respective unadjusted pH cell-free supernatant

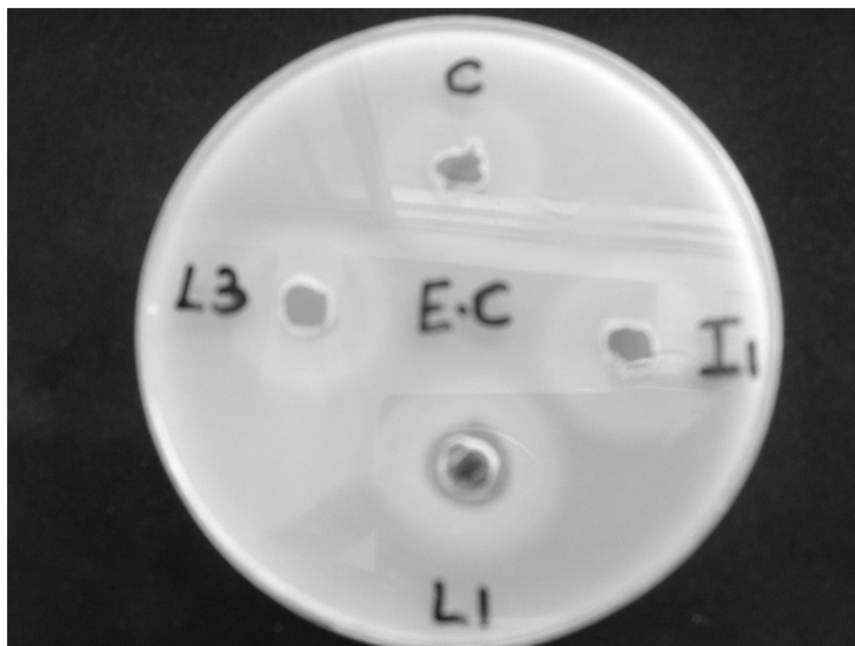


Plate2: Inhibition of *Erwinia chrysanthemi* by *Enterococcus faecalis* Cell-free Supernatant by Agar Well Diffusion Assay.

E.C, *Erwinia chrysanthemi* ; L₁, *Enterococcus faecalis*; I₁, (sterile MRS broth). Wells contain 50 µl aliquot of the *Lactobacillus casei*; L₃, *Lactobacillus cellobiosus*, C, Control respective unadjusted pH cell-free supernatant

The results obtained in this study revealed that the spoiled Irish potato samples contained five bacteria strains belonging to three genera within the limit of the traditional phenotypic method of identification used. Further characterization of the isolated strains using molecular methods would be necessary as further studies. In addition, the isolated bacteria showed multiple antibiotics resistance pattern which is a threat to public health. The inhibitory effects of the cell free supernatant of *Enterococcus faecalis* against *Erwinia* sp, the causal agent of bacterial soft rot is suggestive that lactic acid bacteria can be screened and exploited as biopreservative during storage, therefore preventing potato spoilage and huge economic losses.

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