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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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African Journal of Microbiology Research

Table of Contents: Volume 7 Number 42, October 18, 2013

ARTICLES

Research Articles

African Journal of Microbiology Research

Table of Contents: Volume 7 Number 42, October 18, 2013

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Full Length Research Paper

Variations in structural and plasmid profiles of starved *Shigella* in seawater

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In this study, we incubated three strains of *Shigella* (*Shigella sonnei* (S1), *Shigella boydii* (S2) and *Shigella flexneri* (S3)) in seawater microcosms (at room temperature and at 4°C) for eight months and we studied the modification of the proteins secreted, cytosolic and inner membrane profiles and their composition in fatty acids. Plasmid profiles were also investigated. Plasmids profiles analysis showed that *S. boydii* (S2) and *S. flexneri* (S3) did not lose any of their plasmids. In addition, fatty acids composition of Shigella under stress conditions was modified. Proteins secreted, cytosolic and inner membrane of stressed bacteria was changed and these modifications were manifested by the appearance and/or disappearance of bands.

Key words: *Shigella*, seawater, starvation, plasmids, proteins, fatty acids.

INTRODUCTION

In their natural environment, bacteria are constantly subjected to alternating between good growing conditions and conditions of stress or nutritional deficiencies which they must quickly adapt to survive. Indeed, enteric bacteria such as *Shigella*, disseminated in marine environment, are submitted to multiple physicochemical stresses: high osmolarity, low temperature, nutrient starvation (Moriarty and Bell 1993) and solar light irradiation (Arana et al. 1992). The ability of enteric bacteria to adapt to fluctuations in the ambient osmolarity is of fundamental importance for their survival (Nakamura et al. 1964; Bakhrouf et al. 1994; Ellafi et al. 2012).

In general, microorganisms do not respond to starvation by simply arresting all metabolic activities and stopping growth. Instead, they carry out starvation-induced activities that may include production of degradative enzymes, such as proteases and lipases, and substrate-capturing enzymes, such as glutamine synthetase and alkaline phosphatase. In addition, nutrient-deprived bacteria may try to differentiate into a more resistant state to maintain viability for starvation (Siegele and Kolter, 1992). During nutrient deficiency, bacteria can survive for a long time by sequential changes in cell physiology and gradual changes in morphology (Morita, 1993).

The mechanisms involved in the bacterial tolerance are not fully understood and the majority of studies are focused on the role of stress proteins and the regulation of gene expression in response to environmental changes (Foster and Spector, 1995; Foster, 2000; Dodd and Aldsworth, 2002). However, a link between the membrane fatty acid composition and the bacterial resistance has also been found (Annous et al., 1999; Sampathkumar et al., 2004; Alvarez-Ordonez et al.,

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2008). In general, these authors have shown that cells with a decreased concentration of unsaturated fatty acids or with an increased content of saturated fatty acids have a decreased membrane fluidity, which is linked to a higher heat resistance. Furthermore, Alvarez Ordonez et al. (2008) found that the formation of cyclic fatty acids plays an important role in protecting acid-adapted *Salmonella typhimurium* cells from heat inactivation.

The aim of this work was to study the responses elicited in *Shigella* spp. following their incubation in seawater for eight months, by analyzing their: (i) secreted proteins, (ii) cytosolic and inner membrane profiles, (iii) membrane fatty acid profiles, and (iv) plasmid profiles.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Three *Shigella* strains were used in this study: two *Shigella sonnei* (S1), *Shigella boydii* (S2) and *Shigella flexneri* (S3). All strains were provided from the Monastir hospital in Tunisia and maintained at -80°C in Luria-Bertani broth (LB) supplemented with glycerol (15%, vol/vol). For the experiments, the cells were grown at 37°C in tryptic soy broth (TSB, (Difco)) for 24 h. Natural seawater (100 ml) from the Tunisian coast of Monastir (salinity 4%, pH 8) was filtered through membranes (pore size, 0.22 µm; Millipore Corp., Bedford, Mass.) and autoclaved (115°C for 15 min) in 100 ml Erlenmeyer flasks. *Shigella* cells were washed three times by centrifugation (13000 rpm for 10 min at 20°C) with autoclaved seawater and then suspended in 10 ml of autoclaved seawater (Ellafi et al., 2009). The microcosms (100 ml) were inoculated with these suspensions (approximately 10^9 CFU/ml) and then incubated in a static state at room temperature (22 to 25°C) and at 4°C.

Plasmid extraction and analysis

Isolation of plasmid DNA was done using plasmid mini preparation kit obtained from Biobassic, according to the manufacturer's instruction. Plasmids were detected by electrophoresis in 0.7% agarose gel containing 0.5 µg of ethidium bromide per milliliter and photographed with ultraviolet light illumination.

Analysis of fatty acids

To analyze the total cellular fatty acids, cells recovered from 10 ml of each cell suspension were pretreated following the MIDI protocols (Sasser, 1990). All reagents for saponification, methylation, extraction, and washing were dispensed with autopipets into this same tube, making the hands-on time minimal. Next, the final extracts were analyzed by gas chromatography (column: 30 m × 0.25 mm HP-Innowax; flame ionization detect temperature at 280°C; carrier gas N_2 at 1 ml/min; injector temperature 270°C; oven temperature programmed from 130 to 230°C) using a Hewlett-Packard HP 5890 capillary gas chromatograph linked to an HP Chemstation integrator. The identification of fatty acid methyl esters was performed by external standards (all purchased from Sigma Chemical Co.) submitted to the same processes of manipulation as the biological samples analyzed. The values of fatty acids are presented as area percentage of total fatty acids. Total saturated fatty acids (SFA), total unsaturated fatty acids (UFA) were used to determine the differences among membrane fatty acids of *Shigella* cells grown under the different conditions. The UFA/SFA ratio was used as an indirect indicator of the membrane fluidity. It has been previously reported that membranes with high UFA/SFA ratio show a high fluidity (Casadei et al., 2002).

Extracellular proteins extraction

Extracellular proteins of *Shigella* before and after incubation for eight months in seawater were prepared according to the method described previously (Kaniga et al., 1995). Briefly, the cells were grown at 37°C in tryptic soy broth prepared with seawater (100%) to an optical density at 600 nm of 0.5. Bacterial cells were removed from cultures by centrifugation at 7000×*g* for 20 min and subsequent filtration through a 0.22 µm pore-size filter. Proteins from the cell-free culture supernatants were then precipitated by addition of 10% (v/v) trichloroacetic acid and recovered by centrifugation at 7000×*g* for 20 min. Pellets were resuspended in 4 ml of phosphate-buffered saline (PBS), and proteins were precipitated again by addition of 20 ml of cold acetone. After centrifugation at 7000×*g* for 20 min, the pellets were washed once with cold acetone, dried, and resuspended in 25 µl of PBS.

Proteins secreted (2 µg) were analysed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) with 15% acrylamide in the separating gel and 5% acrylamide in the stacking gel. After separation, the proteins were visualized according to standard procedures by staining with Coomassie brilliant blue G250 (Sigma, Chemical Co., St Louis, MO, USA) and molecular weights were determined by means of commercial markers (High-Range Rainbow; Amersham, Little Chalfont, Buckinghamshire, UK).

Cytosol and inner membrane fractions

The cytosol fractions were isolated as described previously (Kumar et al., 2001). Overnight cultures were centrifuged for 5 min at 7000 *g*. Pellets were washed once with 20 mmol/L Tris, 10 mmol/L EDTA, pH 8 (TE), then resuspended in the same buffer. Bacteria were disrupted by sonication for 1 min, followed by a 2 min rest, then an additional 1 min sonication. Samples were centrifuged for 5 min at 7000 *g* to remove debris, and the resulting supernatant was centrifuged for 1 h at 60 000 *g* at 4°C. The clear supernatant was retained as cytosolic fraction. The pellet was resuspended in TE, and the protein concentration was estimated. Protein concentration was adjusted to 5 mg/mL and solubilized with sodium lauryl sarcosinate 1% w/v (final concentration) at 4°C for 1 h. Samples were centrifuged again for 1 h at 60 000 *g* at 4°C, and the supernatant was taken for analysis of inner membrane fraction. Protein concentrations of cytosol and membrane fractions were estimated and analysed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) with 15% acrylamide in the separating gel and 5% acrylamide in the stacking gel.

Statistical analysis

Statistical analysis was performed using the S.P.S.S. 13.0 statistics package for Windows. The differences in UFA/SFA ratio were examined by the Friedman test, followed by the Wilcoxon signed ranks test. P-values of < 0.05 were considered as significant.

RESULTS

Plasmid analysis

Plasmid profiles of investigated *Shigella* strains analyzed

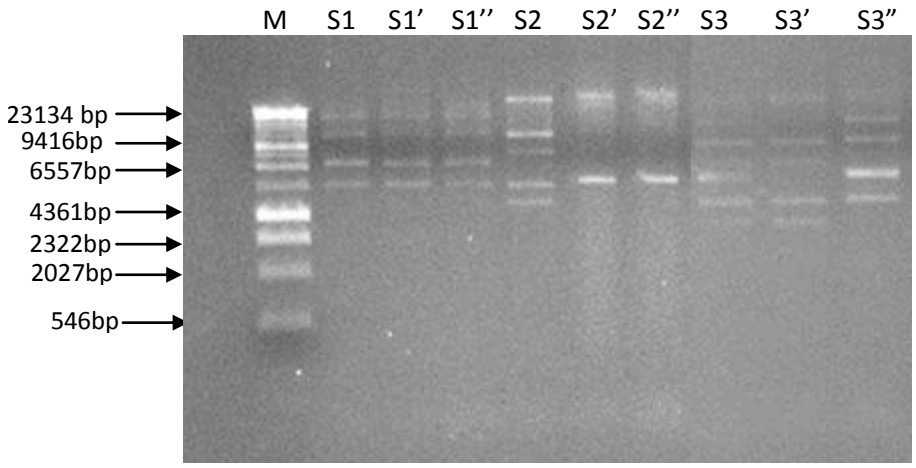

Figure 1. Plasmid DNA profiles of *Shigella* cells exposed to starvation for eight months in seawater. M, λ DNA-*Hin*dIII molecular size marker (Promega); Sn, strain before incubation in seawater; Sn', strain incubated during eight months in seawater microcosms at room temperature. Sn'', strain incubated during eight months in seawater at 4°C.

Table 1. Fatty acids composition of *Shigella* exposed to starvation for eight months in seawater.

Strain	Fatty acid								
	C10 :0	C12 :0	C14 :0	C14 :1	C15 :0 iso	C15:0 antieso	C16:1n7	C16:1n5	C16 :0
S1	1.43	0.26	0.46	0.21	0.44	0.55	4.03	6.52	33.08
S1'	0.34	0.2	0.08	-	0.31	0.42	4.35	5.62	31.87
S1''	0.6	0.53	0.2	0.16	0.08	0.61	4.43	1.06	31.35
S2	1.86	0.43	0.14	0.15	0.06	0.62	4.44	5.62	34.8
S2'	1.03	0.49	0.29	0.04	0.06	0.91	4.79	5.82	23.07
S2''	1.53	0.4	0.12	0.12	0.44	0.45	4.82	6.23	34.82
S3	0.95	0.42	0.12	-	0.32	0.2	1.33	0.71	43.63
S3'	1.09	0.33	0.12	0.12	0.04	0.53	4.59	5.61	35.52
S3''	-	-	-	-	-	0.56	3.57	5.93	36.96

on 0.7% agarose gel showed that all Shigella harbored four to six plasmids, ranging in size from 3 to 23 kb before incubation. After eight months of incubation in seawater, we also observed that S1 conserved its their plasmids. Furthermore, we noted that S2 and S3 lost its many original plasmids, which corresponds to 9, 7.5 and 4 kb sizes for S2 and 3kb for S3 (at 4°C), respectively, after this period of starvation (Figure 1).

Membrane fatty acids composition of Shigella

The membrane fatty acid composition of *Shigella* cultured under the different growth conditions (starved and normal cells) was determined using a chromatographic method (Tables 1 and 2). Twenty (20) fatty acids were found when cells were grown both under normal and stress conditions. The five main peaks were identified as palmitoleic acid (C16:1n7), n-hexadécanoique (C16: 1n5), paltmitic acid (C16:0), vaccenic acid (C18:1n7) and linoleic acid (C18:2n6). Their relative percentages were between 1.33 and 39. 88%. fifteen fatty acids were also detected at lower relative concentrations: C10:0, C12:0, C14:0, C14:1, C15:0 iso, C15:0 antieso, C17:0, C17:0 iso, C17 antieso, C17:1, C18:0, C18:1n9, C18:3n6, C20:1n9 and C20:0. As expected, incubation of Shigella in seawater resulted in differences in membrane fatty acid composition. Indeed, we also observed a significant ($P < 0.05$) decrease in SFA accompanied by a significantly ($P < 0.05$) increase in the UFA. The UFA/SFA ratio observed for control cells (S1, S2 and S3) was higher (1.05; 1.51;1.43) than cells grown under stress conditions (Table 2).

Extracellular proteins analysis

After eight months of incubation in seawater, the

Table 1. Contd.

Strain	Fatty acid										
	C17:0	C17:0 iso	C17:0 antieso	C17:1	C18:0	C18:1n9	C18:1n7	C18:2n6	C18:3n6	C20:0	C20:1n9
S1	0.31	1.16	0.43	0.91	1.14	1.11	24.44	20.34	2.31	0.52	0.23
S1’	0.15	0.77	0.19	0.6	1.19	0.27	30.05	20.95	2 .24	0.2	0.07
S1’’	0.86	0.93	0.5	0.93	1.27	1.88	31.43	20.01	2.71	0.16	0.19
S2	0.53	0.88	0.46	0.66	0.71	3.24	19.06	24.23	1.47	0.53	-
S2’	0.45	1.33	0.94	1.97	3.33	2.35	31.34	14.92	5.72	0.55	0.5
S2’’	0.57	0.9	0.41	0.84	0.67	3.53	20.97	21.06	1.51	0.37	0.15
S3	0.65	0.88	0.13	0.24	0.86	0.71	2.47	39.88	1.26	0.43	4.7
S3’	0.45	0.63	0.32	0.59	0.56	1.88	24.64	20.04	1.43	0.17	1.25
S3’’	0.2	0.59	0.31	0.3	1.03	0.27	20.62	26.3	1.19	0.01	0.05

Sn, Strain before incubation in seawater; Sn’, strain incubated during eight months in seawater microcosms at room temperature. Sn’’, strain incubated during eight months in seawater at 4°C. C10:0, Capric acid; C12:0, lauric acid; C14:0, myristic acid; C14:1, myristoleic acid; C15:0 iso, isopentadecylic acid; C15:0 antieso, antiesopentacyclic acid; C16:1n7, palmitoleic acid; C16:1n5, n-hexadécanoique acid; C16:0, palmitic acid; C17:0, margaric acid; C17:0 iso, isomargaric acid; C17:0 antieso, antiesomargaric acid; C17:1, heptadecenoic acid; C18:0, stearic acid; C18:1n9, oleic acid; C18:1n7, vaccenic acid; C18:2n6, linoleic acid; C18:3n6, gamma-linolenic acid; C20:0, arachidic acid; C20:1n9, eicosenoic acid.

Table 2. Effect of starvation on the fatty acids compostion *Shigella*.

Strain	Fatty acid		
	SFA	USF	USF/SFA
S1	39.78	60.1	1.51
S1’	35.72*	64.28*	1.79*
S1’’	37.09*	62.8*	1.69*
S2	41.02	58.87	1.43
S2’	32.45*	67.45*	2.07*
S2’’	40.68*	59.23*	1.45*
S3	48.59	51.3	1.05
S3’	39.76*	60.15*	1.51*
S3’’	39.66*	58.23*	1.46*

Sn: strain before incubation in seawater; Sn’ :strain incubated during eight months in seawater microcosms at room temperature. Sn’’:strain incubated during eight months in seawater at 4°C. SFA: Total saturated fatty acids; UFA: Total unsaturated fatty acids. *: $P<0.05$.

extracellular proteins of Shigella strains were examined by SDS-PAGE (Figure 2). Before their incubation in seawater, we found that all strains of Shigella had almost the same profile. Shigella secretes a large number of proteins in the extracellular medium. After eight months of incubation in seawater, we noticed a remarkable difference between the profiles of non-incubated and incubated strains. Indeed, we observed a decreased expression for almost all proteins compared to the normal state. At the profile *S. sonnei* (S1), we observed the appearance of four bands corresponding to molecular weights: 80, 47, 30 and 25 kDa and about the disappearance of two bands of size 22 and 57 kDa after stress. We also noted the appearance of new bands after incubation in seawater for S2: 80, 40 and 37kDa and S3: 65kDa. We also noted the disappearance of other bands of sizes 40 and 37 kDa for S2 and 71 and 16kDa for S3.

Cytosol and inner membrane analysis

The proteins were fractionated and subjected to SDS-PAGE. SDS-PAGE of the cytosolic and inner membrane is shown in Figures 3 and 4, respectively. Cytosolic fraction of Shigella grown in the starvation conditions showed significant difference. At the profile S1, we observed the appearance of two bands corresponding to molecular weights: 200 and 60kDa and about the disappearance of two bands of size 22 and 16 kDa after stress. We also noted the appearance of new bands after incubation in seawater for S2: 15 and 5kDa and S3: 64, 22 and 4kDa. We also noted the disappearance of other bands of sizes, 50 and 17 kDa for S2 and for S3: 29kDa. The inner membrane fractions of normal and starved cells of Shigella did not show a significant difference (Figure 4).

DISCUSSION

The results in the present work show that Gram-negative bacteria like *Shigella* are able to adapt and survive under starvation conditions. Many marine bacteria, especially *Shigella* spp., can survive for a long time for starvation by sequential changes in cell physiology and gradual changes in morphology (Jiang and Chai, 1996). Our results show the starved cells of *Shigella* lose many their plasmids, only for S1. These results are in agreement with the report of Gauthier et al. (1988), who demonstrated

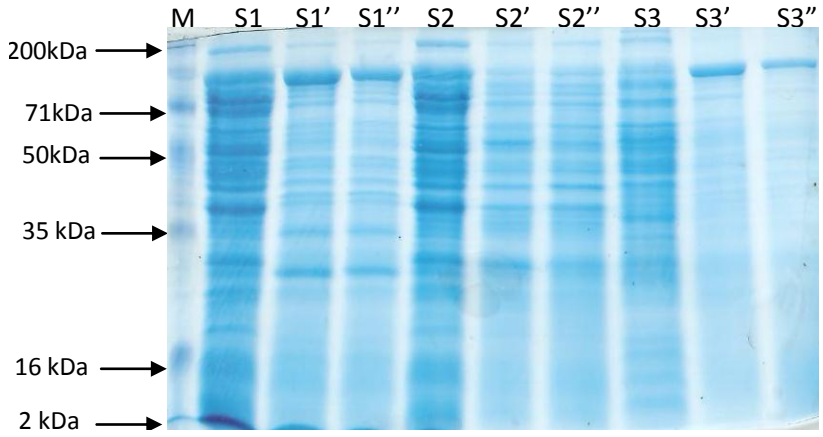

Figure 2. Extracellular proteins of *Shigella* cells exposed to starvation for eight months in seawater. M, molecular weight marker; Sn, strain before incubation in seawater; Sn', strain incubated during eight months in seawater microcosms at room temperature. Sn'', strain incubated during eight months in seawater at 4°C.

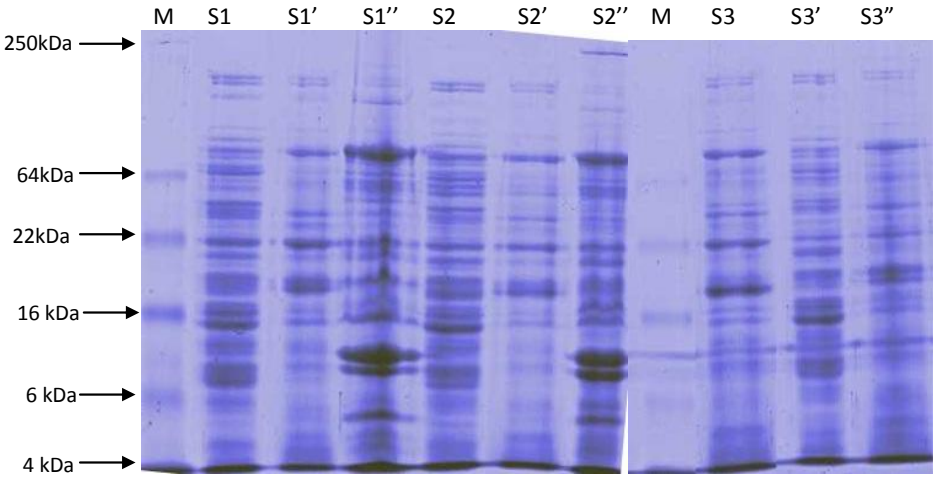

Figure 3. Cytosolic proteins of Shigella cells exposed to starvation for eight months in seawater. M, Molecular weight marker; Sn, strain before incubation in seawater; Sn', strain incubated during eight months in seawater microcosms at room temperature. Sn'', strain incubated during eight months in seawater at 4°C.

that *Escherichia coli* lost plasmid pCS1, encoding synthesis CFAI, after nine days of incubation in seawater. Our results testify to the genetic instability of stressed bacteria. Indeed, some marine bacteria can lose plasmid DNA in seawater while gaining others. This can reflect on the stability of virulence factors of bacteria in marine environment, as well as the transformation of non-pathogenic strain to pathogenic strain and vice versa.

Total saturated fatty acids (SFA) and total unsaturated fatty acids (UFA) were used to determine the differences among membrane fatty acids of Shigella cells grown under the different conditions. The UFA/SFA ratio was used as an indirect indicator of the membrane fluidity. A significant ($P<0.05$) decrease in the UFA/SFA ratio was observed with all the serogroups. These changes in membrane fatty acid composition result in *Shigella* cells with decreased membrane fluidity. These results confirm those found by Ellafi et al. (2009) and Ali et al. (2011) on antibiotic resistance and morphological change after starvation in seawater. It has been previously reported that membranes with high UFA/SFA ratio show a high fluidity (Casadei et al., 2002). Various studies have indicated that growth conditions, such as the composition of the growth medium (Annous et al., 1999), the growth phase of the cells (Russell et al., 1995; Kadner, 1996; Casadei et al., 2002), the incubation temperature (Annous et al., 1999; Casadei et al., 2002; Kadner, 1996; Wang et al., 2005), and the pH value (Russell et al.,

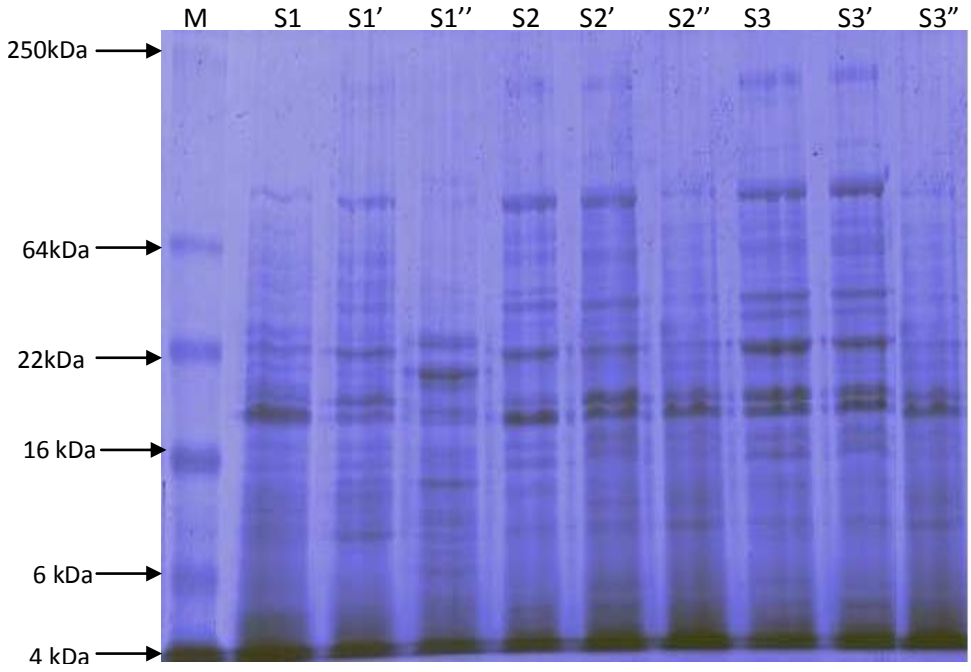

Figure 4. Inner membrane proteins of *Shigella* cells exposed to starvation for eight months in seawater. M, Molecular weight marker; Sn, strain before incubation in seawater; Sn', strain incubated during eight months in seawater microcosms at room temperature. Sn'', strain incubated during eight months in seawater at 4°C.

1995; Brown et al., 1997; Sampathkumar et al., 2004; Wang et al., 2005) markedly affect the bacterial membrane fatty acid composition. However, little is known on the influence of these modifications in membrane composition on the bacterial resistance to subsequent stresses. One of the most important consequences of membrane fatty acid changes in microorganisms is to modulate the activity of intrinsic proteins that perform functions such as ion pumping and nutrient uptake (Russell and Fukanaga, 1990).

Our study shows alterations in total proteins secreted and cytosolic patterns of starved *Shigella* cells after eight months of incubation in seawater microcosm. These alterations were manifested by the appearance and/or disappearance of bands as well as in the level expression of certain proteins. These modifications are probably due to nutrient deficiency in seawater. Indeed, it is clear now that changes in the environment induce several alterations in bacterial function and protein expression. After the beginning of an adverse effect, such as starvation, the synthetic functions of cells became inhibited and cells division is interrupted. In parallel, the production of several proteins increases (Kustos et al., 2007). These proteins include cytolysins, lipases, siderophores, exopolysaccharides, and proteases such as caseinase and gelatinase. These proteases are mainly involved in providing peptide nutrients for the micro-organism. However, the production of bacterial proteases could contribute to the pathogenesis of infections, and therefore they could be considered virulence factors (Secades and Guijarro, 1999). The paucity of food in seawater can also lead to the loss of some features either by repression of the specific enzymes or following modifications at the level of the bacterial wall. The effect of environmental conditions on the production of extracellular proteolytic enzymes could play an important role in the induction or repression of the enzyme by specific compounds (Secades and Guijarro, 1999). Production of extracellular proteases has been shown to be sensitive to repression by different carbohydrate and nitrogen sources (Haulon et al., 1982). Catabolic enzymes responded to both carbon control and nitrogen control in enteric bacteria (Goldberg et al., 1976). In the bacteria *A. hydrophila* (O'Reilly and Day, 1983), *A. salmonicida* (Dalhe, 1971), and *P. aeruginosa* (Jensen et al., 1980) protease production is influenced by carbon and nitrogen sources. Additionally, the temperature can influence the protease production, as occurs in *A. hydrophila* (O'Reilly and Day, 1983).

In the present study, we observe that the starvation of *Shigella* could significantly influence the structural and plasmid profiles of the bacterium. This, in turn, may influence the pathogenesis of *Shigella* infections.

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Full Length Research Paper

Effect of biological treatment and ultraviolet (UV)-C radiation disinfection process on wastewater bacterial community as assessed by denaturing gradient gel electrophoresis (DGGE) fingerprints

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The rotating biological contactor (RBC) process was frequently used for the biological wastewater treatment in order to remove pollutants and to improve the water quality before discharge to the environment. The presence of bacteria species in the secondary treated wastewater indicates the necessity of a tertiary treatment process [ultraviolet (UV)-C radiation disinfection] to reduce the number of living organisms in the water. Denaturing gradient gel electrophoresis (DGGE) method using 16S rDNA was commonly used for a direct comparison of structural diversity among different microbial communities. In the present study, community in treated and untreated wastewater from RBC treatment plant was investigated using DGGE coupled with sequence analysis of 16S rRNA gene fragments from bands of interest. The analysis of the DGGE profiles and the sequence of the dominant DGGE bands showed a variability of the bacterial community with season. DGGE patterns of samples collected in summer were more complex than those collected in winter. In addition, the investigation of the effect of increasing $UV_{253.7}$ germicidal doses on the bacterial community, in secondary treated wastewater effluent, revealed variability in bacterial tolerance to $UV_{253.7}$ radiation. This variability is inter-specific and is dependent on the UV-C dose used and the bacterial specie irradiated. Consequently, this study demonstrated that DGGE method coupled with sequencing provides precise information on RBC and UV-C wastewater treatment process.

Key words: Wastewater, biological treatment, bacterial communities, polymerase chain reaction - denaturing gradient gel electrophoresis (PCR-DGGE), 16S rDNA, ultraviolet (UV)-C radiation disinfection**.**

INTRODUCTION

Wastewater and drinking water are treated to eliminate pathogenic microorganisms and to prevent waterborne transmission. However, previous study indicated that conventional wastewater treatment does not guarantee their complete elimination (Howard et al., 2004). When discharged to environment, untreated or insufficiently treated wastewaters cause several problems, such as eutrophication, oxygen consumption and toxicity (Ding et al., 2011).

The rotating biological contactor (RBC) treatment

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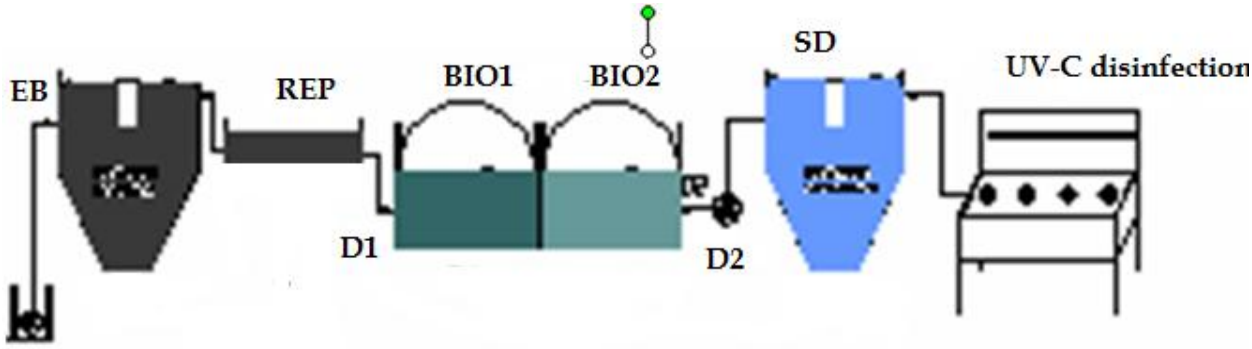

Figure 1. Experimental RBC treatment system. EB: entrance of the primary settlement tank, REP: outlet part of the primary settlement tank, D1: entrance of the first RBC tank, D2: outlet part of the second RBC tank, SD: outlet part of the secondary settling tank, BIO1 and BIO2: biofilm samples taken from the first and the second disc, respectively.

process is considered one of the most frequently used methods to treat municipal wastewater. This process involves allowing the wastewater to come in contact with a biological medium in order to remove pollutants in the wastewater before discharge of the treated wastewater to the environment. In order to improve the microbiological wastewater quality, the ultraviolet (UV)-C radiation is suggested as one of the successful disinfection practices for water treatment. UV-C disinfection of water employs low-pressure mercury lamps. The lamps generate short-wave UV radiation at 253.7 nm which is lethal to micro-organisms including bacteria, protozoa, viruses, yeasts, fungi, nematode eggs and algae. The mechanism of micro-organisms destruction is currently believed to be that in which UV causes molecular rearrangements in DNA and RNA, which is turn blocks replication (Eccleston, 1998). In fact, biological inactivation by UVC light arises from the fact that DNA molecules absorb UV photons between 200 and 300 nm, with peak absorption at almost 260 nm. This absorption creates damage in the DNA by altering nucleotide base pairing; thereby creating new linkages between adjacent nucleotides on the same DNA strand (Ben said et al., 2011).

Community-level studies are relying more and more on culture-independent methods based on the direct analysis of DNA or RNA without any culturing step (Jany and Barbier, 2008). Coupled to sequencing, these methods make it possible to investigate complex microbial communities. Denaturing gradient gel electrophoresis (DGGE) technique based on 16S rDNA gene was commonly used for a direct comparison of structural diversity among different microbial communities. Comparative analyses with nucleotide databases and phylogenetic reconstruction of the amplified 16S rRNA genes from DNA fragments excised from DGGE gels allowed the identification of organisms affected by the population changes (Ding et al., 2011).

In the present study, we aimed to assess bacterial community structure in wastewater and biofilm during the rotating biological contactor treatment process (secondary water treatment process) by using DGGE and 16S rRNA techniques; and to determine the influence of ultraviolet disinfection system (UV-C dose) as tertiary water treatment method on bacterial populations.

MATERIALS AND METHODS

Experimental rotating biological contactor (RBC) treatment system

Wastewater and biofilm samples were collected from Wastewater Treatment Plant located in El Menzah 1, Tunis, Tunisia (http://www.certe.rnrt.tn/station.htm), which receives wastewater of domestic origin. The Rotating Biological Contactor Process is a biological treatment process that involves the biological degradation of the wastewater pollutants and the organic material.

Wastewater and biofilm sampling

Wastewater and biofilm samples were collected from seven different points of the system. Wastewater samples for DNA extraction were collected in sterile bottles and frozen at -20°C for immediate processing. Five individual well-mixed wastewater samples were taken from entrance of the primary settlement tank (EB), outlet part of the primary settlement tank (REP), entrance of the first RBC tank (D1), outlet part of the second RBC tank (D2), and outlet part of the secondary settling tank (SD). Two individual biofilm samples were taken on the first disc (BIO1) and the second disc (BIO2) (Figure 1). Samples were carried out during summer season (July: water temperature was between 30 to 32°C) and winter (November: water temperature was between 20 to 23°C), in order to investigate the community structure changes.

Ultraviolet (UV)-C radiation photoreactor system

The batch laboratory UV-device was built in cooperation with Guy Daric S. A. (Aubervilliers, France). This prototype contained a sliding rack, with an irradiation board that held six Petri dishes (90 mm diameter). A germicidal low-pressure mercury vapor discharge lamp (length= 900 mm, diameter= 13 mm, power of UV emission at 253.7 nm= 55 W) with reflector could be adjusted to different heights above the irradiation board.. Incident intensity UV rays at 253.7 nm were measured using a selective detector for UV joined

to a radiometer (Vilbert-Lourmat, Norme La Vallée, France). Doses were calculated as the product of radiation intensity and time following the formula recognized by Hassen et al. (2000). In order to evaluate community resistance to UV-C light in waste water, one sample collected from secondary treatment, was irradiated (several exposure time: 30, 60, 90, 120 and 180 s) using the photoreactor described above.

Total DNA extraction

The bacterial biomass was collected by centrifugation of 30 ml well mixed wastewater samples at 14000 rpm for 15 min. Each pellet was mixed with 1 ml of lysis buffer (50 mM Tris-HCl, 50 mM EDTA, SDS 3%, pH 8.0). The genomic DNA of each sample was extracted with a modified method described previously (Guillaume et al., 2000).

Total DNA was extracted from biofilm samples using the fast DNA Spin Kit for Soil (MOBIO) according to the manufacture procedure. The quality of extracted DNA was analyzed by electrophoresis in 0.5x TBE (Tris-Borate-EDTA buffer) on 0.8% agarose gel.

PCR amplification of 16S rDNA

Polymerase chain reaction (PCR) amplification targeting bacterial 16S rDNA gene fragments was performed using the universal primers specific to the bacteria domain: 907R (5'-CCGTCAATTCCTTTGATGTTT-3') and 357F (5'-TACGGGAGGCAGCAG-3'). The amplified sequence is corresponding to the V3-V5 hypervariable region of 16S rRNA molecule of Escherichia coli. A 40-bp GC-rich sequence (5' -CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC CC- 3') attached to the 5' end of the forward primer in order to increase separation of DNA bands in DGGE analysis Muyzer and DeWall (1993). PCR experiments were performed as previously described Sass et al. (2001).

Analysis of PCR products by denaturing gradient gel electrophoresis (DGGE)

DGGE analysis was performed by using the INGENY phorU-2 system. DGGE were applied onto 7.5% (W/V) polyacrylamide gels in 1X TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, PH 7,4) with a denaturing gradient ranging from 40 to 60%. Electrophoresis was performed for 18 h at 99 V. The temperature was set at a constant of 60°C. After electrophoresis, polyacrylamide gels were stained with ethidium bromide for 20 min. Each stained gel was immediately photographed on a UV trans-illumination table with a video camera module.

Recuperation of bands from denaturing gradient gel electrophoresis (DGGE) gels and sequence analysis

Dominant DGGE bands were selected and excised from the gel for nucleotide sequence determination. DNA of ach fragment selected was eluted by incubation in 80 µl of sterilized water at 37°C for 7 h with weak shake at 450 rd min^{-1}. Fifteen µl of the supernatant solution was used as a template to reamplify the band of interest using the forward primer 357F without the GC clamp and the reverse primer 907R. A slightly different PCR protocol was used (Marzorati et al., 2006). Thereafter, the amplicons were visualized on ethidium bromide stained agarose gel, purified using the Wizard® SV Gel and PCR Clean-UP System (Promega, Madison, USA) and then sequenced by Center of BioTechnologies Sfax (CBS), Tunisia. The sequences obtained from exciced bands in the DGGE gel were compared with the 16S rDNA sequences in the GenBank database by using the basic local alignment search tool (BLAST, National Centre for Biotechnology Information, US National Library of Medicine) to recover similar sequences and phylogenetically related species. Water bacterial diversity was expressed by the Shannon diversity index (*H*), wich was calculated as described by Steele et al. (2005).

RESULTS AND DISCUSSION

Denaturing gradient gel electrophoresis (DGGE) patterns analysis of wastewater and biofilm in summer and winter

Analysis of wastewater and biofilm DGGE patterns was based on the variation in bands number and intensity. Visual comparison of the DGGE profiles of wastewater samples and biofilm collected from different points of the RBC revealed important variations in microbial community structure between summer and winter. These changes were noted in band position, intensity, and number of bands present in bacterial DGGE patterns. In addition, analysis of DGGE patterns showed that profiles of samples collected in summer (Figure 2A) were more complex than those collected in winter (Figure 2B). As shown in Table 1, the mean value of DGGE band numbers and the Shannon diversity index decreased from ~7 to ~3 and from 1.86 to 1.09, respectively suggesting that bacterial community diversity was affected. The probable reason of community changes may be the perturbation caused by the climatic changes, particularly the temperature (Gilbride et al., 2006; Moura et al., 2009; Ding et al., 2011).

In summer, the results showed that considerable differences in bacterial DGGE data were observed between different points of the process. More bands were detected in samples from the out part of the bioreactor (SD) than those from the entrance of the bioreactor (EB, REP, and D1) and from biofilms (BIO1, BIO2). These results suggested that bacterial populations were accumulated in the secondary settling tank.

In winter, a decrease in bands number and intensity were observed in DGGE community patterns. Summer bacterial community was disappeared and yielded places to novel bacterial populations (B16 and B18). This result suggested that the bacterial community diversity were affected by the period of the year.

Sequence analysis of the dominant bacteria in the secondary treated wastewater

In order to determine dominant bacterial species in the secondary treated wastewater (the outlet part of the secondary settling tank), a number of 8 bands showing the highest intensity from gels were excised, re-amplified, purified, sequenced and subjected to BLAST GeneBank

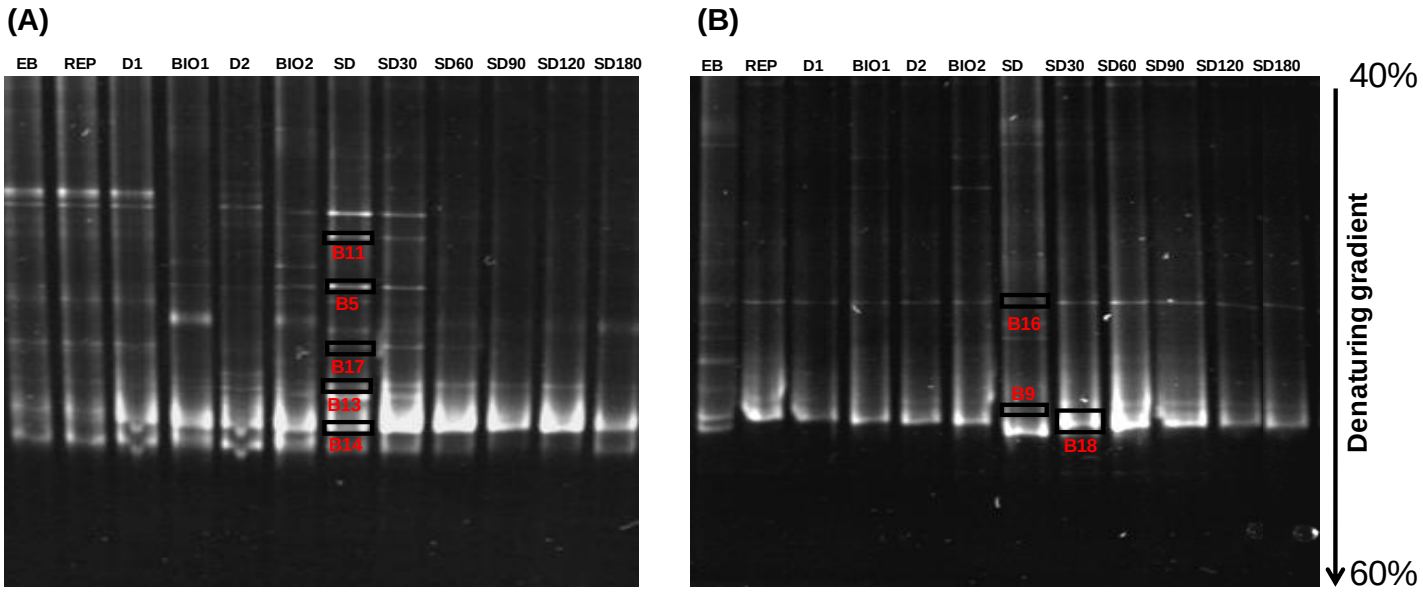

Figure 2. DGGE patterns of V3-V5 region of the 16S rDNA generated from the wastewater and biofilm samples collected in summer (A) and winter (B), from different sites in the RBC system. EB: entrance of the primary settlement tank, REP: outlet part of the primary settlement tank, D1: entrance of the first RBC tank, D2: outlet part of the second RBC tank, SD: outlet part of the secondary settling tank, BIO1 and BIO2: biofilm samples taken from the first and the second disc, respectively. SD30, SD60, SD90, SD120 and SD180: DGGE patterns of 16S rDNA fragments amplified from secondary treated wastewater exposed to increasing UV253.7 Germicidal doses. (SD: output secondary clarifier (T_0), s: seconde) Bands marked were excised and sequenced. The gradient of the urea and formamide ranged from 40 to 60%.

analysis. The results of homology search and the origin of the closet relative for the sequences obtained were showed in Table 2. The recovered fragment sequence of band B5 has a high similarity (99%) to an uncultured *Cyanobacterium* isolated from cultivated soil (Jangid et al., 2011).

Band B9 have 99% similarity on the 16S rRNA gene level with *Salmonella enterica* (Hurrell et al., 2009). Band B11 is 100% similar with *Fluviicola taffensis,* a novel freshwater bacterium of the family *Cryomorphaceae* in the phylum *Bacteroidetes*, isolated from water of the River Taff, Cardiff, UK (O'Sulivan et al., 2005). Band B13 and B17 showed 99% of homology with *Chromobacterium* sp. Strain DS1, a novel cholesterol oxidase producer (Doukyu et al., 2008). In addition these sequences have a high similarity (99%) to *Chromobacterium violaceum* strain 968 (Wesener et al., 2011). The 16S rDNA sequence of band B14 has a high similarity (99%) to *Aquitalea* sp. strain AOLR28 isolated from aerobic sludge granules (Aday et al., 2010). Finalely, two bands B16 and B18 were exclusively obtained from samples collected in winter.

Band B16 is 99% similar to *Burkholderia* sp. strain M27-VN8-1W, which was isolated from Vietnamese soils contaminated with 2,4-Dichlorophenoxyacetic acid (2,4-D) and 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T), (Huong et al., 2007). It is probably that the corresponding organism of band B16 is *Burkholderia xenovorans* which can grow at low temperature, near to 25°C. (Rehmann and Dauguli, 2008; Hughes et al., 2011). The 16S rDNA sequence of band B18 has 100% of homology with *Pantoea agglomerans* which was isolated from activated sludge (Parsley et al., 2010).

Denaturing gradient gel electrophoresis (DGGE) patterns analysis of ultraviolet (UV)-C radiation wastewater treatment

The persistence of pathogens in secondary treated wastewater (the outlet part of the secondary settling tank) stresses the need for tertiary treatment as disinfection UV irradiation at 253.7 nm. For this reason, we treated wastewater samples with varying doses of $UV_{253.7}$. Comparison of DGGE patterns obtained in summer and winter seasons demonstrated reduction of number and intensity of bands by increasing of exposure UV-C dose (Figure 2).

In summer, the secondary treated DGGE pattern enclosed 8 visible bands including 5 bands (B5, B11, B13, B14 and B17), which were sequenced and identified. From UV dose equal to 720 $mW.s.cm^{-2}$ (60s irradiation), the band B5, B11 and B17 corresponding to *Cyanobacterium*, *Fluviicola taffensis* and *Chromobacterium* sp., respectively disappeared completely. For the remaining band B13, high UV-C dose (≥ 2160 $mW.s.cm^{-2}$) reduced considerably bands intensity, suggesting the possible diminution and/or disap-

Table 1. Shannon diversity index calculated from DGGE data. S: samples collected in summer, W: samples collected in winter.

Sample	Band number	Shannon diversity index (*H*)
EBs	10	2.303
REPs	10	2.079
D1s	10	2.079
BIO1s	6	1.792
D2s	8	2.079
BIO2s	9	2.197
SDs	9	2.197
SD30s	9	2.197
SD60s	5	1.609
SD90s	4	1.386
SD120s	4	1.386
SD180s	3	1.099
Average	~7	1.86
EBw	9	2.197
REPw	2	0.693
D1w	2	0.693
BIO1w	4	1.386
D2w	3	1.099
BIO2w	4	1.386
SDw	6	1.792
SD30w	3	1.099
SD60w	2	0.693
SD90w	2	0.693
SD120w	2	0.693
SD180w	2	0.693
Average	~3	1.09

pearance of these populations in UV-C treated wastewater. However, we have noticed the persistence of the high intensity of the band B14 even after the use of UV dose equal to1440 mW.s.cm^{-2}.

In winter, the secondary treated wastewater DGGE pattern enclosed three visible bands including bands B9, B16 and B18. Bands B9 and B18 related to *Salmonella* sp. and *P. agglomerans*, respectively were very weak. However, the use of UV dose equal to 360 mW.s.cm^{-2} (≥ 30 s irradiation) increased the intensity of these two bands, and their diminution was from 1440 and 2160 mW.s.cm^{-2}, respectively (Table 2). The band B16 persisted even after the use of UV dose equal to 2160 mW.s.cm^{-2}.

At wavelength 253.7, UV irradiation can denature DNA (Zimmer and Slawson, 2002), or even cause structural changes inducing loss of vital cellular compounds, which leads to inhibition of replication, loss of reproducibility, and cell death (Liu et al., 1993; Nigro et al., 1998; Ben said et al., 2012). Indeed, obtained results showed variability in bacterial tolerance to $UV_{253.7}$ radiation. This variability is inter-specific and is dependent on the UV dose used. Accordingly, the effectiveness of UV disinfection depends on two factors: (i) the $UV_{253.7}$ doses used and (ii) the irradiated bacterial species. Similar results reported by Hassen et al. (2000) showed that the susceptibility of bacteria to $UV_{253.7}$ radiation was different from one specie to another and that UV is more effective on Gram-negative strains.

An increase in the intensity of bands after UV irradiation was observed for *Salmonella* sp., *Aquitalea* sp. and *P. agglomerans*. This result is probably due to the bacterial reactivation in the presence of visible light. Indeed, in response to moderate and/or non-lethal UV-C dose, bacteria react by mechanisms of DNA repair. Several studies showed that, to a certain extent, DNA damage can be tolerated by the cell until repair occurs (Lindauer and Darby, 1994; Arrieta et al., 2000; Zimmer and Slawson, 2002). The mechanism by which, micro-organism recovers replication activity is called photo-reactivation (Douki et al., 2003). Apart from photo-reactivation numerous light-independent repair mechanisms exist that are regulated by the expression of the single-strand DNA binding protein RecA (Makarova et al., 2000). This result underscored the importance of bacterial mechanisms that could be used to overcome stress conditions generated by moderate UV exposure.

Finally, this study shows that the elimination of the majority of pathogenic bacteria in wastewater required an $UV_{253.7}$ dose higher than 2160 mW.s.cm^{-2} which is recommended for this type of treatment.

The culture-independent technique allows characterization of bacterial communities present in a sample without resorting to traditional microbiological methods (Jany and Barbier, 2008) and has a major advantage as evidenced by its ability to detect non-cultivable bacterial species or difficult to identify by conventional culture methods (Evans et al., 2004). Especially, when the irradiated samples included different bacterial viability form among the same irradiated bacterial specie, as viable but non cultivable (ABNC) bacteria not yet reactivated, active but non cultivable (ABNC) bacteria and VBNC-UV inactivated bacteria (Ben Said et al., 2012).

Consequently, denaturing gradient gel electrophoresis method (DGGE) provided precise information for the effect of increasing $UV_{253.7}$ germicidal dose on the bacterial community in secondary treated wastewater effluent, and could be useful for studying the effect of different wastewater treatment processes on the bacterial community.

Conclusion

Rotating biological contactor has been frequently used for the biological wastewater treatment in order to remove

Table 2. Effect of increasing UV253.7 Germicidal doses on bacterial species of secondary treated wastewater and the partial sequences analysis of (16S rDNA genes) major bands recovered from DGGE pattern.

Bande	rDNA accession number	Closed species	Similarity (%)	Exposure time (s) and UV253,7 doses (mW. S. cm^{-2})					
				0 (s)	30	60	90	120	180
				0 (mW. S. cm^{-2})	360	720	1080	1440	2160
B5	**JQ003547**	Uncultred Cyanobacterium	99	+	+	-	-	-	-
B9	**JN193512**	*Salmonella enterica*	100	+/-	+/-	+	+/-	-	-
B11	**JN193514**	*Fluviicola taffensis*	100	+	+	-	-	-	-
B13	**JN193516**	*Chromobacterium* sp.	99	+	+	+	+	+	+
B14	**JN193517**	*Aquitalea* sp.	99	+	++	++	++	++	+
B16	**JN193519**	*Burkholderia* sp.	99	+	+	+	+	+/-	+/-
B17	**JN193520**	*Chromobacterium* sp.	99	+/-	+/-	-	-	-	-
B18	**JQ003548**	*Pantoea agglomerans* sp.	100	+/-	++	++	++	+	+/-

Intensity of band are (-): absence, (+/-): low, (+): high, (++): very high.

pollutants and improve the water quality. The presence of bacteria species in the secondary treated wastewater indicate the necessity of a tertiary treatment process to reduce the number of living organisms in the water to be discharged. Obtained results show that DGGE patterns of samples collected in summer were more complex than those collected in winter. In addition, variability in bacterial tolerance to $UV_{253.7}$ radiation was revealed. This variability is inter-specific and is depen-dent on the UV-C dose used and the bacterial species irradiated. Consequently, the DGGE technique coupled with sequencing provides precise information on UV-C wastewater treatment process, and could be of great importance to wastewater treatment studies.

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Full Length Research Paper

Genetic diversity of bovine viral diarrhoea virus in Beijing region, China from 2009 to 2010

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The genetic diversity of bovine viral diarrhea viruses (BVDV) from Beijing region, China between 2009-2010, was investigated using a phylogenetic analysis of partial 5'untranslated region (5'UTR) nucleotide sequences from 61 BVDV positive samples, and for 13 selected samples, the N^{pro} region was also investigated. All the sixty-one blood samples were collected from cattle that were BVDV persistently infected (PI) within a BVD eradication program on Beijing farms during 2009 and 2010. Phylogenetic analysis based on 5'UTR sequences indicated that all BVDV positive samples were of the BVDV-1 genotype, but were clustered into four different subtypes: sub-genotype 1b (n = 31), 1m (n = 24), 1c (n = 4) and 1d (n = 2). BVDV-1b and 1 m are the most prevalent sub-genotypes in Beijing. The results also demonstrate that in most herds, certain BVDV-1 sub-genotype predominates. It is the first time that BVDV-1d is reported in China with unclear origin. The result of this study will be useful in constructing an effective vaccination plan to further control BVDV in Beijing region.

Key words: bovine viral diarrhea viruses 1 (BVDV-1), 5'untranslated region (5'UTR), N^{pro}, phylogenetic analysis.

INTRODUCTION

Bovine viral diarrhea virus (BVDV), a member of the genus Pestivirus, family Flaviviridae, causes significant losses in cattle farming worldwide (Vilcek et al., 2001). BVDV has a positive single-stranded RNA genome, approximately 12.3 kb in length, and encodes an open reading frame (ORF) that is translated into a single polyprotein of approximately 4000 amino acids. The ORF is flanked by untranslated regions (5'UTR, 3'UTR) (Meyers and Thiel, 1996). The 5'UTR highly conserved segments provide sufficient sequence data for reliable genetic classification of new BVDV isolates (Toplak et al., 2004).

Two antigenically distinct genotypes of BVDV (BVDV-1 and BVDV-2) are recognized. BVDV-1 was first described in 1954 and is presently found worldwide, whereas BVDV-2 was discovered in the USA and Canada fifteen years ago (Baker et al., 1954; Ridpath et al., 1994), and has recently been sporadically reported in other countries, such as Belgium (Letellier et al., 1999), Germany (Wolfmeyer et al., 1997), Japan (Nagai et al., 1998), Austria (Vilcek et al., 2003), Argentina (Leandro et al., 2001) and China (Zhu et al., 2009). Both genotypes can cause acute and persistent infections with similar clinical manifestations, however, some highly virulent BVDV-2

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Table 1. BVDV positive samples used in this study.

Herd orgin	Collection data	Genotype	Sample and Accession number (5'UTR)
Xijiaoyi	2009	BVDV-1b	BJ09-10 (HQ116542),BJ09-16 (HQ116545),BJ09-17 (HQ650843)
	2009	BVDV-1m	BJ09-12 (HQ650842),BJ09-13 (HQ116543),BJ09-15 (HQ116544)
Zhongyi	2009	BVDV-1m	BJ09-02 (HM769723),BJ09-08 (HQ116540),BJ09-09 (HQ116541)
Lvhe Ermu	2009	BVDV-1b	BJ09-18 (HQ650854),BJ09-19 (HQ650844),BJ09-28 (HQ879781),BJ09-35 (HQ650850),BJ09-38 (HQ650851),BJ09-40 (HQ650857),BJ09-41 (HQ650852),BJ09-43 (HQ650858),BJ09-44 (HQ650859),BJ09-45 (HQ650860),BJ09-46 (HQ650861),BJ09-47 (HQ650862),BJ09-50 (HQ650863),BJ09-52 (HQ650853),BJ09-54 (HQ650855),BJ09-56 (HQ650866), BJ09-57 (HQ650867), BJ09-58 (HQ650868)
	2009	BVDV-1m	BJ09-33 (HQ650849),BJ09-39 (HQ650856),BJ09-51 (HQ650864),BJ09-55 (HQ650865)
Jinyindao	2009	BVDV-1c	BJ09-27 (HQ116552),BJ09-31 (HQ650847),BJ09-32 (HQ650848)
Changyangsi	2009	BVDV-1b	BJ09-03 (HQ116536),BJ09-04 (HQ116537),BJ09-05 (HQ116538),BJ09-06 (HQ116539)
Jinxing	2009	BVDV-1b	BJ09-20 (HQ116546),BJ09-29 (HQ650845),BJ09-30 (HQ650846)
	2009	BVDV-1c	BJ09-21 (HQ116547)
	2009	BVDV-1d	BJ09-22 (HQ116548),BJ09-23 (HQ116549)
Beijiaosan	2010	BVDV-1b	BJ10-01 (HQ879782),BJ10-02 (HQ879783),BJ10-03 (HQ879784)
Xinpu	2010	BVDV-1m	BJ10-04 (HQ879785),BJ10-05 (HQ879786),BJ10-06 (HQ879787)
Jingyuan	2010	BVDV-1m	BJ10-07 (HQ879788),BJ10-08 (HQ879789), BJ10-09 (HQ879790)
Nankou'er	2010	BVDV-1m	BJ10-10 (HQ879791)
Nankousan	2010	BVDV-1m	BJ10-11 (HQ879792),BJ10-12 (HQ879793),BJ10-13 (HQ879794),BJ10-14 (HQ879795),BJ10-15 (HQ879796),BJ10-16 (HQ879797),BJ10-17 (HQ879798)

strains can also cause a severe haemorrhagic syndrome with high mortality rate (Ridpath et al., 1994). Within BVDV-1, BVDV-1a and BVDV-1b were initially described (Ridpath et al., 1994). Vilcek et al. (2001) collected 78BVDV isolates from Austria, France, Hungary, Italy, Slovakia, Spain and UK, and their phylogenetic analysis separated BVDV-1 into at least 11 genetic sub-groups (Vilcek et al., 2001). Subsequently, additional BVDV isolates were reported from many countries and regions, and the meaningful genomic regions of these isolates were sequenced and analyzed. Several other BVDV-1 sub-genotypes, named 1l, 1m, 1n, 1o, 1p, were introduced. In China, occurrence of BVDV infection has been observed since 1980, with most BVDV isolates being classified as BVDV-1b (Mahony et al., 2005). Several BVDV-1c were identified in Xinjiang (Huang et al., 2008), and several isolates were clustered into BVDV-1m (Xue et al., 2010). Xue et al. (2010) also reported that several isolates from China were clustered into a new BVDV sub-genotype 1p.

Pestiviruses are known to be highly variable antigenically and genetically (Paton, 1995). When designing and constructing effective vaccination strategies to control BVD, the genetic diversity of circulating BVDV needs to be considered. After screening persistently infected cows in Beijing dairy herds, we investigated the genetic diversity of BVDV obtained in positive serum samples by performing phylogenetic analysis of partial 5'UTR genome part, and for selected samples the N^{pro} region to check the results was obtained.

MATERIALS AND METHODS

Animals and samples

Animals investigated in this study were from 11 dairy farms in Beijing, which had an area of 16,808 km^2 and about 150,000 cattle. Before this study, BVDV PI cattle screening under a BVDV eradication program were enforced on 15 dairy farms in Beijing during 2009 and 2010, including about 30,000 cattle (Zhang et al., 2012). Sixty one (61) sera samples were collected from all the PI cattle which had been tested positive in an antigen capture ELISA screening test (HerdCheck BVDV Antigen Test Kit/Serum Plus, IDEXX, Sweden) (Shannon et al., 1991) within the program. These positive samples belong to 11 farms, which are located at 7 districts of Beijing, including Fangshan, Tongzhou, Daxing, Haidian, Changping, Shunyi and Miyun District, and most of Beijing's cattle are kept in these areas. The samples were stored at -70°C. All the sera samples examined in this study are listed in Table 1.

RNA isolation

Total RNA was extracted from sera samples using TIANamp Virus RNA Kit (Tiangen Biotech, China) according to the manufacturer's instructions. The extraction was accomplished with 140 µl of sera and 560 µl of TRIZOL, and the RNA was resuspended in 40 µl of DEPC-treated water. The RNA isolation was performed directly on sera, which excluded the possibility of BVDV contamination during cell culture and the authenticity of the results was upheld (Vilcek et

al., 2001; Xue et al., 2010).

cDNA synthesis and PCR

cDNA synthesis was performed using Quantscript cDNA First-strand Synthesis Kit (Tiangen Biotech, China) with random primers. Subsequently, a 288 bp DNA product was amplified from the 5'UTR using GoTaq Green Master Mix (Promega Corporation, USA) for 35 cycles. Utilized primer, 324 and 326, were widely used to amplify a cDNA fragment for genetic diversity analysis of BVDV isolates on 5'UTR (Vilcek et al., 1994, 2001, 2003).The conditions for amplification were 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C. For 13 selected samples, a 428 bp DNA product was amplified from N^{pro} region employing the same method with primers BD1 and BD3 (Vilcek et al., 2001). The conditions for amplification were 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C.

Sequencing and phylogenetic analysis

Amplified products were separated by electrophoresis in 1.5% agarose gel in Tris -Acetate EDTA buffer , and purified using a QIAquick Gel Extraction Kit (QIAGEN, Germany). The resulting DNA were then sequenced in both directions by using primers 324/326 or BD1/BD3 and an ABI 3730XL sequencing device utilizing fluorescent labeled dideoxynucleotide terminators. All the sequences were confirmed as BVDV by blasting the specified sequences against existing sequences in the NCBI Genbank. Nucleotides sequences were aligned using the Clustal W V.12.10. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0, employing the Neighbor-Joining method with 1000 Bootstrap replications (Tamura et al., 2007).

RESULTS AND DISCUSSION

The genetic diversity of BVDV field positive samples was surveyed by determining the nucleotide sequences of the 5'UTR of 61 positive samples and the N^{pro} region of 13 selected samples, obtained in Beijing between 2009 and 2010. The 5'UTR phylogenetic tree was constructed with the nucleotide sequences of the 61 positive samples, and 36 representative strains retrieved from GenBank. The N^{pro} phylogenetic tree was created using the nucleotide sequences of the 13 selected samples and 24 representative strains retrieved from GenBank. The 13 selected samples are clustered into the four sub-genotypes by the 5'UTR phylogenetic tree. We selected these samples, and constructed the N^{pro} tree to independently test the results obtained from the 5'UTR tree.

As expected from previous analyses (Vilcek, 2001; Mahony et al., 2005) both phylogenetic trees produced consistent results (Mahony et al., 2005; Vilcek et al., 2001). All the 61 BVDV field positive samples investigated in this study were of the BVDV-1 genotype (Figures 1 and 2). Of the total, 31 positive samples were of the BVDV-1b sub-genotype, 24 were of the BVDV-1m sub-genotype, 4 were of the BVDV-1c sub-genotype, and two were of the BVDV-1d sub-genotype. To the best of our knowledge, this is the first time BVDV-1d has been reported in China, and the first time BVDV-1m, BVDV-1b and BVDV-1c has been reported in Beijing. Based on these results, the BVDV-2 genotype was absent in Beijing during this period. From the distribution of 61 positive samples obtained (Table 1), we can see that in most herds (8 out of 11) there is one predominant sub-genotype. The alignment of these sequences reveals that mutations were usually located in two variable regions, 208-228 and 298-328 of the NADL strain, which is consistent with the results obtained by Vilcek et al. (1997).

Xue et al. (2010) identified BVDV-1p from cattle in Beijing, however, in the current study no samples clustered into the BVDV-1p sub-genotype (Xue et al., 2010). Two most prevalent sub-genotypes in Beijing are BVDV-1b and 1m. A Blast search of the NCBI Genbank, indicated that these BVDV-1b and 1m sequences are similar with the strains reported in China previous to this study (Vilcek et al., 1997).

Four positive samples were clustered into the BVDV-1c sub-genotype, which is the predominant sub-genotype in Australia. A Blast analysis of the BVDV-1c positive samples against NCBI Genbank non-redundant database identifies VR924 and VR1000 (Mahony et al., 2005) as the most similar sequences which were both collected from Australia. The two herds exhibiting BVDV-1c as the prevalent sub-genotype, the Jinxing and Jinyindao herds had imported cows from Australia in 2005 and 2006. However, when importing cattle, a BVDV antigen capture ELISA was conducted to confirm that no BVDV PI animal was introduced. So it is an interesting question: how was BVDV-1c introduced? Interestingly, two farms, Zhongyi and Lvhe Ermu, imported cows from Australia without developing BVDV-1c as the prevalent sub-genotype (Li et al., 1983). The self-clearance action of BVDV infection should be considered. In order for BVDV infection to persist in a herd for an extended period of time (without re-introduction), one or more sero-negative animals need to be in early pregnancy while there are PI animals in the herd. Otherwise, self-clearance will occur (Lindberg and Alenius, 1999). Consequently, we should acknowledge that PI animals may not be identified if they are aborted, stillborn, experience an early death or are traded before a testable age.

After blasting, the most similar sequence to the two BVDV-1d positive samples was isolate 2900/83 (Tajima et al., 2001), initially reported in Germany. So far as we know, frozen semens imported from Germany were used in Beijing dairy farms. However, how BVDV-1d was introduced is still unknown and worth further investigation.

BVDV PI cattle screening is a very important step in BVDV eradication programs, but if we want to keep the spread of BVDV under control, designing and constructing effective vaccination strategies is also essential. Data pertaining to BVDV genotypes circulating in Beijing

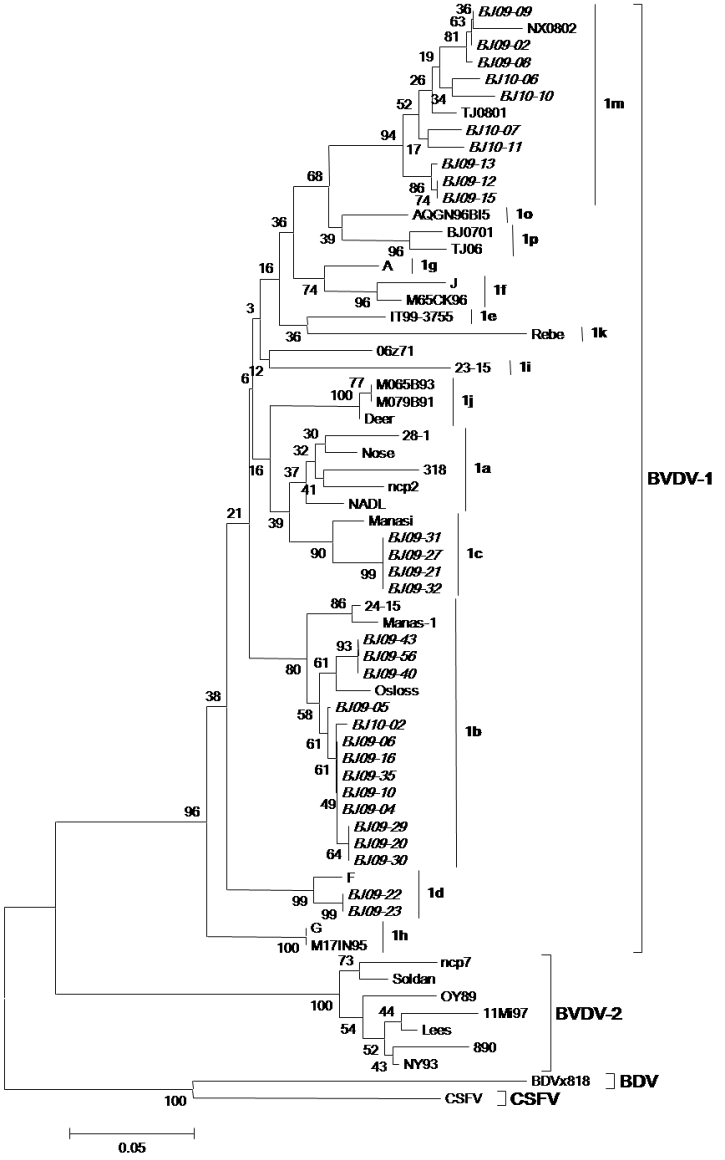

Figure 1. Phylogenetic tree based on amplification of the 5'UTR region (128-372 in NADL) of 61 BVDV positive samples (in italic) and 36 representative strains. 32 positive samples being identical with some samples shown were removed from the tree. The phylogenetic tree was created using the MEGA program by means of Neighbor-Joining. Numbers over the phylogenetic branches indicate the percentage of 1000 bootstrap replicates that support each branch. Bar indicates 0.05 nucleotide substitutions per site. The GenBank accession numbers of the representative strains are as follows: ncp7 (AY443026), Soldan (U94914), 890 (L32886), NY93 (AF039173), 11/Mi/97 (AJ293603), Lees (U65051), OY89 (AB003621), 24-15 (AF298060), 318 (AF298062), ncp2 (AY443027), Nose (AB019670), 28/1 (AF298061), M065B/93 (U97409), M079B/91 (U97410), M65CK/96 (U97456), M17IN/95 (U97431), TJ06 (GU120246), BJ0701 (GU120247), TJ0801 (GU120255), NX0802 (GU120253), Manas-1 (EU555288), Manasi (EU159702), NADL (AJ133739), Osloss (M96687), F (AF298065), IT99-3755 (AJ318616), J (AF298067), A (AF298064), G (AF298066), 23-15 (AF298059), Deer (AB040132), Rebe (AF299317), 06z71 (DQ973181) and AQGN96BI5 (AB300691). The three was rooted by two outgroup: BDVx818 (AF037405) and CSFV (m31768).

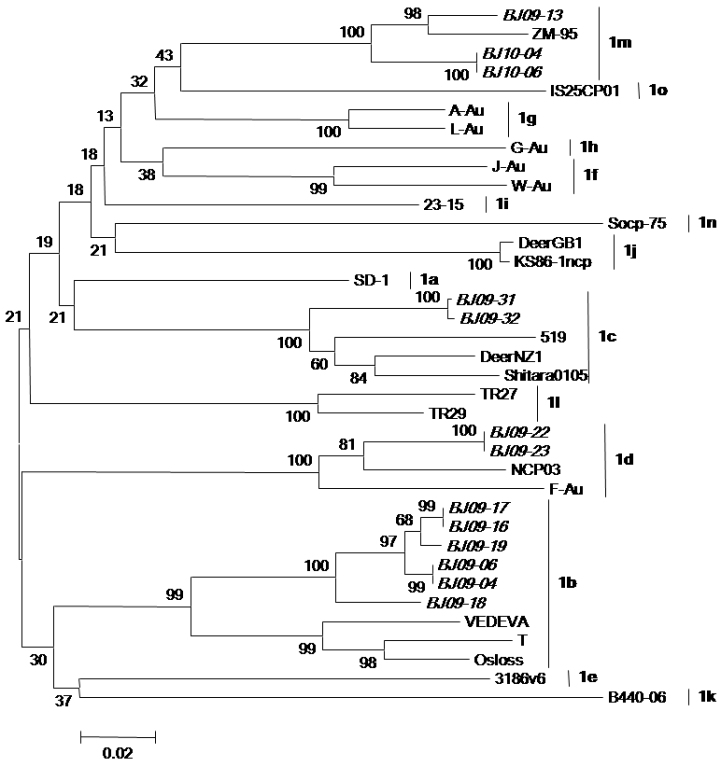

Figure 2. Phylogenetic tree created for the N^{pro} gene region (389-770 in NADL) of 13 selected BVDV positive samples (in italic) and 24 representative strains. This phylogenetic tree was constructed by the same method with the 5'UTR tree. Bar indicates 0.02 nucleotide substitutions per site. The GenBank accession numbers of the 13 selected BVDV positive samples are JN542492-JN542504. The GenBank accession numbers of the representative strains are as follows: SD-1 (M96751), 519 (AF144464), DeerNZ1 (U80903), Shitara0105 (AB359926), F-Au (AF287284), 3186v6 (AF287282), J-Au (AF287286), W-Au (AF287290), A-Au (AF287283), L-Au (AF287287), G-Au (AF287285), 23-15 (AF287279), DeerGB1 (U80902), B440-06 (EU224257), TR27 (EU163975), TR29 (EU163977), Osloss (M96687), VEDEVA (AJ585412), Ncp03 (AB359927), Socp-75 (AB359929), KS86-1ncp (AB078950), IS25CP01 (AB359931), ZM-95 (AF526381) and T (AF287289).

is indispensable for a better understanding of the pathogenesis and epidemiology of BVDV infections. On the basis of this study, the BVDV-1b and BVDV-1m sub-genotypes should be considered first when constructing a vaccine to control the BVDV infections in Beijing. Consequently, further studies needs evaluation of the antigenicity of Beijing isolates belonging to different sub-genotypes.

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Full Length Research Paper

Microbial pollution of the Mezam river system and its health impact in Bamenda (North-West Cameroon)

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A two-year study was carried out from June 2009 to May 2011 to investigate the microbial quality of the Mezam river system in Bamenda and its health impact, given the prevalence of waterborne diseases among the population who use the river water for various purposes, notably domestic and agricultural activities. River, spring and tap water samples were collected monthly and analysed quantitatively for faecal bacteria indicators of pollution and qualitatively for specific pathogens. The study shows that most of the sites were heavily polluted with faecal bacteria (12 to 2822 cfu/100 ml) that consistently exceeded the WHO recommended range for potability. These bacteria often comprised the pathogens *Salmonella* and *Shigella* which seemed to be endemic. They all tended to be highest in the dry season and at the onset of the rainy season. The incidence of waterborne diseases showed a seasonal pattern similar to the seasonality of the causative agents in water samples. The most impaired segments were the Ayaba and Mughed tributaries which receive inputs from urban and domestic activities, as well as the Nkimefeu tributary which receives direct waste discharge from the dressing of carcasses at the town slaughter house. The population which is dependent on the river water are thus exposed to health risks which could be reduced by minimizing the discharge of both liquid and solid wastes into water channels.

Key words: Faecal bacteria, market gardening, pathogens, river pollution, wastewater.

INTRODUCTION

Surface water plays an important role in the transmission of pathogenic agents discharged through human and animal faeces. These agents may find their way into the water via domestic wastewater, surface runoff from agricultural land and pastures during rainfall, or by direct deposition of faecal matter with access to stream channels (Eyles et al., 2003; Collins et al., 2005). They can transfer to humans by various routes, like recreation, irrigation of fruits and vegetables and drinking water (Davies-Colley et al., 2004). An understanding of the nature and dynamics of the microbial community in such surface waters is a necessity, since the indiscriminate increase in pollution factors has made natural purification capacities insufficient. In particular, knowledge of the levels of microorganisms in water is important for indexing the health hazard associated with its use, adopting meaningful interventions and improving bacterial water quality (Entry and Farmer, 2001).

The Mezam river sub-basin in Bamenda, West Cameroon, harbours a growing urban community that relies on the Mezam river for household chores, irrigation of market gardens and as a source for public watersupply (Tita et al., 2012). It also serves as recreational swimming pool for small children from nearby homes and

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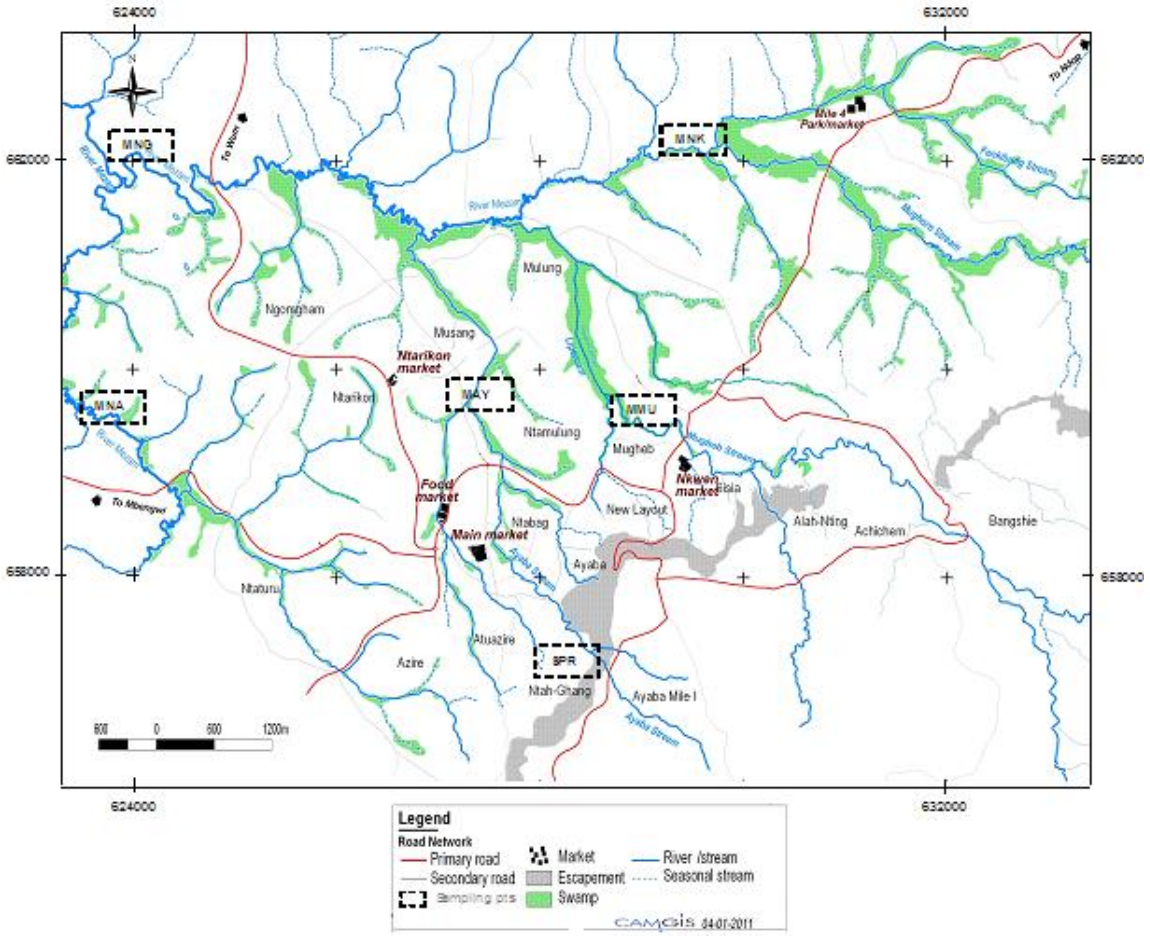

Figure 1. Map of Mezam river system in Bamenda showing sampling points.

schools. The increasing demand on this water resource and the resultant contamination from human activities generate a number of pollution problems thus increasing the risk of outbreaks from waterborne diseases. Katte et al. (2003) and Kuitcha et al. (2010) have illustrated that serious public health problems prevail in Dschang and Yaoundé (Cameroon), respectively, due to the biological pollution of wells, backwaters and rivers resulting from poor domestic and industrial waste management.

In fact, a number of studies have highlighted the great threat to public health in urban centres in many developing countries caused by microbial contamination and associated increase in waterborne enteric diseases (Ilorin, Nigeria: Kolawole et al., 2011; Abidjan, Ivory Coast: Coulibaly et al., 2004; Blantyre, Malawi: Palamuleni, 2001).

Hospital records from the Bamenda Health District show a high prevalence of waterborne diseases in Bamenda, and isolated cases of cholera occur from time to time. There is, however, a complete lack of scientific information on bacteriological water quality in this locality for appropriate intervention.

The main objective of this study was to evaluate the microbial status of the Mezam river system in Bamenda and its health impact on the population. Specifically, the study aimed to determine the types and levels of faecal bacteria and to identify the factors responsible for the degradation of the water resources. Such a study is important as it shall provide a framework for practical measures to guide water management in the sub-basin and, through this, mitigate and control the impact of pollution on the population.

MATERIALS AND METHODS

Study area

The study area is the Mezam river sub-basin in Bamenda (North West Cameroon), situated between latitudes 5°43' and 7°10' N and longitudes 9°35' and 11°12' E. The Mezam River is a second order perennial stream fed by several small streams and springs which take their rise from the Bamenda Escarpment. Water samples were collected for bacteriological analyses at six sites (SPR, MNA, MAY, MMU, MNK and MNG) in the Mezam river system (Figure 1). Details of the sites are given in Table 1.

Table 1. Location and characteristics of sampling sites in the Mezam river system in Bamenda.

Site N^{o}	Code	Location	Characteristic
1	SPR	Spring situated at the foots of the escarpment and constitutes reference point.	Presence of a few farm and grazing lands.
2	MNA	Naaka stream, western boundary of Bamenda town.	Rural tributary for the most part. Water drains in from the Mbatu plain, an intensive agricultural area in the basin.
3	MAY	Ayaba tributary	Urban tributary. Drains through thickly populated commercial centre and residential area.
4	MMU	Mugheb tributary	Urban tributary. Drains waste from the Nkwen market and its neighbourhood.
5	MNK	Nkimefeu tributary, northern boundary of Bamenda town.	Receives waste successively from fish farms, carwash, market gardens, soap factory and town slaughter house.
6	MNG	Mezam river at Ngomgham Situated at the outlet of the Mezam River from Bamenda.	Drains the Municipal open waste discharge at Mile 8.
7	MTW	Tapwater	Treated water from Mezam River distributed to population.

Water sampling

Sampling was carried out monthly from June 2009 to May 2011 for bacteriological analyses. On each sampling occasion, 1-l samples of water were collected from a well-mixed, flowing section of the river using sterile glass bottles and transported in an ice bucket to the Centre Pasteur Laboratory in Yaoundé, where they were analysed quantitatively for bacteria indicators of faecal contamination within 24 h. Every other month, qualitative analyses for specific indicators and pathogens were also carried out. During these occasions, tap water (MTW) was simultaneously sampled for both quantitative analysis of faecal bacteria and qualitative determination of pathogens.

Quantitative analysis of bacteria indicators of pollution

Heterotrophic bacteria and *Clostridium* were quantified by incurporation in plate count agar and beef liver agar, respectively, and incubated at 37°C for 24 h. Total coliforms, faecal coliforms, faecal streptococci and *Pseudomonas* were enumerated using the membrane filter technique (APHA, 1998). After filtration, the membrane was placed over lactose agar and cultured for 24 h at 37°C (total coliforms) or 44°C (faecal coliforms). To estimate faecal streptococci, the membranes were cultured over Slanetz medium for 48 h at 37°C. *Pseudomonas* was estimated on Cetrimide agar for 24 to 48 h at 37°C. The results are expressed in colony forming units (cfu) per 100 ml.

Qualitative analysis of specific indicators and pathogens

Escherichia coli were identified by inoculating characteristic total coliform colonies in MacConkey broth. Growth characteristics together with reaction to indole methyl red confirmed the presence of *E. coli* (Marchal et al., 1991). Meanwhile, Enterococci were detected by incubating faecal streptococci colonies on Enterococci specific agar and confirmed by negative catalase test. The pathogens *Salmonella*, *Shigella* and *Vibrio* were identified on Hectoen agar after enrichment on Rappaport (APHA, 1998), and presumptive colonies were confirmed biochemically (search for galactosidase, urease and indole oxydase, respectively) (Marchal et al., 1991).

Waterborne diseases

Data on waterborne diseases were obtained from the Mezam Health District Service and through structured interviews involving 500 households randomly selected from all the quarters in the city of Bamenda. Respondents were required to furnish information on their sources of water for domestic chores and the occurrence of waterborne diseases in their respective families.

Statistical analysis

The Duncan test was used to evaluate significant differences among sampling sites and the probability for significance set at $p \leq 0.01$. All calculations (mean, standard deviation and t-tests) were performed using Microsoft EXCEL statistical package for win XP-2002 and SPSS version 10.1 statistical calculation program (SPSS Inc., Chicaco IL).

RESULTS

Distribution of faecal indicator bacteria in the Mezam river system

The microbial survey carried out on water samples from the Mezam river system showed that apart from tap water, all the sites sampled contained all the indicator bacteria analysed, and each exceeded the WHO (2001) recommended limits for potable water (Table 2). They were dominated by coliforms and *Clostridium* spores. Further analysis of the coliforms revealed the presence of faecal coliforms in all the samples. Faecal streptococci were also numerous while *Pseudomonas* sp was generally the least represented.

Statistical analysis indicated that faecal indicator bacteria counts were significantly different among sites ($p \leq 0.01$). The highest mean coliform counts (>1600 cfu/100 ml) were recorded downstream at MNG and MNK, as well as MAY and MMU located on the urban tributaries (Table 2). Faecal coliforms and streptococci were also most prolific at these urban sites, whereas upstream at MNA, bacteria counts were less than 100 cfu/100 ml. *Pseudomonas* and *Clostridium* also presented a similar trend. All the indicators were much

Table 2. Geometric mean of bacteria counts in water samples from the Mezam river system in Bamenda (n = 24 except at MTW where n = 12).

Indicator bacteria	Sites in the Mezam river system							*DWQ
	SPR	MNA	MAY	MBA	MMU	MNG	MTW	
Heterotrophic bacteria (cfu/100 ml)	1485[a]	23951[b]	123721[c]	143363[c]	130054[c]	169633[c]	31	<20
Total coliforms (cfu/100 ml)	82[a]	994[b]	1750[c]	1648[c]	1904[c]	2468[c]	<1	<1
Faecal coliforms (cfu/100 ml)	21[a]	68[b]	201[c]	281[d]	175[c]	382[d]	<1	<1
Faecal streptococci (cfu/100 ml)	12[a]	122[b]	168[b]	231[c]	335[d]	311[d]	<1	<1
Pseudomonas sp (cfu/100 ml)	20[a]	25[a]	59[b]	146[c]	41[b]	165[c]	>10	<1
Clostridium spores (cfu/100 ml)	13[a]	432[b]	1753[c]	1521[c]	511[b]	2822[c]	>10	<1

Values in the same row followed by the same letter are not significantly different at $p \leq 0.01$; *Drinking water quality (WHO, 2001).

fewer but nonetheless present in spring water (SPR) at the river source. In tap water (MTW), total coliform, faecal coliform and faecal streptococcus counts were within the recommended limit for drinking water (<1 cfu/100 ml) but heterotrophic bacteria, *Pseudomonas* and *Clostridium* spores exceeded their respective limits.

Temporal variations in faecal indicator bacteria in the Mezam river system

The faecal bacteria exhibited great temporal variations at all the sites: high counts were recorded during periods of low and rising water levels (February to May), corresponding to the dry season and the onset of the rainy season, respectively (Figure 2). The counts were much lower in periods of high and falling water levels (August to November). *Clostridium* levels were particularly high and more or less constant (1000 to 1200 cfu/100 ml) but increased two to four fold between February and April. On its part, *Pseudomonas,* which was almost absent during high discharges, registered a sharp increase between January and March.

Occurrence of specific indicators and pathogens in the Mezam river system

E. coli and Enterococci were present in all the water samples analysed except tap water. The pathogens *Salmonella* and *Shigella* were detected at various sites and at different times. *Salmonella* was more frequently (63.3%) detected than *Shigella,* and *Vibrio* sp. was detected only once at MNK (7.69%) (Table 3). None of the pathogens was identified in tap water. Analysis of the isolation frequency of *Salmonella* showed that more than 65% of the *Salmonella*-positive samples were recorded between January and May which corresponds to the dry season and the onset of rains, respectively (Figure 3).

Incidence of waterborne diseases in Bamenda

Household interviews conducted in Bamenda town showed that in most households, the inhabitants repeatedly suffer from various waterborne diseases, and records from the Bamenda Health District Service indicated that between 2008 and 2010, 29695 patients consulted for diarrhoeal diseases, while 8544 were diagnosed for dysentery and 9166 for typhoid fever (Figure 4). Many more people suffered from diarrhoea between December and May while dysentery and typhoid infections were more common from March to June. Meanwhile, less than 20% of the respondents use tap water for drinking while more than 60% use either spring, well and/or river water for domestic activities that include cooking, washing, bathing and even drinking (data not shown). River water is also extensively used for the irrigation of market vegetables.

DISCUSSION

Total coliforms and heterotrophic bacteria are used to evaluate the hygienic status of water and any presence of the coliform group in water indicates contact with sewage (Markosova and Jezek, 1994). Faecal coliforms such as *E. coli* are prevalent in the digestive tracts of warm-blooded animals and it is believed that there is a correlation between their presence and pathogenic organisms since they have very similar survival characteristics to those of the well-known pathogenic members of the family, *Salmonella* and *Shigella* (Van Kessel et al., 2004). They thus serve as indicators for these pathogens and associated animal wastes that enter a water body, and their presence is definite evidence of faecal contamination and the potential risk of zoonotic pathogens (Entry and Farmer, 2001). Faecal streptococci, particularly *Enterococcus* spp, and *Clostridium* spores also show faecal contamination, the former indicating recent contamination as they do not multiply in the environment, and the latter representing more ancient contamination (Payment and Franco, 1993).

Spatial distribution of faecal indicator bacteria in the Mezam river system

The distribution of faecal bacteria in the Mezam river

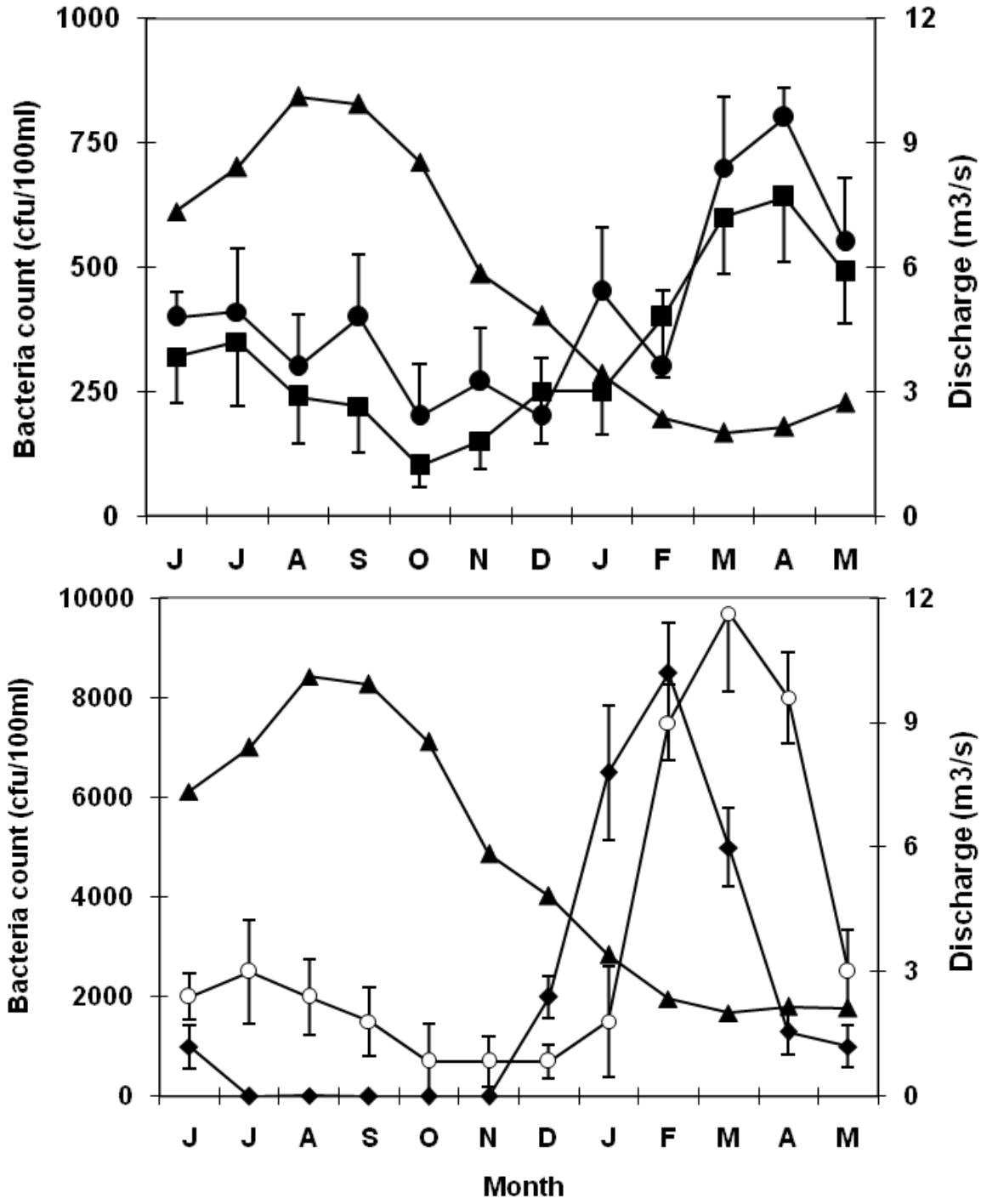

Figure 2. Temporal variations in faecal bacteria levels in water samples from the Mezam river system in Bamenda: (■) faecal coliform, (●) faecal streptococci, (◆) *Pseudomonas* spp, (o) Clostridium spores) with respect to river discharge (▲(n = 12).

system seemed to reflect input from the surrounding land. The lowest counts were measured in SPR, whereas the highest concentrations were obtained at MAY, MMU, MNK and MNG. Bacteria levels at MNA were low to moderate depending on the type of bacteria and the season. MNA is situated downstream of a flood plain where riparian vegetation provides a significant buffer zone such that inputs from human wastes and grazing stock are likely to be reduced (Eyles et al., 2003). Besides, the river gradient is low in this section and this, together with the in-stream vegetation that is almost unique to the segment, may favour the settling of faecal

Table 3. % positive samples for pathogens identified in water samples from the Mezam river system in Bamenda (n = 12).

Pathogen	Sites in the Mezam river system						
	MRS	MNA	MAY	MMU	MNK	MNG	MTW
Salmonella sp.	7.69	23.07	61.53	71.42	76.92	84.61	0
Shigella sp.	0	7.69	15.38	28.57	15.38	23.07	0
Vibrio cholerae	0	0	0	0	7.69	0	0

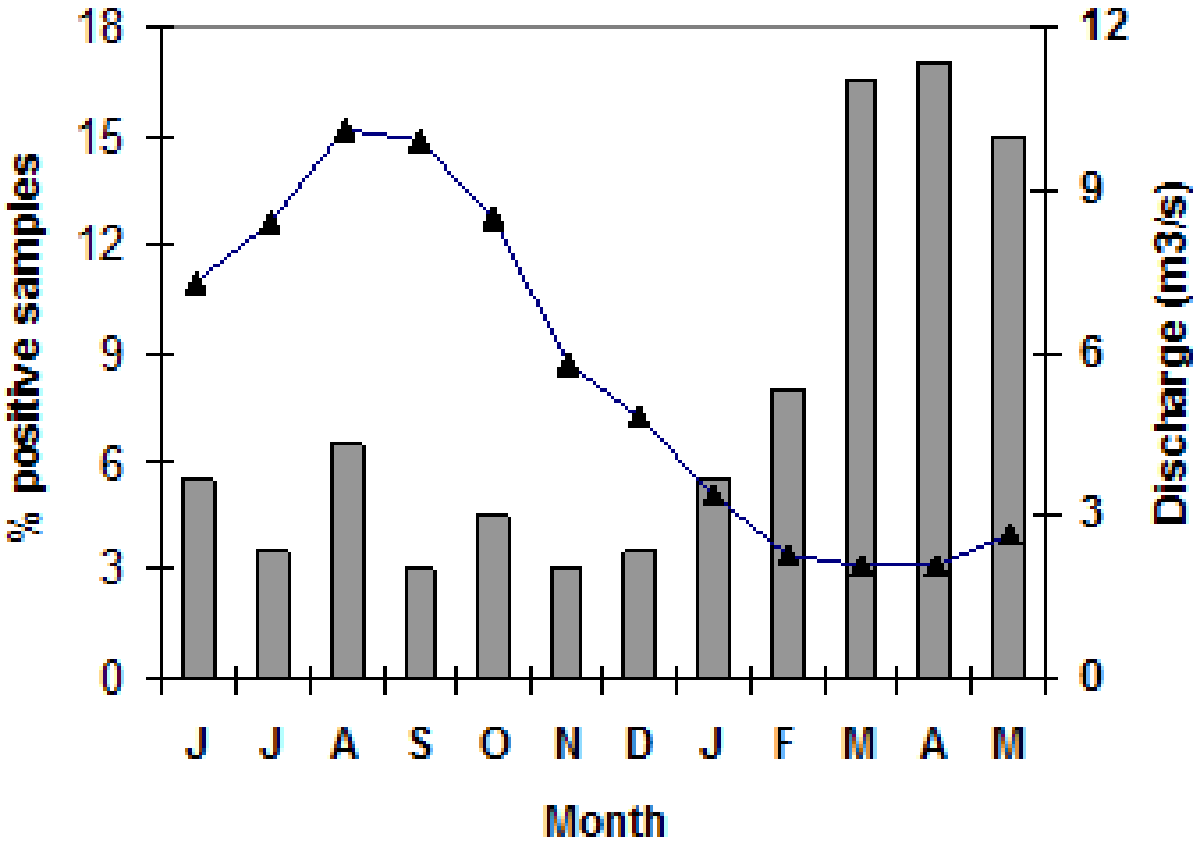

Figure 3. Temporal distribution of *Salmonella*-positive samples ( ) in the Mezam river system in Bamenda with respect to river discharge ( )(n = 12).

indicator bacteria and so reduce water column levels (Tita et al., 2012; Mitsch and Gosselink, 2000).

MAY and MMU are urban sites and receive numerous direct sewage inputs from residential areas along the watercourses as there is no central treatment system for Bamenda town (> 350 000 inhabitants). In fact, many homes do not have latrines and the stream simply serves this purpose. Just upstream from the MMU sampling point, pipes can be seen discharging from some homes, while huge quantities of garbage are deposited either near the river or directly into the river channel. MNK is situated downstream from the town slaughter house whose wastes are discharged directly into the river channel. Furthermore, a number of small animal farms are found in the back yards of many homes, while the presence of a carwash and soap factories attracts a concentration of people for whom sewage facilities are generally not available. Finally, MNG located at about 7 km downstream from MMU, receives not only the bacteria loaded water from the latter, but also input from the urban tributaries, as well as that from the Municipal open waste discharge that extends into the river channel.

Temporal variations in faecal indicator bacteria in the Mezam river system

Faecal contamination of the river was generally higher

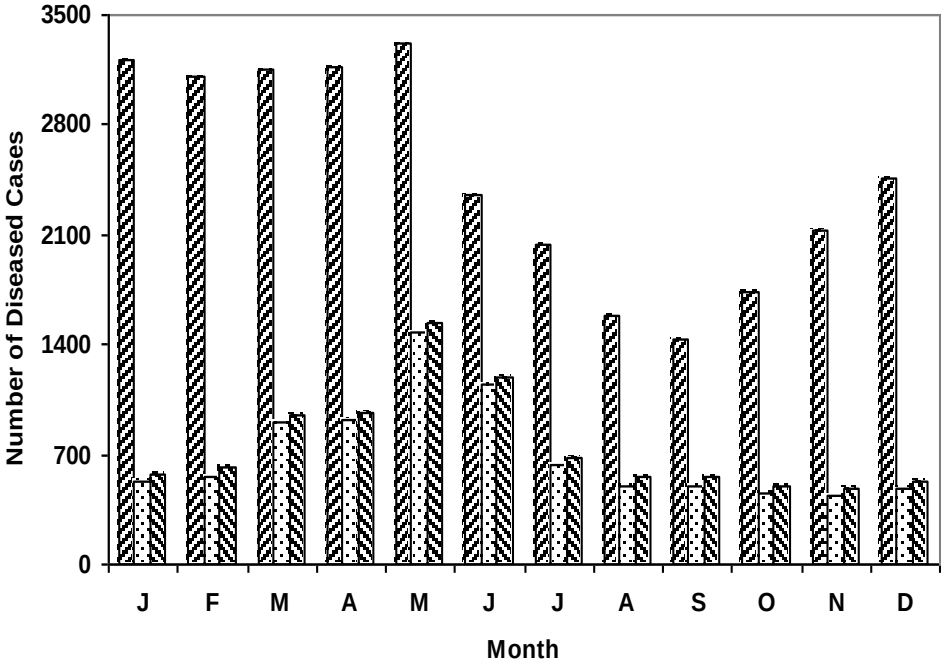

Figure 4. Incidence of waterborne diseases in the Bamenda Health District (2008-2010): (▨) diarrhoea; (▩) dysentery; (▧) typhoid fever.

during low and rising water levels and tended to reduce under high and falling discharge rates, corresponding to the dry season/onset of rains and rainy season/end of rains, respectively. In the dry season, water levels are much reduced and bank vegetations cleared for the cultivation of market vegetables using river water for irrigation. This makes the streams more accessible to both humans and animals. This may increase faecal bacteria levels in the water column through direct deposition of faecal matter and re-suspension from sediments, compounded by minimum dilution due to low river flow (Castillo et al., 2004). It is also possible that as the water velocity reduces in the dry season, faecal bacteria accumulate and settle as a result of greater contact between water and sediment which enables significant sediment-water exchange (Mitsch and Gosselink, 2000). Moreover, a lot of faecal matter and associated wastes accumulate on land due to the lack of rainfall. The first rains wash them into surface water and the upsurge in bacteria counts at this time could thus be a combination of contributions from such land stores and those re-suspended from sediments.

On the other hand, regular rainfall flushes faecal matter from land as they are deposited, and with the increased volume of water in river channels, there is maximum dilution resulting in lower counts (Castillo et al., 2004). However, storm events typically resulted in elevated bacteria counts, whether during low or high discharge regime. Storm events are characterised by turbulent rolling waves that result in channel mixing and entrainment of particles in the wave front (Wilkinson et al., 2006).

Besides, the large volumes of water generated may lead to ingress of the stream water into encroaching garbage and waste dumps, as well as the overflow of poorly maintained septic tanks. Faecal bacteria levels are therefore greatly increased by re-suspension from the streambed and allocthonous inputs from runoff and dissolution of actively decomposing waste matter from far afield. As the rainy season dwindles to an end, there is reduced input from land stores through runoff and, with decreasing flow, settling out of sediment particles with associated bacteria is enhanced. The sediments thus gradually switch from being in-channel sources of faecal bacteria to serving as sinks. Consequently, the water column bacteria counts remain low during this phase of the hydrograph.

Implications for public health

Freshwater quality criteria for domestic supply require that faecal bacteria levels should not exceed a geometric mean value of 100 cfu/100 ml while the drinking water criterion is <1 cfu/100 ml (WHO, 2001). Many segments of the Mezam river system are thus highly polluted and either unacceptable for public water supply, or require fairly expensive treatment before use. The presence of *Clostridium* spores in tap water suggests that highly persistent micro-organisms like protozoan cysts may

have survived during treatment, while high counts of heterotrophic bacteria are indicative of the availability of nutrients in the water, which may result in aesthetic problems or in the presence of opportunistic pathogens (Payment et al., 2003). Indeed, heterotrophic bacteria are used to assess the suitability of water for use in the manufacture of food and drink products, where high counts may lead to spoilage. This is of particular concern in the present study area where local homemade beverages such as “foléré”, “ginger”, “Alaska”, “kossam” and corn beer are very common. On the other hand, the presence of *Pseudomonas* spp. in high concentrations should be of great concern today with the increasing number of HIV/AIDS patients as it causes opportunistic infections in debilitated patients (Baron and Hollander, 1993).

The pathogens *Salmonella*, *Shigella* and, to a much lesser extent, *Vibrio* were identified at the studied sites. A few significant correlations ($p \leq 0.01$) existed between the presence of *Salmonella* and the levels of some of the faecal indicator bacteria but generally, no direct relationship could be established between the presence of pathogens and levels of faecal indicators. However, it is worth noting that the pathogens were more frequently detected at the urban sites that were also quite remarkable for their chronically elevated levels of faecal bacteria.

The prevalence of waterborne diseases in Bamenda is similar to the situation described by Djuikom et al. (2006) and Katte et al. (2003) in some urban and precarious quarters of Yaoundé and Dschang, respectively. Diarrhoeal diseases are waterborne and common in communities where living standards are low, and wastewater and excreta disposal facilities inadequate. Typhoid is caused by *Samonella* spp. and bacillary dysentery by *Shigella dystentriae*. *Salmonella* was shown in this study to be the most prevalent and endemic of the three classic bacterial agents of intestinal infections investigated. The incidence of the diseases seemed to follow a seasonal pattern that was almost reflected in the seasonality of the agents in water samples. Although the Bamenda Health District lacks the technical facilities to identify many diarrhoea agents and their serogroups, there seems to be strong circumstantial evidence that the enteric diseases in general and typhoid fever in particular, could be transmitted, at least in part, through the use of contaminated water from the Mezam river system.

Conclusion

The high levels and variety of faecal bacteria recorded in this study as well as the presence of pathogens in both river and spring waters clearly show that the Mezam River in Bamenda receives faecal contaminants on a continual basis. The urban tributaries are highly impacted by commercial and domestic activities but the Nkimefeu tributary is the most impacted due to direct waste discharge from the dressing of carcasses at the town slaughter house. The implications of these findings are that people who are dependent on the river water for domestic or agricultural uses may be exposed to public health risks. The evidence of the data and processes discussed indicate that the risk could be reduced by minimising the discharge of both liquid and solid wastes into water channels. This may be achieved by encouraging the establishment of thick and perennial bank vegetations to limit direct access to the river channel, accompanied by the provision of waste collection and disposal facilities by the Municipality. This should be reinforced with the periodic inspection of homes to ensure the effective and proper installation of latrines and individual sewage evacuation systems.

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Full Length Research Paper

The plant growth promoting bacterium *Bacillus* sp. CaSUT007 produces phytohormone and extracellular proteins for enhanced growth of cassava

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Bacillus sp. **strain CaSUT007, a plant growth promoting rhizobacterium isolated from cassava, was investigated for the secretion of compounds that might be involved in plant growth promotion. Extracts containing phytohormone and extracellular proteins were made from the cell-free fluid of CaSUT007 broth cultures. These extracts, along with a whole culture of CaSUT007 and the raw fluid and cellular fractions from a CaSUT007 culture, were applied separately to cassava stakes. The stakes were planted into pots of soil maintained in a greenhouse condition. Under this condition, all of the extracts including phytohormones and extracellular proteins increased root and shoot lengths and cassava biomass as compared to negative control. Our results indicate that the culture extracts, when applied to cassava stakes, increased root and shoot lengths by more than 30%, and increased fresh and dry weights by more than 25% compared to the distilled water control. Thus, photohormone and extracellular proteins secreted by CaSUT007 can influence plant growth and development. Analysis of the photohormone and extracellular proteins extracts revealed indole-3-acetic acid and peptides to be the primary compounds.**

Key words: Cassava, growth promotion, Bacillus, extracellular proteins, phytohormone.

INTRODUCTION

Bacillus sp. CaSUT007, a strain of plant growth promoting rhizobacteria (PGPR) isolated from a cassava farmer field in northeast of Thailand, can enhance growth promotion of cassava crop and inhibit the fungal pathogens causal agent of cassava diseases (Buensanteai et al., 2011). When applied as a cassava stakes treatment, it can also promote the growth of cassava (Buensanteai et al., 2011). The mechanisms by which CaSUT007 promotes plant growth are not well understood.

In addition to CaSUT007, strains classified in genus *Bacillus* sp. have been reported to be effective for the

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biocontrol of phytopathogens and for plant growth promotion (Araujo et al., 2005; Kloepper et al.,2004; Idriss et al., 2002; Buensanteai et al., 2011). Extrapolating from the accumulative literature on these species and on other species of PGPR, numerous mechanisms are possible in CaSUT007: as antagonist against pathogens, alteration of nutrient availability and direct interactions with plants. All of these interactions could potentially lead to plant growth promotion (Buensanteai et al., 2011).

As a starting point towards understanding how CaSUT007 causes enhanced plant growth, we focused this study on factors secreted by CaSUT007 that might directly affect the physiology of cassava plants. In this context, the production of auxins, indolic compounds with phytohormone activity, by *Bacillus* spp. is well known (Araujo et al., 2005; Kloepper et al., 2004; Idriss et al., 2002; Buensanteai et al., 2008). Indole-3-acetic acid (IAA), the main auxin in plants, controls important processes including cell enlargement and division and tissue differentiation. Regulation of these processes requires a balance between auxins and other phytohormones. Thus, IAA production by strains of PGPR can contribute sufficient auxin to the plant's auxin pool to have profound effects on these processes and potentially allow the microorganisms to redirect a plant's physiology and biochemistry for their own benefit (Idriss et al., 2002; Leveau and Lindow, 2005; Patten and Glick, 2002; Buensanteai et al., 2011).

Another large group of bacteria-secreted compounds is extracellular proteins. Strains in the *Bacillus* group secrete high levels of extracellular proteins as enzymes and secondary metabolites (Buensanteai et al., 2008). Some proteins secreted by *Bacillus* spp. are involved in key ecological functions such as biofilm formation (Buensanteai et al., 2008), but extracellular proteins, as a group, have not yet been investigated in the context of plant growth promotion.

The mechanisms of CaSUT007 promotion of plant growth are not well understood. Then, the goal of this study was to determine whether or not compounds secreted by CaSUT007 have a direct role in regulating plant growth, which promotes plant growth under control conditions. One objective was to determine whether indoles and extracellular proteins produced by CaSUT007 in liquid culture could enhance growth of cassava when applied to cassava stakes. Another objective was to analyze the extracellular proteins and phytohormone extracts from CaSUT007 to identify the primary components.

MATERIALS AND METHODS

Culture conditions and preparation of extracellular extracts

Cells of *Bacillus* sp. strain CaSUT007 stored in nutrient glucose broth with 10% glycerol at -80°C were revived by streaking onto nutrient glucose agar (NGA) and cultured at 28 ± 2°C for 48 h. To prepare cultures for extraction of extracellular factors, the strain was transferred to 500 mL volumes of nutrient broth containing 2% glucose (NGB) and incubated for 48 h at 28 ± 2°C with constant shaking at 180 rpm. The cultures were centrifuged at 13,000 rpm at 4°C for 20 min and the supernatants were passed through 0.2 nm nitrocellulose filters and retained for further extraction.

Extracts containing indoles were made from the cell-free culture supernatant and fluid was extracted three times with ethyl acetate after adjusting the pH to 2.8 and then evaporated at 40°C for 20 min. The material was solubilized in sterile distilled water for use in the experiments.

Extracellular proteins were extracted from CaSUT007 culture supernatant by acidifying the fluid to pH 2.0 with concentrated HCl and allowing the formation of a precipitate at 4°C overnight. The precipitate was collected by centrifugation (12,000 rpm, 4°C, 15 min), washed three times with distilled water, and dried by vacuum lyophilization. The dried extracellular protein was extracted three times with 100% methanol for 3 h. The methanol was removed with a rotary evaporator under reduced pressure, yielding a brown-colored crude extracellular protein extract. The extract was solubilized in sterile distilled water prior to use in experiments.

Plant bioassays for growth promotion

The experiment was conducted to evaluate CaSUT007 culture fluid extracts for effects on cassava growth. Stakes of cassava cv. Kasetsart50 were surface disinfested by treatment with 95% ethanol for 2 min, followed by soaking in 20% (v/v) solution of commercial bleach for 20 min. The stakes were then washed with sterile distilled water 5 times in order to remove the bleach. Before planting, cassava stakes were dipped thoroughly in a CaSUT007 liquid treatment. The treatments included a whole culture of CASUT007 in NGB and cell-free fluid from a CASUT007 culture in NGB. Sterile distilled water was used as the control. Cell concentrations in the whole culture were adjusted with sterile distilled water to 1×10^8 cfu mL^{-1}, based on absorbance. Other treatments included extracts of extracellular proteins (250 µg mL^{-1}) and indoles (50 µg mL^{-1}) extracted from CaSUT007. Distilled water was used to dilute the raw extracts to these concentrations, which roughly correspond to those found in NGB cultures with 1×10^8 cfu CaSUT007 mL^{-1}. In the experiments conducted under greenhouse condition, treated stakes were planted in pots (30 cm diameter) containing soils from the cassava field in Nakhon Ratchasima province, Thailand. There were 10 replicate pots per treatment with one stake per pot. The pots were watered daily with water and kept in a greenhouse with a 12-h photoperiod. At 14 days after cassava stakeling emergence, stakelings were harvested for measurements of root and shoot lengths, along with fresh and dry weights. The experiment was conducted two times.

Data from each experiment was subjected to analysis of variance using SAS version 9.1. Separation of treatment means was accomplished by Duncan's Multiple Range Test, and all tests for significance were conducted at $P \leq 0.05$.

Analysis of indoles

Supernatants from two types of cultures of CaSUT007 were extracted and assayed for indoles. In one, CaSUT007 was grown in NGB for 12, 48 and 96 h. The indole extract was extracted from cell-free culture supernatants as described above. Indole concentrations were determined based on the method described by Patten and Glick (2002) with slight modifications. For the colorimetric Salkowski assay for indoles, 1 mL of culture fluid was mixed with 4 mL of Salkowski reagent and incubated at room temperature for 20 min. The absorbance was measured at 535 nm using a spectrophotometer (Crozier et al., 1988). The quantity of indoles was determined by comparison with a standard curve using

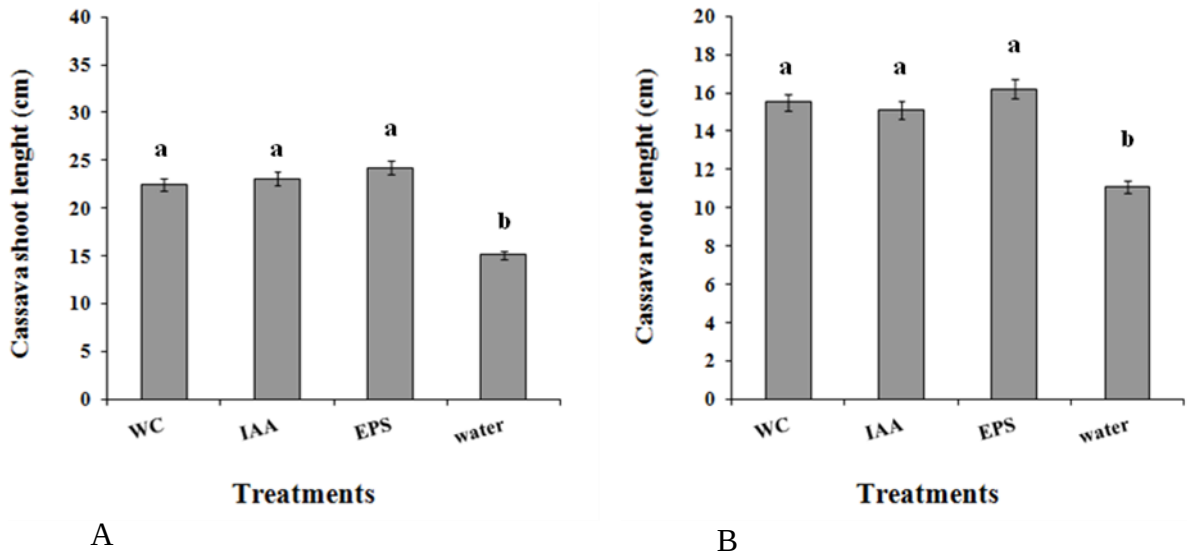

Figure 1. Effects of 48 h cellular fractions and extracellular extracts from cultures of *Bacillus* sp. CaSUT007 on the growth of cassava under greenhouse conditions, as measured at 21 days after induction in: A) shoot length; B) root length. WC CaSUT007 = whole culture of CaSUT007; IAA = indole extracted; EPS = extracellular proteins extracted. The data are the average of four replications (three plants per replication) for each treatment. Error bars represent the standard deviation. For each growth parameter, different letters indicate significant differenced ($P \leq 0.05$) among treatments.

purified IAA in the concentration range of 0 to 50 µg mL^{-1}.

Analysis of peptides

A lipopeptide-enriched extract was obtained from 20 mL of the crude peptide extract from cultures of CaSUT007 in NGB by purification on an ISOLUTE C-18 CE type cartridge following a modification of the method described by Jacques et al. (1999) and Araujo et al. (2005). The enriched extract was dissolved in butanol and loaded onto a column of a reverse-phase HPLC system. The system was operated at a flow rate of 2.0 mL min^{-1} with 90% methanol as the mobile phase. HPLC spectra were detected by a UV monitor at 210 nm. The presence of surfactin-type lipopeptides was determined on the basis of retention times compared with those of purified surfactin standards (Sigma S3523).

RESULTS

Effects of CaSUT007 extracellular factors on plant growth

Crude extracellular proteins, and indole extracts from cultures of strain CaSUT007 were effective in promoting the growth of cassava under greenhouse conditions. These culture extracts, when applied to cassava stakes, increased root and shoot lengths, by more than 30% (Figure 1), and increased fresh and dry weights by more than 25% (data not shown) compared to the distilled water as negative control. Treatment with the extracellular proteins, and indole extracts had similar effects on cassava growth as stake treatments with a whole culture of CaSUT007 (Figure 1). There was signi-ficant effect of cassava stake treatment with CaSUT007 and its products compared with distilled water for any cassava plant growth parameter (Figure 1). Similar results were obtained when the experiment was repeated.

Indole-3-acetic acid analysis

Strain CaSUT007 secreted IAA as the major auxin, as determined by Salkowski assay, when cultured in NGB medium, with the highest IAA concentration (31.0 µg mL^{-1}) detected at stationary phase at 48 h (Figure 2).

Extracellular proteins as a peptide production

When the extracellular proteins extract from the culture fluid of CaSUT007 was analyzed by HPLC, high concentrations of bioactive non-polar antibiotics were detected (Figure 3). Surfactin-type peptides were identified in the extract on the basis of their retention times being similar to those of purified surfactin standards, such as surfactin produced by *Bacillus* sp. ATCC21332. C18 homologues represented together more than 50% of the total amount of peptides present in the extract.

Based on HPLC peak areas of the peptide extract compared with values obtained for standards, the total amount of surfactins produced by strain CASUT007

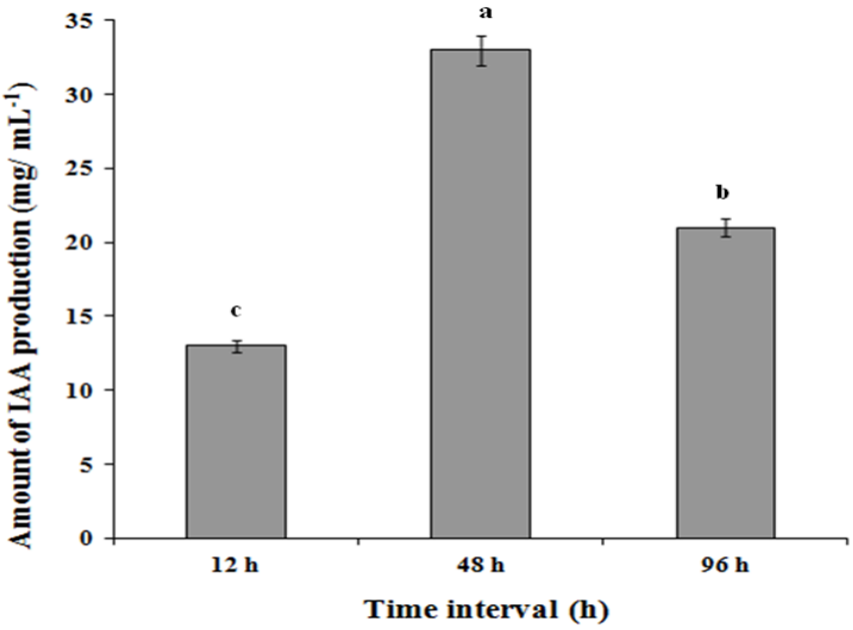

Figure 2. Concentrations of indole-3-acetic acid secreted by *Bacillus* sp. CaSUT007 grown in NGB medium at difference time interval. The concentration values were standardized to 10^8 cfu mL^{-1} of cells in the medium. The data are the means and standard deviations of three replicates. Error bars represent the standard deviation. Bars with the same letter are not significantly different ($P \leq 0.05$).

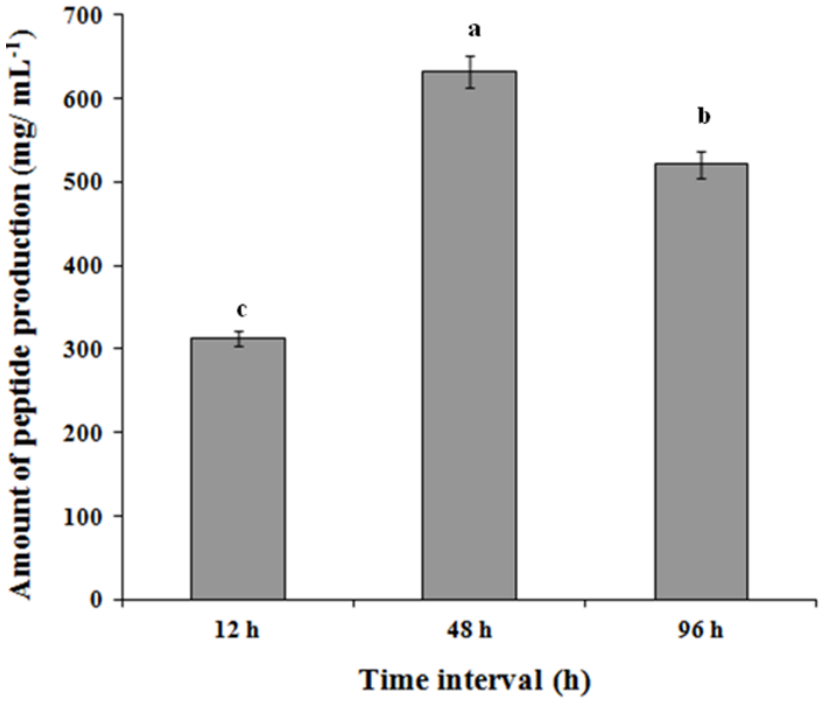

Figure 3. Amounts of peptide secreted by *Bacillus* sp. CaSUT007 grown in NGB medium at difference time interval. The concentration values were standardized to 10^8 cfu mL^{-1} of cells in the medium. The data are the means and standard deviations of three replicates. Error bars represent the standard deviation. Bars with the same letter are not significantly different ($P \leq 0.05$).

was 570 ± 7.267 mg L^{-1} at 48 h (mean and standard deviation calculated from three independent cultures).

DISCUSSION

This study represents the analysis of metabolite production by *Bacillus* sp. CaSUT007 in relations to cassava growth promotion. We found that CaSUT007 culture fluid extracts containing secreted indoles, and extracellular proteins individually can influence the growth of cassava to similar degree as cells of CaSUT007 washed free of preformed exoproducts. Whether or not the same type of compounds are secreted by CaSUT007 cells while existing in the rhizosphere remains to be determined, but nevertheless, the results are consistent with the hypothesis that strain CaSUT007 promotes the growth of cassava by secretion of several types of bio-compounds.

Based on the fact that each of the crude extracts affected cassava growth and development under greenhouse condition, we surmise that components within each extract had direct effects on cassava, perhaps acting as signaling compounds. Because IAA was the predominant component in the indole extract and its role in plant growth stimulation by plant growth promoting bacteria has been well established (Kutschera and Briggs, 1987; Lambrecht et al., 2000; Patten and Glick, 2002; Martinez-Morales et al., 2003; Araujo et al., 2005; Idriss et al., 2002; Spaepen et al., 2007; Buensanteai et al., 2008), it was most likely that it is the compound responsible for the activity of that extract. The effects of the indole extract on root development apparent in the experiments are consistent with the effects of exogenous IAA. The peak concentrations of IAA detected in the indole extract of CaSUT007 were higher than that reported for any other strains of plant growth promoting bacteria (Araujo et al., 2005; Leveau and Lindow, 2005; Kang et al., 2006). Although small peptide was a major component of the peptide extract, its role in causing the growth promotional effects of that extract was confirmed. If purified small peptide proves to have this effect, then this would be a new function for a secondary metabolite that is better known for its effects on plant cell division (Jacques et al., 1999; Bonmatin et al., 2003; Ongena et al., 2007). There is evidence that certain extracellular proteins can stimulate plant growth by synergizing plant growth action, activating certain plant enzyme systems, or affecting plant cell elongation or division, thereby increasing water or nutrient uptake or excretion of plant factors such as riboflavin (Ernst et al., 1971). But other than a report that extracellular proteins can enhance growth promotion in soybean (Buensanteai et al., 2008), there is precedence for extracellular proteins having a direct effect on plants leading to elevated growth (Vessey, 2003). This study represents the first analysis of the extracellular proteome of a *Bacillus* species in relations to cassava growth enhancement (Zavahir and Seneviratne, 2007).

Ultimately, the approaches used in this study could increase understanding of the modes of action by which *Bacillus* sp. CaSUT007 enhances cassava growth. With such information, we could potentially enhance the efficacy of CaSUT007 strains or better exploit such strains as sources of new bio-products in the near future.

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African Journal of Microbiology Research

Full Length Research Paper

The possibility of using *Sansevieria aethiopica* (Thunb) leaf extracts in combinations with gentamicin for the treatment of oral enterococcal infections

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Sansevieria aethiopica **(Thunb) has been reported to be used for the treatment of oral infections in Eastern Cape of South Africa. Based on ethnobotanical survey, the plant was selected for the possible synergistic effects of its acetone, ethanol and methanolic extracts with gentamicin on the planktonic and sessile cells of *Enterococcus faecalis* ATCC 29212 and *Enterococcus faecalis* KZN. *In vitro* interactions between the plant extracts and gentamicin were studied using checkerboard macrodilution method and anti-biofilm activity of the iso-effective combinations was determined by semi-quantitative adherence assay. Acetone extract of *S. aethiopica* has the highest inhibitory activity. The minimum concentration of gentamicin that inhibited the two isolates was the same (0.016 mg/ml). Different iso-effective points were observed with fractional inhibitory concentration indexes ranged between 0.375 and 1.9313. Out of the iso-effective points observed only four were synergistic while one was addictive. The maximum biofilm reduction was observed when the two antibacterial agents were combined. We therefore suggest that the extracts of the plant at the test concentrations can be used in combination with gentamicin for oral hygiene.**

Key words: *Enterococcus faecalis, Sansevieria aethiopica,* biofilm, planktonic cell, dental infection, medicinal plant.

INTRODUCTION

The prevalence of dental caries in school aged children is up to 90% and the majority of adults are also affected (Petersen et al., 2005) and chronic diseases like diabetes and HIV have been reported to enhance oral infections (Petersen, 2003). Oral infection is caused by different bacteria out of which *Enterococcus faecalis* is emerging. *E. faecalis* has been mentioned with increased frequency with regard to oral with post-treatment diseases. Enterococci are Gram positive coccus bacterium and a part of the normal flora in the oral cavity and gastrointestinal tract. *E. faecalis* has been reported as the most common bacterium recovered from different oral infections with failed treatment and persistence (Rams etal., 1992; Colombo et al., 2002; Rocas et al., 2004). The pathogen survives in situations where conventional therapy has eliminated other bacteria; this attribute has resulted in the emergence of drug resistance strain of the bacterium (Sundqvist et al., 1998).

E. faecalis accounts for around 80% of all infections caused by enterococci (Sahm, 2000). Enterococci pose increasing problems in medicine due to an increased resistance to various antibiotics. It account for 10% of the dental infections among healthy individuals (Smyth et al., 1987) and higher rate of 60% was observed among long-term patients (Murray, 1990).

The genus *Enterococcus* has been implicated in 60% of dental infections among school children and 75% of patients with endodontic infection (Gold *et al*., 1975). Sedgley et al. (2004) reported the presence of *E. faecalis* in 29% of oral rinse samples, 55% of tongue dorsum and 22% of gingival sulcus samples from 41 endodontic subjects. The genus is well known for biofilm formation

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which serves as an important reservoir of cells that can repopulate colonized sites hence lead to persistency (Costerton et al., 2011; Li and Yu-mei, 2011). Biofilms are responsible for persistent pathogens on infection sites (Costerone *et al*., 1999; Anderson et al., 2004; Li and Yu-mei, 2011).

The use of plant for oral hygiene is age long. The Babylonians recorded the use of chewing sticks in 7000 BC and its use ultimately spread throughout the Greek and Roman Empires (Almas and al-Lafi, 1995). It has been documented that medicinal plants confer considerable antibacterial activity against various bacterial pathogens including those responsible for dental infections (Jonathan et al., 2000).

Sansevieria aethiopica (Thunb) is a medicinal plant belonging to the family *Asparagaceae.* It is a perennial shrub with tough, semi-succulent and erected leaves (Van Wyk et al., 2000). The hard, mottled leaves arise from thick fleshy rhizomes and like other members of the genus, it flourishes under low light conditions. *S. aethiopica* is called *isikholokotho, isikwendle* or *isitokotoko* in South Africa where it has been long recognized as a remedy for the treatment of dental infections, otitis, ulcers among others (Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996; Newton, 2001; Von Koenen, 2001).

The aim of this study was to investigate the *in vitro* interactions between extracts of *S. aethiopica* and gentamicin and the activities of the resultant iso-effective combinations against biofilm of *E. faecalis* ATCC 29212 and *E. faecalis* KZN.

MATERIALS AND METHODS

Plant material

Plant material was collected in March 2012 from a single population of *S. aethiopica* growing around Alice Township in Nkokobe Municipality, Eastern Cape Province (32°47' S, 26°50' E and altitude 589 m). The species was authenticated by Prof. Donald S. Grierson, University of Fort Hare, Alice, South Africa and a voucher specimen (DavMed, 2012/2) was prepared and deposited in the Giffen Herbarium of the University of Fort Hare, Alice, South Africa for future reference. Plant sample was dried in the oven at the temperature (40°C).

The dried leaves of the plant sample were pulverized. Powdered plant material (40 g each) was separately extracted in acetone, ethanol and methanol for 48 h on an orbital shaker (Stuart Scientific, Manchester, UK). The extracts were filtered through Whatman No. 1 filter paper. The extracts were evaporated to dryness under reduced pressure at 40°C using a rotary evaporator (Laborota 4000-efficient, Heldolph, Germany). Individual crude extracts was diluted using 50% dimethylsulphoxide to give 50 mg/mL stock solution (Taylor et al., 1995). This was then diluted to the required concentrations for the bioassay.

Test organisms

Two strains of *E. faecalis* (*E. faecalis* KZN and *E. faecalis* ATCC 29212) used in this study were obtained from the Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa. Each strain was maintained on Nutrient Agar (Biolab, South Africa) plates. The grown cultures were used for preparation of bacterial suspensions in sterile distilled water with densities adjusted to 0.5 McFarland standard.

Antimicrobial susceptibility tests (AST)

The checkerboard method was used to examine the effects of combinations of *S. aethiopica* extract and gentamicin against strains of *E. faecalis*. Twofold serial dilutions of crude extract of *S. aethiopica* and gentamicin were added to make up to final concentrations of 0.0488 to 50.0mg/ml and 1.0 to 64.0 µl/l, respectively in Mueller Hinton broth. The tubes were incubated for 24 h at 37°C. The tube with least concentration and with no sign of growth was taken for MIC of each of the antibacterials and the iso-effective combinations. Fractional inhibitory concentration (FIC) index, which represents the sum of the FICs of each drug tested, where the FIC is determined for each drug by dividing the MIC of each drug when used in combination by the MIC of each drug when used alone.

$FICI = FIC_{Ext} + FIC_{Gen} = (C_{Ext}\ comb/MIC_{Ext}\ alone) + (C_{Gen}comb/MIC_{Gen}\ alone)$

Where, MIC_{Ext} *alone* and MIC_{Gen} *alone* were respectively the MICs of extract and gentamicin when acting alone, and C_{Ext} comb and C_{Gen} comb are concentrations of extract and gentamicin at the iso-effective combinations respectively. The interpretation of FICI was made as follows: synergistic (<0.5), additive (0.5 to 1.0), indifferent (>1), or antagonistic (>4.0) according to White et al. (1996).

Biofilm eradication test

Mueller Hinton broth (5 ml) was prepared in the test tubes and inoculated with then test organism. The tubes were incubated at 37°C for 6 h after which the MICs of the *S. aethiopica* extracts and the iso-effective combinations were added to separate tubes except the control. The tubes were further incubated aerobically for 24 h at 35°C. After the incubation, the contents of tubes were discarded and the tubes washed three times with sterile distilled water. The remaining attached bacteria were fixed with methanol after 15 min of which the tubes were emptied, air dried and stained with 10 ml of 1.0% crystal violet for 5 min. Excess stain was rinsed off with water. After the plates were air dried, the dye bound to the adherent cells was extracted with 10 ml of 33% (v/v) glacial acetic acid (Merck, Darmstadt, Germany) per tube. The optical density of each tube was measured at 570 nm using UV-3000 PC spectrophotometer (Optima Scientific). The percentage reduction in biofilm was calculated as

$\%Biofilm\ reduction = (A_C - A_T)/A_C) \times 100$

Where, A_C = absorbance of the control, A_T = absorbance of the test

RESULTS AND DISCUSSION

In this study, *S. aethiopica* leaf extracts in combination with gentamicin were screened against the *E. faecalis* strains planktonic and sessile cells. *S. aethiopica* alone and in combinations with gentamicin produced good inhibitory activity on the isolates. The result of MIC study is represented in Table 1. Against *E. faecalis* KZN, methanolic and ethanolic extracts had lower inhibitory activity, compared to acetone extract. The MICs of ethanolic extract of *S. aethiopica* were 0.3906 and 0.1953 mg/ml against *E. faecalis* KZN and *E. faecalis* ATCC 29212 respectively. The minimum concentration of genta-

Table 1. Combined activity of extracts of *S. aethiopica* and gentamicin on planktonic cells of *E. faecalis* strains.

Extract	*E. faecalis* KZN				*E. faecalis* ATCC 29212			
	MIC A	MIC B	FICI		MIC A	MIC B	FICI	
			MIN	MAX			MIN	MAX
Ethanolic	0.3906	16	0.625	0.1249	0.1953	16	0.75	1.2499
Methanolic	0.3906	16	0.625	1.1249	3.1250	16	0.375	1.9313
Acetone	6.250	16	0.375	1.0625	0.7812	16	0.625	1.0625

MICs values were expressed in mg/ml.

Table 2. Numbers of the iso-effective combinations and interactions of extracts of *S. aethiopica* and gentamicin on planktonic cells of *E. faecalis* strains.

Test organism	Extract	Iso-effective combinations (n)	Synergy	Addictive	Indifference	Antagonism
E. faecalis KZN	Ethanolic	5	0	0	5	0
	Methanolic	5	0	0	5	0
	Acetone	8	2	0	6	0
E. faecalis ATCC 29212	Ethanolic	5	0	1	4	0
	Methanolic	8	2	0	6	0
	Acetone	6	0	0	6	0

micin that inhibited the two isolates was the same (0.016 mg/ml). Our report is similar to that of Van Wyk and Gericke (2002) and Aliero et al. (2008) that reported that *Sansevieria* spp. are very rich in antimicrobial compounds. The plant has also reported to inhibits bacteria associated with otitis (Hutchings et al., 1996). Philip et al. (2011) reported antibacterial activity of closer member of Genus; *Sansevieria roxburghiana* on common pathogens. They also reported, in agreement with our results, that the methanolic is more active on *E. faecalis* than acetone extract of the plant. The *S. aethiopica* extracts could be good alternatives against enterococcal infections. Enterococcal oral infections are developing resistance against antibiotics and different researchers have suggested alternates to antibiotic treatment of the infections (Rocas et al., 2004; Stuart et al., 2006; Zehnder and Guggenheim, 2009).

The FIC index is the most frequently used method to determine the interaction between antifungal drugs. The interpretation is based on the value got as synergistic interaction of anti-bacterial which has FIC that is less than one. This means that the action is better when in combination than they are separately (Isenberg, 1992). FIC results were provided to allow re-evaluation of the results and also indicate whether the degree of interaction (Eliopoulos and Eliopoulos, 1988). Though bulk of the thirty six iso-effective points observed were indifferent, only four showed synergistic interactions and one was addictive, none was antagonistic as shown in Table 2. The FIC indexes calculated for different iso-effectives concentrations ranged between 0.375 and 1.9313. The interaction between the combinations of *S. aethiopica* extracts and gentamicin against two strains of *E. faecalis* were predominantly indifference. FICI values greater than 1 were considered as indifference and as the value decreases the interaction tends towards synergism. The FIC index is based on the assumption that neither the *S. aethiopica* extract nor gentamicin would interact with itself, therefore the effect of a self-drug combination will always be additive, with an FIC index of 1. An FIC index lower or higher than 1 indicates synergy or antagonism, respectively, because less or more of either of the extracts of gentamicin would be required in order to produce the same effect as either alone.

As shown in Tables 3 and 4, at the MIC of ethanolic extract, there was reduction in biofilm formed by the two test organisms. The maximum biofilm reduction was observed when two antibacterial agents were combined as shown in Table 5. The inhibitory effect of the extracts (alone) on the biofilm of the two enterococci was noticed to be more pronounced than the gentamicin except for the methanolic extract on the biofilm of *E. faecalis* ATCC 29212. The combinations of extracts and gentamicin achieved inhibition of cells in suspension and eradication of the biofilm of the test bacteria. The recognition that most oral infections developed from a biofilm explains why some dental infections like periodontitis have been

Table 3. Effect of ethanolic extracts of *S. aethiopica* in combination with gentamicin on biofilm of *E. faecalis* strains.

Isolate	Ethanolic extract-gentamicin combination		
	Extract (mg/ml)	Gentamicin (mg/ml)	Biofilm eradication (%)
E. faecalis KZN	0.3906	0	29.31
	0.3906	0.001	12.07
	0.1953	0.002	24.14
	0.1953	0.004	32.76
	0.0977	0.008	15.52
	0.0488	0.016	31.03
	0	16 (MIC)	17.24
E. faecalis ATCC 29212	0.1953 MIC	0	32.11
	0.1953	0.001	35.78
	0.1953	0.002	38.53
	0.0977	0.004	11.01
	0.0977	0.008	21.10
	0.0488	0.016	24.77
	0	0.016 (MIC)	15.60

Table 4. Effect of methanolic extracts of *S. aethiopica* in combination with gentamicin on biofilm of *E. faecalis* strains.

Isolate	Methanolic extract combinations		
	Extract (mg/ml)	Gentamicin (mg/ml)	Biofilm eradication (%)
E. faecalis KZN	0.3906 (MIC)	0	44.68
	0.1953	0.001	46.81
	0.1953	0.002	41.13
	0.0977	0.004	47.52
	0.0488	0.008	46.10
	0.0488	0.008	15.60
	0	(MIC)	15.92
E. faecalis ATCC 29212	3.1250 (MIC)	0	33.33
	1.5625	0.001	38.10
	1.5625	0.002	24.76
	1.5625	0.004	26.67
	0.7813	0.004	21.90
	0.3906	0.004	29.52
	0.1953	0.008	19.05
	0.0977	0.016	8.58
	0.0488	0.016	15.23
	0	16 (MIC)	20.95

reported to be very difficult to prevent and treat (Kishen et al., 2004; Rocas et al., 2004; Arias-Moliz et al., 2009). All the *S. aethiopica* extracts were effective at reducing the *E. faecalis* biofilms at concentrations that will inhibit the planktonic cells of the pathogens. Gentamicin, like other aminoglycoside antibiotics, affects Gram positive bacteria by production of transferase enzyme and impairment of permeability of cell membrane (Simjee et al., 1999;

Table 5. Effect of acetone extracts of *S. aethiopica* in combination with gentamicin on biofilm of *E. faecalis* strains

Isolate	Acetone extract combination		
	Extract (mg/ml)	Gentamicin (mg/ml)	Biofilm eradication (%)
E. faecalis KZN	6.250 (MIC)	0	34.23
	6.250	0.001	41.18
	3.1250	0.002	32.29
	1.5625	0.004	40.34
	0.7812	0.004	31.09
	0.3906	0.008	32.77
	0.1953	0.016	8.40
	0.0977	0.016	20.17
	0.0488	0.016	35.29
	0	0.016 (MIC)	37.82
E. faecalis ATCC 29212	0.7812 (MIC)	0	26.45
	0.7812	0.001	25.00
	0.7812	0.002	38.02
	0.3906	0.004	42.98
	0.1953	0.008	42.15
	0.0977	0.008	31.40
	0.0488	0.016	33.88
	0	0.016 (MIC)	17.36

Katzung, 2006). It also impaired 30S ribosomal subunit and inhibits protein synthesis by causing misreading of mRNA and complete inhibition of protein synthesis at low and high levels, respectively (Kaye, 2004; Poole, 2005). Medicinal plants have different sites of actions due to the different bioactive compounds (Cushman et al., 1991; Qin et al., 2010). The actions of these antimicrobial must have complemented each other to achieve this activity. The variation in the activities of the combinations may be due to different bioactive compounds present in the *S. aethiopica* extracts as suggested by Gatsing et al. (2010).

The *S. aethiopica* extracts reduced the pre-formed biofilm at varying degrees, though there was no total eradication at the MICs (of both the extracts and gentamicin) and also at the iso-effective concentrations. This is in agreement with the work of George *et al*. (2005), Arias-Moliz *et al*., (2009, 2010) and Nett et al., (2008) that reported that higher concentrations than MIC are needed to clear already established biofilm due to the protection offered by the glycoprotein. Pathogens within a biofilm environment behave very differently from free-floating bacteria (Lima et al., 2001; Kishen et al., 2008; Arias-Moliz et al., 2010; Balaei-Gajan et al., 2010). Protective extra-cellular slime matrix makes bacteria extremely resistant to antibiotics, antimicrobial agents and host defense mechanisms (Colombo et al., 2002; Rocas et al., 2004; Rolland et al., 2006; Daneshmehr et al., 2008).

The combinations of the agents were able to break the barrier created by the biofilm. This is necessary before dental pathogen embedded in the biofilm could be eradicated or reduced. The efficacies of the extracts of *S. aethiopica* (singly and in combination with gentamicin) justifies its usage in oral hygiene and also suggests it as an important candidate for the formulation of paste or tincture for oral hygiene and treatment of enterococcal infections. However, isolation and characterization of the active ingredients in this plant together with their mechanisms of actions on pathogens are still open for further investigations.

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Full Length Research Paper

Using phenotypic based approaches to compare *Escherichia coli* isolates from human, livestock, fish and environmental sources within the Lake Victoria basin of Kenya

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The study compares *Escherichia coli* recovered from human, fish products, domesticated animals and the environment within the Lake Victoria basin on the basis of their antimicrobial susceptibility profiles. A total of 134 *E. coli* isolates were isolated from the collected samples. 52.2% of the *E. coli* isolates were found to be resistant to at least one antibiotic. Isolates originating from fish and soil showed the highest levels of resistance (100%). Based on the discriminant analysis (DA), most of the fish isolates were misclassified into soil category, probably due to the groups displaying similar Multiple Antibiotic Resistance (MAR) profiles. On the other hand, human isolates had the highest score of 0.55. The findings suggest that soil may be an important source of bacterial contamination of fish. Similarly resistance to antibiotics is widely prevalent among human, environment and domesticated animals within the Lake Victoria basin.

Key words: *Escherichia coli*; Environment; Discriminate; Antimicrobial resistance.

INTRODUCTION

Lake Victoria is the second largest lake in the world with an area of 68,800 km^3 and is shared between Kenya (6%), Uganda (43%) and Tanzania (51%), with a shoreline of about 3,440 km long and a catchment of 193,000 km^2 which has been estimated to have over 30 million people within the three countries (Okedi, 2005). Lake Victoria and its basin provide fresh water for domestic, industrial, agricultural and recreational use to the riparian communities. The communities in the Lake Victoria basin interact with the lake ecosystem on a daily basis in fishing as well as collecting water for domestic and commercial purposes. The state of the lake is directly linked to their livelihoods and has a major influence on water borne and related communicable diseases (Tanzarn et al., 2005).

The Lake Victoria basin has been shown to bear a great burden of diarrhoeal infections (Brooks et al., 2003; Brooks et al., 2006), and *Shigella*, *Vibrio cholerae*, *Salmonella*, diarrhoeagenic *Escherichia coli*, and *Campylobacter* are some of the pathogens that have been associated with this diarrhoeal infection. Brooks et al. (2003) has reported that drinking water from Lake Victoria (the major source of water for all use in the community under study) and also sharing of latrines between multiple households increased risk of diarrhoea. However, no attempts have been made to demonstrate

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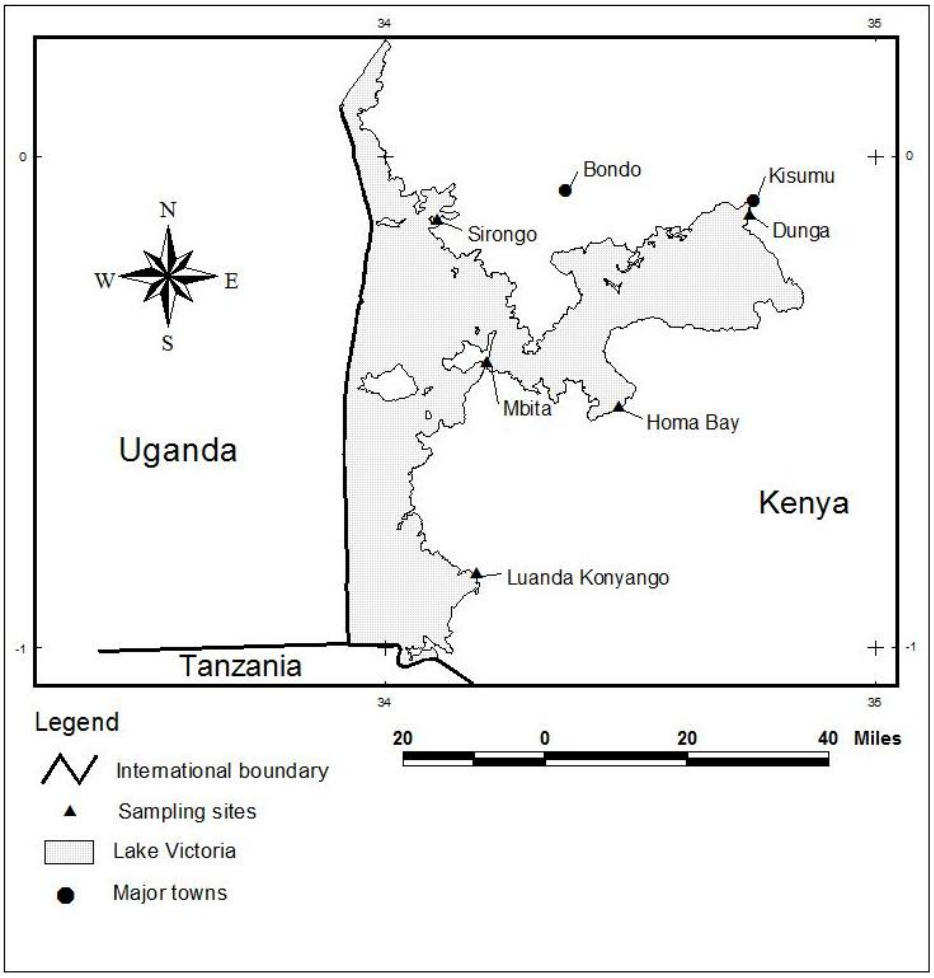

Figure 1. Map of Lake Victoria (Kenya) showing the study sites.

the distribution, occurrence, and possible reservoirs of *E. coli* species within the Lake Victoria basin thus negligible information exist about their antimicrobial resistance patterns.

Strain discrimination or strain typing has become an important aspect for epidemiological studies. Typing methods fall into two categories; phenotypic methods and genotypic methods. Phenotypic methods are cheap and easy to use and have been used successfully in strain typing (Scott et al., 2002). They include antibiotic resistance (AR) based analysis, and immunological methods. Multiple antibiotic resistance (MAR) analysis has been used to differentiate bacteria (*E. coli* or faecal *Streptococci*) from different sources using antibiotics commonly associated with human and animal therapy, as well as animal feed (Wiggins, 1996). The use of this method is based on the underlying principle that the bacterial flora present in the gut of various types of animals are subjected to different types, concentrations, and frequencies of antibiotics, and over time, selective pressure within a specific group of animal selects for flora that possess specific "fingerprints" of antibiotic resistance (Scott et al., 2002). This study therefore aimed at discriminating *Escherichia coli* recovered from human, fish products, domesticated animals and the environment within the Lake Victoria basin of Kenya. Discriminating such indicator bacteria from different sources may make it possible to compare and determine possible reservoirs and sources of contamination.

MATERIALS AND METHODS

Study site and design

This study was conducted within the Lake Victoria basin of Nyanza province in Western Kenya, targeting both the rural and urban communities. Generally, fishing, cattle rearing and subsistence farming are the principal occupation for the rural communities within the study areas (Figure 1). The samples were collected from five fish landing beaches along Lake Victoria (Sirongo, Dunga, Homa Bay, Mbita town, and Luanda Konyango) of Western Kenya. The study sites were chosen based on fish production and proximity to an urban town and human activities. Human stool specimens were

collected from Kisumu District Hospital also in western part of Kenya. The study was based on a repeated cross-sectional study design, and took a prospective approach. The samples were collected between January 2010 and June 2012.

Sample collection and processing

Freshly deposited faeces from domesticated animals (within a radius of 500 m from the fish landing site) were picked using a sterilized spoon and placed in a sterile container (Greiner Bio-one). Water samples were collected by submerging pre-sterilized pipette to depth of 30 cm below the surface and extracting a 100 ml sample which was dispensed in sterilized 250 ml Pyrex glass bottles; three water samples were collected from each sampling site (but at different points from the shores, namely at the shores- (0, 100 and 150 m). Fish samples of 500 g each were purchased from fishermen at landing sites for freshly landed samples, whereas sundried fish products were sourced from markets. Soil samples were collected aseptically using a sterilized spoon from six points and pooled together to form a representative sample for that site. Human stool specimens were transported on Cary Blair medium (HIMedia Lab. Pvt. Mumbai, India). All the samples were transported on ice in insulated containers to Maseno University Biomedical laboratory for analyses.

Upon arrival at the laboratory in Maseno, fish samples were processed according to FAO (1992). 25 g of fish was cut and homogenized aseptically in 225 ml buffered peptone water (HIMedia Lab. Pvt. Mumbai, India) followed by direct plating onto selective media MacConkey agar (HIMedia Lab. Pvt. Mumbai, India). Soil samples were processed as described by van Elsas and Smalla (1997) briefly, two spoonfuls of the pooled soil sample were transferred into a pre-sterilized, Whirl Pak bag. 100 ml of sterile phosphate-buffered water was then added and mixed for 2 min and the mixture filtered through a pre-sterilized 28 µm - pore - size nylon filter. The filtrate was then used to recover the *E. coli* by direct plating on MacConkey agar (HIMedia Lab. Pvt. Mumbai, India). Water was processed by taking 10 ml of sample and adding it to 90 ml of buffered peptone water followed by plating on MacConkey agar as described by Anazoo and Ibe (2005). Fecal samples were processed by direct plating on MacConkey agar as described by Kariuki et al. (2002).

All incubations were at 37°C for 18 h. Characteristic colonies were based on morphological characteristic and subjected to biochemical test triple sugar iron agar, lysine iron agar, citrate agar and indole all from HIMedia Lab. Pvt. Mumbai, India. The isolates were further confirmed to genera or species level using API 20 E (BioMerieux, France). All *E. coli* isolates were stored at - 20°C in tryptic soya broth plus 15% glycerol.

Susceptibility test with six antibiotics namely ampicillin (10 µg), tetracycline (30 µg), cefuroxime (30 µg), nalidixic acid (30 µg), chloramphenicol (30 µg) and gentamicin (10 µg) (Oxoid Inc, UK) was performed using the standard Kirby-Bauer disk diffusion method on Mueller Hinton (HIMedia Lab. Pvt. Mumbai, India). The plates were then incubated at 37°C for 18 to 20 h. The diameters (in millimetres) of clear zones of growth inhibition around the antimicrobial agent disks, including the 6 mm disk diameter was measured by using precision callipers (Clinical and Laboratory Standards Institute (CLSI), 2002) A standard reference strain of *E. coli* (ATCC 25922) was used as a control. The breakpoints used to categorize isolates as resistant to each antimicrobial agent were those recommended by CLSI (2002).

Data was entered in Ms Excel spread sheet Windows EP professional 2003 and analyzed by Minitab version 14. Data for the antimicrobial agent resistance of each bacterial isolate were reported as the diameter of the zone of inhibition (in millimeters). Descriptive statistics were generated to assess the distributions of the diffusion zones, and nonparametric tests (Kruskal-Wallis tests) were carried out to test for differences in diffusion zones between different groups. Discriminant function models were generated for the different species classification groups using Minitab 14. Only the linear discriminant analysis (DA) model could be performed with cross-validation. The cross-validation classification table was used to calculate the percentage of misclassified isolates and determine the average rate of correct classification.

RESULTS

Of the 134 isolates examined, 52.2% were found to be resistant to at least one antibiotic. Among the eight sources, all *E. coli* isolates originating from fish and soil sources showed (100%) resistance to at least one antimicrobial agent tested. However isolates originating from humans showed the highest level of resistance when comparing resistance to two or more antibiotics (78.8%). Apart from chicken, isolates from livestock recorded resistance levels below 22%. Among isolates recovered from cattle, no isolate recorded resistance to more than one antibiotic. Based on MAR indices calculated in this study, human isolates demonstrated the highest score of 0.55; others that scored above 0.2 were isolates from fish and soil sources. Cattle isolates recorded the lowest MAR indices of 0.04, followed by Donkey and goat isolates recording scores of 0.07 (Table 1).

As shown in Table 2, 18 distinctive antibiotic resistance patterns were observed altogether. The most frequent antimicrobial resistance observed among the isolate was that against tetracycline (21) followed by a combination of tetracycline - ampicillin (15); and ampicillin (9). The other frequent co- resistance was that against tetracycline - ampicilin - nalidixic acid (4) which was common among human isolates. Among the domesticated animals, no resistance to nalidixic acid and cefuroxime was observed. Resistance to nalidixic acid and cefuroxime was observed frequently among human isolates but also to some extent among fish and water isolates. Resistance to chloramphenicol and gentamicin was also only observed among isolates recovered from human and presented as co-resistance with other antibiotics.

Using the Kruskal Wallis test no significant differences were observed among chloramphenicol ($p = 0.075$) and gentamicin ($p = 0.11$). However, there were significant differences among the other four antibiotics tetracycline, ampicilin, nalidixic acid and cefuroxime ($p < 0.0001$). By using discriminant analysis (DA) with the 134 *E. coli* isolates, the average rate of correct classification (ARCC) for all isolates was 41% (Table 3). However when all isolates were reclassified into five host groups namely livestock, fish, human, soil and water, ARCC improved to 58.2%; and to 52.2% with cross validation (Table 4). When all isolates were reclassified into two host groups (human and non-human), ARCC rose to 78.4%; and to 76.1% with cross validation (Table 5). Soil isolates were well classified 100% followed by donkey and goat isolates at 60% and 56.3%, respectively, while chicken,

Table 1. Classification of *E. coli* sources based on percentage levels of resistance to the six antibiotics tested and Multiple Antibiotic Resistance indices.

Source (n)	% Level of resistance		
	At least one antibiotic	More than one antibiotic	MAR index*
Human (52)	88.5	78.8	0.55
Fish (13)	100	38.5	0.24
Soil (3)	100	33.3	0.22
Water (9)	67	22.2	0.17
Chicken (13)	38.5	23.1	0.1
Donkey (5)	20	20	0.07
Goat (16)	18.8	12.5	0.07
Cattle (23)	21.7	0	0.04

*MAR index was calculated using the formula *a*/ (*b* - *c*); where, *a* is the aggregate antibiotic resistance score of all isolates from the sample, *b* is the number of antibiotics, and *c* is the number of isolates from the sample. Source: Krumperman (1983).

Table 2. Antibiotic resistance patterns of *E. coli* isolates from investigated sources.

Pattern	No. of isolates	Source
Tet	21	Fish, soil, water, chicken, cattle, goat, human
Tet-Amp-Na	4	Human
Tet-Amp-Na-Gn-Cxm	3	Human
Amp-C	2	Human
Tet-Amp-Na-C-Cxm	2	Human
Tet-Amp-Na-Cxm	2	Human
Tet-Na	2	Human
Tet-Na-Cxm	2	Fish, water
Amp-Cxm	1	Water
Amp-Na	1	Human
Amp-Na-C	1	Human
Na-Cxm	1	Fish
Tet-Amp-C	1	Human
Tet-Amp-Na-C	1	Human
Tet-Amp-Na-Gn	1	Human

Tet, Tetracycline; Amp, ampcillin; C, chloramphenicol; Na, nalidixic acid; Gn, gentamicin; Cxm, cefuroxime.

Table 3. Discriminant analysis of disc diffusion zones of *E. coli* isolates from various sources.

Source (n)	(%) of database isolates assigned to each source category							
	Ch(13)	Ca(23)	D(5)	F(13)	G(16)	H(52)	S(3)	W(9)
Chicken	15.4	4.3	0	15.4	0	3.9	0	0
Fish	15.4	4.3	0	23	6.2	19.2	0	0
Goat	15.4	26.1	0	0	56.3	1.9	0	0
Human	7.7	0	20	7.7	0	44.3	0	11.1
Soil	7.7	8.7	0	38.5	12.5	11.5	100	33.3
Water	0	0	0	15.4	0	9.6	0	22.2

n = 134; ARCC = 41%; Ch = chicken, Ca = cattle, D = donkey, F = fish, G = goat, H = human, S = soil, W = water.

Table 4. Discriminant analysis of disc diffusion zones of *E. coli* isolates based on classification of livestock, fish, human, soil and water sources.

Source (n)	No. (%) of database isolates assigned to each source category				
	Livestock (57)	Fish (13)	Human (52)	Soil (3)	Water (9)
Livestock	78.9	15.4	11.5	0	22.2
Fish	7	23	19.2	0	0
Human	3.5	7.7	46.2	0	11.1
Soil	10.5	38.5	11.5	100	33.3
Water	0	15.4	11.5	0	33.3

n = 134; ARCC = 58.2%; classification with cross validation ARCC = 52.2%

Table 5. Discriminant analysis of disc diffusion zones of *E. coli* isolates based on classification of human and non human sources.

Source (n)	(%) of database isolates assigned to each source category	
	Human (52)	Non human (82)
Human	73.1	18.3
Nonhuman	26.9	81.7

n = 134; ARCC = 78.4%; classification with cross validation ARCC = 76.1%.

water and fish isolates were classified poorly at 15.4%. 22.2% and 23.0%, respectively.

DISCUSSION

The aim of this study was to differentiate *Escherichia coli* recovered from various sources including the environment within the Lake Victoria basin of Kenya. A total of 134 *E. coli* isolates were recovered from eight known host sources along the shores of Lake Victoria and phenotypic approaches employed to discrimimate *E. coli* sources. The study found that other than chloramphenicol and gentamicin, all the other antibiotics tested showed significant difference among the disk diffusion zone distributions among the sources ($p < 0.0001$). There could be many factors contributing to this observation, like indiscriminate use of antibiotics by patients visiting the Kisumu District hospital where specimen were collected from, natural occurrence of some antibiotics in the environment like soil (Rysz and Alvarez, 2004) and exchange of extrachromosomal material in the environment (Andersson and Hughes, 2011).

Based on the MAR indexing of *E. coli* and antibiogram patterns observed in this study, the results show that human isolates have higher rates of antimicrobial resistance which agrees with other studies conducted within the Lake Victoria basin (Brooks et al., 2001; Brook et al., 2003). However livestock showed low MAR indices as compared to other studies elsewhere (Krumperman, 1983; Sayah et al., 2005). In fact in this study, out of 23 *E. coli* isolates recovered from cattle, no multiple resistance was observed, whereas fish and soil isolates showed intermediate indices of 0.24 and 0.22, respectively. The low antimicrobial resistance profile observed among isolates recovered from cattle could be due to the fact that cattle farmers in the study site do not practice in intensive farming. Therefore the animals are not exposed to antibiotics as growth promoters. The intermediate MAR indices among fish and soil cannot be explained by this study and calls for more investigations.

However using the MAR indices, at least the *E. coli* sources could be classified into four groups, one group consisting of human isolates with the highest levels of resistance 0.55. This group consists of isolates with high levels of multiple antimicrobial resistance of over 40%. A second group that consisted of fish and soil isolates with MAR indices of 0.24 and 0.22, respectively, the group had multiple antimicrobial resistance of between 30 to 39%. The third group consisted of water, chicken, goat and donkey isolates with MAR indices of 0.17 and 0.1 for water and chicken sources and 0.07 for the latter two. These sources have multiple antimicrobial resistance ranging between 12.5 to 23.1%. The last group was that of cattle isolates with MAR indices of 0.04; this group did not record any multiple antimicrobial resistance. The low occurrence of MAR indices among livestock within the Lake Victoria basin compared to those reported in developed countries (Krumperman, 1983; Sayah et al., 2005) could be an indication of low use of antibiotics among livestock in this region. However the difference among the members of the livestock group sampled in this study could be attributed to the different environments where

the animals find food. Goats, chicken and donkey feed around the human settlements, whereas cattle graze away in the fields not close to human settlements.

According to Krumperman (1983), using MAR indexing, *E. coli* isolate can be categorized into two groups based on risks and public health concerns. Sources with MAR indices with 0.2 and above can be classified as high risk food sources whereas those below 0.2 as low risk food sources. In this study; fish products, within the Lake Victoria basin could be classified as high risk food source. The classification of fish and soil together using the MAR indices, serves to augment the finds by Abila and Jensen (1997), that poor fish handling practices such as drying fish on top of soil, and absence of acceptable sanitary condition along the fishing landing ports a long Lake Victoria may be responsible for high fish post harvest losses.

By using DA with the 134 *E. coli* isolates, the average rate of correct classification (ARCC) for all isolates was 41%. Among the eight sources, chicken, water and fish were classified poorly (15.4, 22.2 and 23.0%, respectively), most likely because of their disk diffusion zones distribution, which may have been influenced by the environmental interactions with the host groups. Most of the fish isolates were misclassified into soil category because these groups displayed similar MAR profiles and antimicrobial susceptibility inhibition zones, further supporting the earlier argument that poor fish handling and sanitary practices may be responsible of fish contamination.

When all isolates were reclassified into five groups by grouping all the domesticated animals as livestock, the ARCC improved to 52.2% with cross validation. However, fish and water were misclassified (38.1% and 33.5%). When classified into two groups namely human and non-human, the ARCC improved to 76.1%. The study demonstrates that the average rate of correct classification increased with reduction of number of groups to be discriminate analysis. Kaneene et al. (2007) also demonstrated that ARCC improved by reducing the numbers of species classifications and antimicrobial agents.

Conclusion

The results of this study confirm the occurrence of multiple antibiotic resistant *E. coli* strains from different sources within the Lake Victoria basin, which is a major public health threat worldwide. Using the two phenotypic approaches, the study was able to discriminate *E. coli* isolates from the different sources sampled in this study. The MAR indexing was able to classify the sources based on the levels of multiple resistance to antibiotics, whereas DA utilized disk diffusion zone expressed by *E. coli* isolates from the different sources. However it appears that including a large number of isolates within the database may be necessary to improve its average rate of correct classification of isolates by their sources. The findings from this study appear to point that soil may be an important source of bacterial contamination of fish products within the Lake Victoria basin and human an important reservoir of multiple antibiotic resistant *E. coli* strains.

The study therefore recommends the adoption of the two techniques to aid in risk assessment, establishment of relationships and interaction of indicator organisms like the *E. coli* among the different food sources within the Lake Victoria region. The two techniques are cheap and can easily be employed within region.

To control the continued emergence of antibiotic resistance within the Lake Victoria basin, studies with comprehensive collection of samples targeting more sources are urgently required to enhance our understanding of mechanisms involved in the dissemination of resistance to antimicrobial agents.

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Full Length Research Paper

Evaluation of antibacterial activity of *Laurus nobilis* L., *Rosmarinus officinalis* L. and *Ocimum basilicum* L. from Northeast of Algeria

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***Rosmarinus officinalis* L., *Laurus nobilis* L. and *Ocimum basilicum* L. are widespread herbs in Algeria. The essential oils of the three species were extracted from leaves by hydrodistillation. The yields were respectively 0.36, 0.6 and 0.71%. The aim of this study was to evaluate the antibacterial activity of these essential oils against twenty bacterial strains: *Enterococcus faecalis ATCC 29212, Staphylococcus aureus* ATCC 25923, MRSA ATCC 31 *(Méthicilino), Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus avium, Escherichia coli* ATCC 25922*, Salmonella* OMA 04, *E. coli, Klebsiella oxytoca, Klebsiella pneumoniae, Proteus mirabilis, Enterobacter* sp., *Citrobacter freundii, Pseudomonas aeruginosa, Acinetobacter baumanii, Serratia marcescens, Salmonella* sp., *Shigella* sp. and *Providencia alcalifaciens.* The essential oils were used in different concentrations. The diffusion tests on solid medium were efficient in all tested bacterial strains except *Pseudomonas aeruginosa*. The activity was more pronounced with the essential oil of Laurel. Indeed, the results of diffusion tests showed zones of inhibition as follows: Laurel, 8.4 to 22.4 mm; Rosemary, 8.4 to 16.4 mm and Basil, 7 to 19.9 mm. This study shows bacteriostatic effect of the three oils on all tested bacteria. The minimum inhibitory concentration (MIC) was determined by the dilution on solid medium method.**

Key words: *Rosmarinus officinalis* L., *Laurus nobilis* L., *Ocimum basilicum* L., essential oils, antibacterial activity, minimum inhibitory concentration (MIC), Algeria.

INTRODUCTION

In recent years, multiple antibiotic resistances of pathogenic bacteria have been exacerbated by the excessive and inappropriate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases (Davis, 1994; Service, 1995). Renewed interest has grown in medicinal plants to counter resistance and find an alternative to antibiotics (Kalemba and Kunika, 2003; Juliani and Simson, 2002;

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Falerio et al, 2003).

Medicinal plants contain many phytochemicals composants such as flavonoids, alkaloids, tannins and essential oils. Among these plants, *Rosmarinus officinalis*, *Ocimum basilicum* and *Laurus nobilis* are widespread in the Mediterranean Basin (Quezel and Santa, 1963).

The three plants were harvested in El Kala National Park ranked Biosphere Reserve by UNESCO in 1990. Its area is 76,438 Ha (Aouadi, 1989). It has a rich flora of about 850 species (De Belair, 1990) and characterized by a sub-humid Mediterranean climate.

Ocimum basilicum and *Rosmarinus officinalis* belong to Lamiaceae that include the most commonly used medicinal plants in the world as a spice and as a source of extract with strong antibacterial and antioxidant properties.

Rosemary is a shrub (Atik Bekkara et al., 2007), with 0.8 to 2m height (Trujano-Gonzalez et al., 2007) and rich in essential oils (1 to 2.5%). It has three chemotypes: cineol, camphor and verbenone (Santoyo et al., 2005; Graven et al., 1992); it contains also triterpene derivatives (2-4%), flavonoids, tannins and saponins.

Basilicim Ocimum (Basil) is an aromatic plant (20 to 60 cm high), used as antispasmodic, aromatic, carminative, digestive, galactagogue, stomachic and tonic (Chiej, 1984; Lust, 1983; Duke and Ayensu, 1985). It contains 0.4 to 0.7% of essential oil, phenolic acids like rosamarinic acid, lithospermic acid B, vanillic acid, hydroxybenzoic acid, syringic acid, ferulic acid, protocatechuic acid, caffeic acid and gentisic acid, chicoric acid (Bais et al., 2002; Lee and Scagel, 2010); flavonoids and tannins (Grayer et al., 1996); cinnamic acid ester, triterpenoids and steroidal glycosides (Siddiqui et al., 2007).

Laurus nobilis belongs to Lauraceae. This aromatic tree is 2 m to 10 m high, it contains about 1.3% essential oils and polar flavonoids mono and sesquiterpenes (Novák, 1985; Appendino et al., 1992; Dall'Acqua et al., 2006), alkaloids (Kivçak and Mert, 2002), glycosylated flavornoids (Fiorini et al., 1998) and megastigmane and phenolic components (De Marino et al., 2004). It is known to have various pharmacological effects, including antimicrobial (Fraga, 2003), cytotoxic (Barla et al., 2007) and immune modulating (Park et al., 1996) activities.

The aim of this work was to evaluate the antibacterial activity of essential oils of these three plants against 20 bacterial strains: *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, MRSAATCC 31 *(Méthicilino), S. aureus, Staphylococcus epidermidis, Enterococcus avium, Escherichia coli* ATCC 25922, *Salmonella* OMA 04, *E. coli, Klebsiella oxytoca, Klebsiella pneumoniae, Proteus mirabilis, Enterobacter* sp., *Citrobacter freundii, Pseudomonas aeruginosa, Acinetobacter baumanii, Serratia marcescens, Salmonella* sp., *Shigella* sp. and *Providencia alcalifaciens.*

MATERIALS AND METHODS

Plant material

The leaves of *Laurus nobilis* L., *Rosmarinus officinalis* L. and *Ocimum basilicum* L. were collected in April 2012 at El Kala National Park located at latitude 36° 52' north and longitude 8° 27' East. Laurel is spontaneous in the park; however, Basil and Rosemary are cultivated. The plants were dried in the shade in order to preserve the integrity of their molecules.

Extraction of essential oils

The extraction of essential oils was carried out by hydro-distillation using Likens Nickerson apparatus for 2 h. We introduced 100 g of dry leaves in a flask filled with 3/4 distilled water and then heated to boil. The water and oil are separated during the condensation of vapour loaded onto the oils (Chiej, 1984). Essential oils have been recovered in small opaque bottles and kept away from light, at a temperature of 4°C. The yield was expressed in percentage.

Evaluation of antibacterial activity

Bacterial strains

The bacterial strains tested were provided by the Laboratory of Medical Microbiology, Faculty of Medicine Annaba. They are: Gram-positive Enterococcus faecalis ATCC 29212, *Staphylococcus aureus* ATCC 25923, MRSAATCC 31 (Méthicilino), *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus avium.*

Gram-negative Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Salmonella OMA 04, Escherichia coli, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterobacter* sp, *Citrobacter Freundii*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Serratia marcescens*, *Salmonella* sp, *Shigella* sp *Providencia alcalifaciens*. The bacterial strains were maintained and grown in a nutrient agar medium.

Disk diffusion method

The antibacterial activity was tested using the disk diffusion method (Davis, 1994). Bacterial cultures were reactivated by sub culturing on nutrient agar and incubated for 24 h at 37°C. From these, pure cultures were prepared by releasing bacterial inoculum strains in physiological water. The homogeneous suspension was equivalent to 0.5 Mc Farland, so an OD of 0.08 to 0.10 was read at 625 nm.

Each essential oil was used at different concentrations: pure oil, diluted oil in DMSO (Dimethyl sulfoxide) to ratio 1/2, 1/4 and 1/8. Discs of 6 mm in diameter, previously sterilized, were used. 10 µl of essential oils was put on each disc and placed on agar. A witness disc (soaked in DMSO) was incubated under the same conditions to ensure that DMSO was devoid of antibacterial activity.

After incubation for 24 h in an oven at 37°C, reading was done. The effect of essential oils on bacteria was estimated by the appearance of clear zones around the discs. The diameter of the halo of growth inhibition was measured and expressed in mm (including the diameter of the disc of 6 mm).

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) is the smallest concentration of essential oil, in which no growth is visible compared to the control without extract. It was evaluated on twelve tested strains by disc diffusion test. We used the dilution method on solid medium (incorporation) (Billerbeck et al., 2002; Marino et al.,

2001).

Serial dilutions of essential oils were performed with DMSO for 2 h. Each dilution was incorporated into *Mueller-Hinton* medium, maintained, super cooled and poured into Petri dishes. The concentrations (in percent), of essential oils used are respectively: 1, 0.5, 0.25, 0.01, 0.125, 0.06 and 0.03. Witness discs containing culture medium and only DMSO were also prepared.

Seeding was done as a deposit of bacterial suspension. After incubation at 35°C for six days, the growth was compared to the control.

RESULTS AND DISCUSSION

Essential oil yield

The yields of essential oils from the dry matter of *Rosmarinus officinalis* L.*, Laurus nobilis* L*. and Ocimum basilicum* L. were respectively 0.36, 0.6 and 0.71%. These results are lower than those found in other regions of Algeria. The yield of Rosemary in Algiers found by Djeddi (2007) was 0.82% and Laurel in Tlemcen was 1.2% (Haddouchi et al., 2009). These differences may result from the high moisture that characterizes the study's area; because it is known that maximum yields are obtained by dry weather. Concerning Basil, the yield obtained is normal because it develops in its natural habitat. Harvesting for the three plants was conducted during the vegetative stage, which has generated a relatively low yield (Bruneton, 1993).

Antibiotic activity

The antibacterial activity of the three essential oils and MIC values are grouped in Tables 1 and 2.

All organisms are sensitive to the three oils except *P. aeruginosa* (Gram negative) which is more and more responsible for nosocomial infections. It has an intrinsic resistance to a wide range of antibiotics (April et al., 1992) and also to essential oils. This resistance is due to the impermeability of the wall of this bacterium (Djeddi et al., 2007; Dorman and Deans, 2000; Duke and Ayensu, 1985).

The essential oil of *L. nobilis* L. has demonstrated a strong activity on the majority of tested strains; the highest sensitivity was in *Enterobacter* sp. that has an inhibition diameter of 22.4 mm, 16.8 mm pure oil and 1/8 dilution. The most resistant strain was *P. aeruginosa.* These results are in concord with those of Dadalioglu and Evrendilek (2004). 1,8 cineole has a part in this activity since it has antimicrobial activity against several strains such as *E. coli, P. aeruginosa* and *Staphylococcus aureus* (Sivropoulou et al., 1997).

The synergy between terpenes (linalool), lactones, oxides (1, 8 cineole) and monoterpenes (camphene, alpha-pinene) gives to the essential oil of Laurel a good antibacterial activity. The MIC equals 2.72 (10^3 micrograms/ml) except in *E. faecalis* ATCC 2921, *S. aureus, S. epidermidis, P. mirabilis and S. marcescens* where it was 1.36 (10^3 micrograms / ml).

The essential oil of *Rosmarinus officinalis* also has an inhibitory power. The most sensitive strain is *Shigella sp.* (16.4 mm and 11.3 mm). The MIC values are quite high, ranging from 3.43 (10^3 mg / ml) in *E. faecalis ATCC 29212, S. Aureus, S. Epidermidis, E. coli ATCC 25922 Proteus mirabilis, C. freundii, S. marcescens* and *Shigella* sp. to 6.85 (10^3 mg / ml) in *Salmonella OMA 04 Enterobacter* sp., *A. baumannii* and *M.R.S.A ATCC31*.

Our results are in agreement with those found by other authors such as Santoyo et al. (2005), Faleiro et al. (2003) and Gachka (2007) Fiorini et al. (1998) with respect to the resistance of *Pseudomonas aeruginosa* against this oil. Celikta (2007) found a moderate activity against *E. faecalis* and *Proteus* sp., however Jiang et al. (2011) obtained pronounced antibacterial activity.

Santoyo et al. (2005) and Graven (1992) attributed the antimicrobial properties of the essential oil of *R. officinalis* to the presence of α-pinene, 1,8-cineol, borneol and camphor. Even minor components have a significant contribution to the antibiotic activity (Wang et al., 2012).

Finally, with the essential oil of *Ocimum basilicum,* we found good inhibition zones. The most sensitive bacterial strain (*Shigella* sp.) presented an inhibition diameter ranging between 12.2 mm and 19.9 mm.

Suppakul et al. (2003) reported, also, that Basil has good antimicrobial activity against a wide range of microorganisms. This activity is due in part to the presence of linalool (Koutsoudaki et al., 2005; Sartoratotto et al., 2004; Sokovic and Van Griensven, 2006; Suppakul et al., 2003). The MIC is equal to 9.5 (10^3 mg/ml).

This study shows that Gram negative bacteria and Gram positive bacteria are both sensitive to the three essential oils.

It is known that Gram negative bacteria are more resistant to essential oils than Gram positive bacteria (Loäpez et al., 2005; Marino et al., 2001). This resistance is due to the nature of these group of cellular membranes of bacteria, because their external structures make them to have highly hydrophobic surfaces (Smith-Palmer et al., 1998). One important characteristic of essential oils and their components is their hydrophobicity, which allows them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and making them more permeable (Sikkema et al., 1994).

Dorman and Deans (2000) indicate that the antimicrobial activity depends, not only, on the chemical composition of the essential oil, but also on lipophilic properties and power of functional groups or aqueous solubility. The mixture of compounds with different biochemical properties can improve the effectiveness of essential oils.

Conclusion

R. officinalis L., *Laurus nobilis* L. and *O. basilicum* L. are

Table 1. Diameter of inhibition of essential oils against the bacterial strains (mm).

Bacterial strain	*Laurus nobilis* L				*Rosmarinus officinalis* L				*Ocimum basilicum* L			
	B	1/2	1/4	1/8	B	1/2	1/4	1/8	B	1/2	1/4	1/8
E. faecalis ATCC 29212	12.5	11.3	11.3	11.1	11.3	10.3	12.5	9.6	9.4	9.4	11.2	11
S. aureus ATCC 25923	15	9.1	8.3	7.4	14	12.4	8.7	8.4	9.75	9.7	9.5	9
M.R.S.A ATCC 31	17.6	13.3	9.5	9.3	14.3	11.4	11.2	11	13.5	12.2	11.2	10.1
S. aureus	15.6	12.6	10.3	9.9	12.9	11.5	10.7	10.1	16	14.5	13.5	11.1
S. epidermidis	13.1	12	8.4	8.2	12.7	10.8	10.8	10.6	12.9	13.3	10.8	9.4
Enterococcus avium	12.8	12.1	10.7	10.3	10.4	8.2	8.1	8.1	10.2	10.1	9.4	9
E. coli ATCC 25922	15.9	12.05	11.85	11.75	13.5	10.2	10.3	8.9	13.8	13.5	11	10.7
Salmonella OMA 04	14	11.2	9.1	/	11.5	9.8	9.8	9	12	9.8	9.2	8.9
E. coli	14.1	11.5	11.3	11	15.9	12.15	11.45	11	11.8	10.65	9.05	9
Klebsiella oxytoca	18	13.05	13.35	11.2	11	13.25	11.6	8.85	10	10.2	9.45	8.5
Klebsiella pneumoniae	17.6	15.6	13.7	13	12	11.6	11.3	10.8	18.8	17.2	13.3	10.1
Proteus mirabilis	16.25	12.6	11.45	10.7	11.1	9.2	12.9	9.8	12.6	11	13.1	11.3
Enterobacter sp.	22.4	22	20.4	16.8	12.7	11	9.4	9	13.6	13.1	13	11.3
Citrobacter Freundii	15	14.3	13.1	12.2	8.7	8.3	8.2	8.2	8.4	8.2	7.3	7
P. aeruginosa	/	/	/	/	/	/	/	/	/	/	/	/
Acinetobacter baumannii	16.8	16	14.1	12.5	9.2	9.1	9.1	9	14.5	11.3	9.5	9.4
Serratia marceseus	16.4	12.1	11.8	10.3	11.5	9.3	8.9	8.4	12	10.4	9.2	8.7
Salmonella sp.	17.1	14.1	11.5	10.8	12.5	11.6	11.4	10.4	15.3	10.8	10.7	10.1
Shigella sp.	21.1	19.3	18.4	16.3	16.4	14.3	12.6	11.3	19.9	13.7	12.4	12.2
Providencia alcalifaciens	16.2	14.4	13.2	11.1	11.4	10.5	10.2	10.1	16	12.1	11.3	9.8

Table 2. Activity of essential oils incorporated in the solid medium (MIC).

Percentage	1%			0.5%			0.25%			0.125%		
Extract (H.E)	B	R	L	B	R	L	B	R	L	B	R	L
Concentration (10^3 µg/ml)	9.5	6.85	5.4	4.95	3.43	2.72	2.47	1.71	1.36	1.23	0.85	0.68
Gram positive												
E. faecalis ATCC 29212	-	-	-	-	-	-	+	+	-	+	+	+
M.R.S.A ATCC 31	-	-	-	+	+	-	+	+	+	+	+	+
S. aureus	+	-	-	+	-	-	+	+	-	+	+	+
S. epidermidis	-	-	-	+	-	-	+	+	-	+	+	+
Gram negative												
E. coli ATCC 25922	-	-	-	+	-	-	+	+	+	+	+	+
Salmonella OMA 04	-	-	+	+	+	+	+	+	+	+	+	+
Proteus mirabilis	-	-	+	+	-	+	+	+	+	+	+	+
Enterobacter sp.	+	-	-	+	+	-	+	+	-	+	+	+
Citrobacter Freundii	-	-	+	-	-	+	+	+	+	+	+	+
Acinetobacter baumannii	+	-	+	+	+	+	+	+	+	+	+	+
Serratia marcescens	-	-	-	-	-	-	-	+	+	+	+	+
Shigella sp.	-	-	-	-	-	-	-	+	-	+	+	+

-: No culture; +: presence of culture

widespread herbs in Algeria. The samples used have been harvested in the National Park of El Kala where Basil and Rosemary are cultivated and Laurel is spontaneous. The essential oils from leaves of Rosemary, Laurel and Basil were extracted by hydro-distillation using Likens Nickerson apparatus for 2 h.

The aim of this study was to evaluate the three essential oils against 20 bacterial strains. For 12 strains we have determined the minimum inhibitory concentration (MIC). The three oils showed good antibacterial activity against both Gram negative and Gram positive bacteria. Laurel oil is the most efficient, *Shigella* sp. has

the highest sensitivity to the three oils and *Pseudomonas aeruginosa* is the most resistant to them. Among the three oils, Laurel gives the lowest MIC against *E. faecalis* ATCC 29212, *Enterobacter* sp., *Shigella* sp., *S. aureus* and *S. Epermidis* (0.25%).

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Full Length Research Paper

Investigations on aflatoxigenic fungi and aflatoxins contamination in some nuts sampled in Algeria

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In this study, 40 nuts samples (almonds, pistachios, hazelnuts, peanuts and walnuts) available in the Algerian market were investigated for aflatoxigenic fungi and aflatoxins contamination. An evaluation of mould biodiversity revealed *Aspergillus* link to be the most frequent genus, while species belonging to the section *Nigri* (30.6%) and to the section *Flavi* (27.9%) were predominantly isolated. 78.6 and 52.4% of the 420 isolates of *Aspergillus* section *Flavi* examined produced cyclopiazonic acid (CPA) and aflatoxins (AFs), respectively. The majority of the aflatoxigenic strains (90.5%) were identified as *Aspergillus flavus*. In 90% of the nuts samples, aflatoxins were detected by HPLC with widely fluctuating contamination levels. Their concentrations ranged from 0.2 to 25.82 µg/kg. The highest levels were found in peanuts, pistachios and walnuts.

Key words: Aflatoxins, Algeria, *Aspergillus* section Flavi, nuts.

INTRODUCTION

Risk of *aflatoxin* (AFs) contamination hits top values in such commodities as nuts. *Aspergillus flavus* and *A. parasiticus* are the most important aflatoxigenic species naturally occurring in agricultural commodities (Pildain et al., 2008; Pitt and Hocking, 2009). These species are distributed worldwide in soil and air, and they can primarily colonize plants in the field and secondarily transfect harvested or stored plant products. Favorable temperature and humidity join inadequate storage practices contributing to mycotoxin contamination of foods and their spoilage. Toxigenic species diagnoses are important, since they could give an indication of the future potential due to the presence of mycotoxins.

Aflatoxins incidence in foods and feeds is closely monitored and regulated in more than 100 countries. Legal limits of *aflatoxin* B1 (AFB1) and total AFs for peanuts and nuts in Algeria are 10 and 20 µg/kg, respectively (FAO, 2004). The purpose of this study was to analyze the incidence and biodiversity of aflatoxigenic species belonging to *Aspergillus* section *Flavi*, as well as to survey current aflatoxins levels in Algerian commercialized nuts. Our aim was to achieve an evaluation of the health risk regarding the consumption of these products.

MATERIALS AND METHODS

Sample collection

A total of 40 nut samples (eight for each type of nut) composed of pistachios, roasted hazelnuts, shelled almonds, shelled peanuts and unshelled walnuts were purchased from retail shops and local markets of different locations of Algeria during March 2010. Almonds and peanuts samples were taken from retail already shelled. For pistachios, the shell is ajar, therefore it is highly

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vulnerable to contamination. 500 g of representative samples were taken from each collected sample after through mixing. The samples with shells (pistachios, roasted hazelnuts and walnuts) were manually shelled after disinfection with alcohol 70%. Finally, 200 g of each sample were finely powdered by a brief high speed Waring Blender in order to avoid sample overheating. Aliquots of 100 g of nut were used for the mycological analysis, and the remainder was stored at -20°C for the aflatoxins analysis.

Standard and reagents

All reagents (potassium chloride, phosphoric acid, hydrochloric acid) were of pro analysis (PA) grade. All solvents (methanol, acetonitrile, *n*-hexane, chloroform) were of high-performance liquid chromatography grade. They were purchased from Merck (Darmstadt, Germany). Deionized water was used for the preparation of all aqueous solutions and for HPLC. Standard toxins, aflatoxins (AFB1, AFB2, AFG1, AFG2) and cyclopiazonic acid (CPA), and Ehrlich's reagent (1 g of 4-dimethyl-aminobenzaldehyde in 75 ml ethanol and 25 ml concentrated HCl) were supplied by Sigma Chemicals (France). The working solutions were prepared according to the AOAC procedure (AOAC, 2000).

Isolation and identification of aflatoxin-producing fungi

Fungal isolation and culture conditions

Dilution plating (surface-spread method) (Pitt and Hocking, 1997) was used for colony counting. 10 g of each milled nuts sample were homogenized in 90 ml 0.1% peptone-water solution for 30 min in an orbital shaker. Serial decimal dilutions up to 10^{-4} were made and 0.1 ml aliquots were inoculated in triplicate onto the Dichloran Rose-Bengal Chloramphenicol Agar medium (DRBC) (King et al., 1979). All Petri dishes were incubated for 3 to 7 days at 28°C in the dark. Stock cultures of the representative strains were maintained for further examination in 20% glycerol at - 20°C.

Morphological characterization of the isolates

For each isolate, spores were suspended in 500 µL of 0.2% agar, and this suspension was used for inoculations on 9 cm diameter Petri dishes containing 20 mL of Malt Extract Agar (MEA) (malt extract, 20 g; glucose, 20 g; peptone, 1 g; agar, 20 g; distilled water, 1000 ml; pH, 6.5) and Czapek-Dox Agar (CZ) (sucrose, 30 g; K_2HPO_4, 1 g; $NaNO_3$, 2 g; KCl, 0.5 g; $MgSO_4 \cdot 7\ H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $ZnSO_4 \cdot 7H_2O$, 0.01 g; $CuSO_4 \cdot 5H_2O$, 0.005 g; Agar, 20 g; distilled water, 1000 ml; pH, 6.2). Cultures were incubated for seven days in the dark, at 25°C, and then analyzed for colony color, eventual presence, color and size of sclerotia, head seriation and conidial morphology. Identification followed the taxonomic keys and guides available for the *Aspergillus* genus (Pitt and Hocking, 1997). Isolates were also cultured on CZ medium at 42°C, and colony diameter was measured after 7 days of incubation (Ehrlich et al., 2007; Kurtzman et al., 1987).

Aflatoxigenic ability of the isolates

For a preliminary screening of aflatoxins production, strains were inoculated at a central point on a 6 cm diameter Petri dish containing 10 ml of Coconut Agar Medium (CAM) supplemented with 0.3% β-cyclodextrin (Fente et al., 2001), and incubated for 5 days in the dark at 28°C. Cultures were tested for 365 nm UV light fluorescence and for bright orange-yellow colony reverse coloring expression under daylight. Thin layer chromatography (TLC) was used as a screening method to confirm the positive samples essentially as described by Calvo et al. (2004). The limit of detection was 50 ng/ml. Aflatoxins quantification was determined using High Performance Liquid Chromatography (HPLC). A post-column derivatization electrochemically generated bromine (Coring Cell) and a fluorescence detector (Spectra Physic 2000) with 362 nm for excitation, and 435 nm for emission) were used. The HPLC column used was a reverse phase RP C18 ProntoSil analytical column (250 x 4 mm, 3 µm particle size) preceded by a C18 pre-column (Ultrasep 10 x 4 mm). The mobile phase consisted of distilled water, acetonitrile, methanol (6:2:2, v/v/v) with 119 mg/l of KBr and 110 µl/l of 65% HNO_3. The injection volume was 20 µl and flow rate was 1 ml/min.

Cyclopiazonic acid detection

The isolates were tested for cyclopiazonic acid (CPA) production on CYA medium following the method described by Pildain et al. (2004). To determine the detection limit, a series of different concentrations (0.5, 1, 10, 25 and 50 µg/ml) of CPA dissolved in methanol was prepared and a volume of 20 µl of each was applied to a silica-gel, which was previously impregnated with a solution of oxalic acid (2% in methanol) for 2 min and dried. The plates were run in the same direction with ethyl acetate, 2-propanol, ammonium hydroxide (45:35:20, v/v/v). After pulverization of the plates with Ehrlich's reagent, the CPA was detected under daylight as an intense purple spot. The detection limit of the TLC technique was 1 µg/g.

Extraction of AFs from nut samples

Aflatoxins levels were determined according to the methodology proposed by El Adlouni et al. (2006) and Nguyen et al. (2007). A sub-sample of 20 g of thoroughly homogenized nuts was finely powdered and added to 20 ml of 4% potassium chloride solution acidified to pH 1.5 with sulfuric acid. The mixture was homogenized and extracted with 180 ml acetonitrile on an orbital shaker for 20 min, and filtered through Whatman no 4 filter paper.

Purification of the extract

The *n*-hexane (100 ml) was added to the filtrate and shaken for 1 min. After separation, the upper phase (*n*-hexane) was discarded. 50 ml of deionized water and 100 ml of chloroform were added to the lower phases. The mixture was shaken for 10 min and, after separation, the lower phase (chloroform) was collected. The upper phase was re-extracted three times with 20 ml of chloroform using the above conditions. Then, 50 ml of 5% sodium bicarbonate was added and shaken for 10 min to the pooled chloroform extracts. The upper phase (bicarbonate) was collected, acidified to pH 1.5 with concentrated hydrochloric acid and allowed to stand about 20 min. The acidified solution was extracted three times with chloroform (100, 50 and 50 ml).

The pooled chloroform phases were evaporated to near dryness under vacuum using a rotary evaporator placed in a 40°C water bath. The extract was re-suspended in 1 ml of methanol, sonicated and filtered through a 0.2 µm Minisart cartridge (Sartorius AG Goettingen, Germany). The analysis was performed using the previously described method.

Recovery experiments

Recovery experiments were performed by spiking aflatoxin-free peanut, almond and pistachio samples (20 g of ground sample) with two concentration levels (5 and 20 µg/kg) with AFB1, AFG1, AFB2

Table 1. Occurrence of moulds*, *Aspergillus*, *Aspergillus* section *Flavi* and *Aspergillus* section *Nigri* in 40 samples nuts collected
from markets in Algeria.

Nuts (n=8)	Total number of fungi ± SD (cfu/g)	*Aspergillus* (%)[a]	*Aspergillus* section *Flavi* (%)[b]	*Aspergillus* section *Nigri* (%)[b]
Pistachios	$16 \times 10^4 \pm 13.7 \times 10^3$	99.3	34.0	55.3
Roasted hazelnuts	$6 \times 10^2 \pm 2.8 \times 10^2$	66.0	38.3	25.3
Shelled almonds	$38 \times 10^3 \pm 2.3 \times 10^3$	99.8	15.4	64.4
Shelled peanuts	$16 \times 10^3 \pm 1.3 \times 10^2$	57.0	38.1	5.0
Unshelled walnuts	$4 \times 10^3 \pm 3 \times 10^2$	18.0	13.8	3.1
Mean		68.0	27.9	30.6

SD, standard deviation; [a]Calculated as a percentage of the total fungi; [b] Calculated as a percentage of the total *Aspergillus*. *The commonly isolated fungi were species of *Aspergillus*, *Penicillium* and *Mucor*.

and AFG2. Spiking was carried out in triplicates and a single analysis of a blank sample was also carried out. Aflatoxins concentrations were determined by HPLC analysis using the previously described method.

RESULTS

Mycological analysis

The fungal strains isolated from 40 samples of nuts (pistachios, roasted hazelnuts, shelled almonds, shelled peanuts and unshelled walnuts) collected from Algiers retail shops and local markets are shown in Table 1. The mean counts of the fungal colonies ranged from 6×10^2 to 16×10^4 cfu/g. Pistachios, shelled almonds, shelled peanut and unshelled walnuts were highly contaminated by total fungi. The commonly isolated fungi were species of *Aspergillus*, *Penicillium* and *Mucor*.

The most repre-sented genus in contaminated samples was *Aspergillus*, which was isolated in all analyzed samples with the mean percentage of 68.0%. The species isolated belonged to the *Aspergillus* section *Nigri, Flavi, Circumdati* and *Terrei.* Highest frequencies were recorded in shelled almonds (99.8%) and pistachios (99.3%).

Distribution and morphological characterization of isolates of *Aspergillus* section Flavi

Regarding *Aspergillus* section *Flavi* isolation, the mean percentage found was 27.9% of the total *Aspergillus* statistic. Colonization by species belonging to *Aspergillus* section *Flavi* was higher in roasted hazelnuts (38.3%), shelled peanut (38.1%) and pistachios (34%) (Table 1). Based on morphological characteristics, we found three distinct morphotypes among the 420 isolates studied. The morphotype 1 (386 isolates, 92%) represents yellow-green colonies and smooth to finely rough globose conidia. The morphotype 2 was represented by four isolates (1%) with dark-green colonies and rough conidia. Colonies of the two morphotypes of isolates tinged of a bright orange died on the reverse side of *Aspergillus flavus/A. parasiticus* Agar (AFPA) plates and could grow at 42°C. The isolates of morphotype 3 (30 isolates, 7.2%) showed dark-brown color producing ornated and brown conidia on AFPA medium; these isolates could not grow at 42°C.

Aflatoxins production by isolates of *Aspergillus* section Flavi

The incidence of aflatoxigenic strains is shown in Table 2. Among 420 isolates, 220 (52.4%) were aflatoxigenic. The incidence of aflatoxigenic strains was 56.2, 51.8, 44, 40 and 30% for pistachios, roasted hazelnuts, shelled almonds, shelled peanuts and unshelled walnuts, respectively. Analysis of aflatoxins production by fluorescence in CAM showed a good correlation with the TLC results.

Indeed, we found that all strains producing blue fluorescence pattern on CAM with brilliant orange-yellow reverse coloration under daylight showed an intense blue and green fluorescence spot for AFB and AFG, respectively. All of the morphotype 2 (4 isolates) were found to be strongly aflatoxigenic. The isolates belonging to the morphotype 1 with small “S” sclerotia (< 400 µm), were stronger producers than large”L” sclerotia (> 400 µm) isolates. The isolates of morphotype 3 were non-aflatoxigenic.

Chemotypes in *Aspergillus* section Flavi isolates

Based on mycotoxin production patterns (AFB, AFG and CPA) and sclerotia size, the 420 strains were classified into seven chemotypes (Table 3). Chemotype I (30.7%) for CPA producers; (ii) chemotype II (19.3%) for non-producers with “L” sclerotia; (iii) chemotype III (39.3%) for AFBs and CPA producers; (iv) chemotype IV (1.2%) for

Table 2. Occurrence and aflatoxins-producing ability of 420 isolates of *Aspergillus* section *Flavi* isolated from nuts collected from markets in Algeria.

Nut	Number of strains	Number of aflatoxigenic strains[a] (%)
Pistachios	130	73 (56.2)
Roasted hazelnuts	85	44 (51.8)
Shelled almonds	150	66 (44)
Shelled peanuts	55	22 (40)
Unshelled walnuts	50	15 (30)
Total	420	220 (52.4)

[a] For a preliminary screening of aflatoxins production, cultures were observed for fluorescence on CAM under long-wave UV light (365 nm) after 3, 5 and 7 days and then confirmed by TLC. The limit of detection (LOD) of TLC method for AFB and AFG was 50 ng/ml.

Table 3. Chemotype patterns of *Aspergillus* section *Flavi* strains isolated from nuts collected from markets in Algeria based on aflatoxins and cyclopiazonic acid producing ability, and on sclerotia size.

Chemotype	Mycotoxin			Fluorescence on CAM and TLC (color)	Sclerotia number of strains		Percentage (%)[c]
	AFB1	AFG1	CPA				
I	-	-	+	-[a]	-	150	30.7
II	-	-	-	-	L[b]	95	19.3
III	+	-	+	++ (Blue)	-	193	39.3
IV	+	-	-	+ (Blue)	L	6	1.2
V	+	-	+	++ (Blue)	S[b]	38	7.9
VI	+	+	+	+++ (Blue; Green)	S	4	1.0
VII	+	+	-	+++ (Blue; Green)	-	4	1.0

AFB1, aflatoxin B1; AFG1, aflatoxin G1; CPA, cyclopiazonic acid. [a] +++, Strong signal; ++, medium signal; +, weak signal; −, not detected. The aflatoxigenic isolates produced amounts of AFs ranging from 0.50 to 2000 µg/g of CAM. [b]The large strain (L) having sclerotia >400 mm in diameter and the small strain (S) with sclerotia <400 mm.[c] Percentage of the 420 isolates.

AFB “L” sclerotia; (v) chemotype V (7.9%) for AFB, CPA and “S” sclerotia producers; (vi) chemotype VI (1%) for AFB, AFG, CPA and “S” sclerotia producers; (vii) chemotype VII (1%) for AFB and AFG producers. The four isolates belonging to the chemotype VII have a distinctly darker green colonies and rough conidia, which produce AFB and AFG but not CPA. Isolates belonging to chemotypes VI and VII produced greater amounts of AFs (up to 2 mg/g). The chemotype I, II, III, IV, V and VI represents the morphotype I with yellow-green colonies and smooth to finely rough globose conidia. The chemotype VII represents the morphotype 2. The isolates with dark brown (morphotype 3) were included in chemotype I.

Performance of the methods

The results of recovery of aflatoxins are summarized in Table 4. The average recoveries were between 72.6 and 91.8%. The performance characteristics were within the acceptable margins indicated in the Commission Regulation No. 401/2006 (EC, 2006) for methods of sampling and analysis for the official control of mycotoxins. The limit of detection and limit of quantification were determined by spiked nuts samples with 5 µg/kg of AFB1, AFG1, AFB2 and AFG2, based on signal-to-noise ratio of 3:1 for LOD and 10:1 for LOQ. The limit of detection was established in 0.05 µg/kg for AFB1 and AFG1, and 0.2 µg/kg for AFB2 and AFG2. The limit of quantification, were 0.1 µg/kg for AFB1 and AFG1, and 0.5 µg/kg for AFB2 and AFG2.

Aflatoxins content in nuts

Of the 40 nuts samples analyzed by HPLC, 36 (90%) were contaminated with aflatoxins (Table 5). AFB1 was detected with concentrations ranging from 0.2 to 20.52 µg/kg. The high levels of AFB1 (20.52, 8.72 and 6.34 µg/kg) and AFs (25.82, 13.45 and 8.76 µg/kg) were found in shelled peanuts, pistachios and unshelled walnuts, respectively. In almonds and roasted hazelnuts samples, only AFB1 was detected with levels ranging from 1.65 to

Table 4. Recoveries of aflatoxins B1, B2, G1 and G2 from spiked non-infected almond, peanut and pistachios samples fortified with 5 and 20 µg/kg.

Aflatoxin	Spiking level (µg/kg)	Mean recovery (%)[a] ± RSD (%)[b]		
		Almond	Peanut	Pistachio
AFB1	5	84.1 ± 2.5	86.4 ± 3.1	80.2 ± 4.1
	20	88.1 ± 4.9	87.8 ± 3.5	91.8± 2.9
AFB2	5	86.2 ± 2.7	86.2 ± 2.7	78.1 ± 2.9
	20	85.7 ± 2.1	88.3 ± 3.5	89.6 ± 1.9
AFG1	5	87.4 ± 1.8	72.6 ± 4.5	82.5 ± 4.7
	20	81.6 ± 2.5	89.3 ± 2.1	88.5 ± 2.5
AFG2	5	78.5 ± 3.2	75.7 ± 1.6	76.5 ± 5.3
	20	81.6 ± 5.6	79.2 ± 4.6	81.7 ± 2.8

[a]Number of replicates: N =3; [b] RSD, relative standard deviation.

Table 5. Occurrence of aflatoxins in nuts samples (n=40) collected from markets in Algeria and analyzed by HPLC.

Nuts sample	No. of samples (No. of positive samples)	Range of AFB1 (µg/kg)	Mean ± SD (µg/kg)	Range of AFs (µg/kg)	Mean ± SD (µg/kg)
Pistachios	8 (7)	0.28 - 8.72	4.45 ± 2.64	0.41 - 13.45	6.70 ± 3.40
Roasted hazelnuts	8 (7)	0.20 - 2.81	1.33 ± 0.88	0.20 - 2.81	1.33 ± 0.88
Shelled almonds	8 (8)	1.65 - 4.00	2.12 ± 1.56	1.65 - 4.00	2.12 ± 1.56
Shelled peanuts	8 (8)	0.20 - 20.52	6.30 ± 3.64	0.34 - 25.82	7.10 ± 3.80
Unshelled walnuts	8 (6)	0.20 - 6.34	3.42 ± 1.35	0.60 - 8.76	4.90 ± 2.44

The LOD were 0.05 µg/kg for AFB1and AFG1, and 0.2 µg/kg for AFB2 and AFG2. The LOQ were 0.1 µg/kg for AFB1 and AFG1, and 0.5 µg/kg for AFB2

4.00 µg/kg and 0.20 to 2.81 µg/kg, respectively. Of the 40 samples analyzed, two peanuts samples (11.26 and 20.52 µg/kg) were above limit as recognized in Algeria (10 µg/kg for AFB1 and 20 µg/kg for AFs) (FAO, 2004).

DISCUSSION

High contamination of the majority of nuts samples can be sustained by storage without packaging and marketing under non-hygienic conditions when temperatures reach 30°C. Our results show very high frequencies of *Aspergillus* particularly in shelled almonds (99.8%) and pistachios (99.3%). We recorded 66 and 57% in roasted hazelnuts and shelled peanut, respectively. In nuts, *Aspergillus* species, and particularly *A. flavus* and *A. Niger*, have been frequently reported (Ihejirika et al., 2005; Horn and Dorner, 2009; Rodrigues et al., 2013). Species belonging to *Aspergillus* section *Flavi*, and especially, *A. flavus* can act as an endophyte in peanuts or invade the peanut fruits (Horn and Dorner, 1998; Bankole et al., 2005). As reported by Bayman et al. (2002) and Rodrigues et al. (2013), we observed the dominance of the black *Aspergillus* strains in almonds (64.4%) and pistachios (55.3%). The association of *A. flavus* and *A. niger* group in nuts can indicate the co-occurence of mycotoxins, particularly AFs and ochratoxin A. This association between these species has been reported by Rodrigues et al. (2012). Because of the effect of roasting, roasted hazelnuts samples were contaminated with lower total number of fungi dominating by *Aspergillus* section *Flavi* (38.3%) and *Aspergillus* section *Nigri* (25.3%). Of the 420 strains examined by TLC technique, 220 (52.8%) aflatoxins producers were detected. The incidence of aflatoxigenic strains was 52.4% for nuts analyzed. The percentage of aflatoxigenic strains of *A. flavus* has been shown to vary with the nature of substrate and environmental factors (Horn, 2003; Klich, 2007). There are few studies concerning the aflatoxigenic contamination incidence in nuts comercialized in Algeria. Fernane et al. (2010a) found 56.5% aflatoxigenic isolates of *A. flavus*. For *A. flavus* isolated from pistachios samples taken in Spain, 70.8% were identified as aflatoxigenic (Fernane et al., 2010b). *Aspergillus* section *Flavi* isolates have been found to be extremely diverse in terms of aflatoxins, CPA and sclerotial production (Vaaamonde et al., 2003; Razzaghi-Abyaneh et al., 2006; Giorni et al., 2007). Strains belonging to chemotypes I, II, III and IV were classified as typical *A. flavus*. These strains represent 90.5% of a total

aflatoxigenic isolates. Thus, our results confirm that the most common aflatoxin-producing fungi belong to *A. flavus* group. Based on the sclerotia size, isolates belonging to chemotype V were classified as atypical *A. flavus.* Chemotype VI for AFB, AFG, CPA and "S" sclerotia producers have been classified as *A. minisclerotigenes* or *A. parvisclerotigenus* (Frisvad et al., 2005; Pildain et al., 2008). In this study, only four isolates of *A. parasiticus* and four isolates of "S" type strain producers AFB and AFG were isolated from peanut and pistachios. Peanuts are regarded as one of the major habitats for *A. parasiticus* (Barros et al., 2006). This species were recorded at high proportions (48% of all *Aspergillus* section *Flavi*) in portuguese almonds (Rodrigues et al. 2009, 2011). However, this species is more restricted geographically as compared to *A. flavus* (Frisvad et al., 2007).

The aflatoxins were found in 36 out of 40 (90%) nuts samples. The high levels of AFB1 (20.52 and 8.72 µg/kg) and AFs (13.45 and 25.82 µg/kg) were found in pistachios and shelled peanuts samples, respectively. In pistachio samples, the incidence of contamination with AFs was 87.5% (7 of 8). Only one pistachio sample analyzed (12.5%) exceeded the maximum limit (10 µg/kg) set by Algerian regulations. The incidence of contamination of pistachios reported by literature is variable. According to a report from Morocco, 45% of pistachio nut samples contained AFs (Juan et al., 2008). In Saudi Arabia, 34% pistachio nut samples were contamined with AFs and two samples contained 411 and 126 µg/kg of AFB1 (El tawila et al., 2013). In Qatar, 27.7% of analyzed pistachio nut samples were contaminated with AFs with levels up to 289 and up to 81.6 µg/kg (Abdulkadar et al., 2000). Pistachios could be contaminated with AFs in every stage, from maturity until storage (Georgiadou et al., 2012). Only AFB1 was detected in roasted hazelnuts and shelled almonds. Roasted hazelnut samples showed a weak contamination with AFB1compared to other nuts analyzed due probably to the effect of roasting. For almonds, few studies have been reported concerning AFs contamination of these nuts compared to the other nuts. Luttfullah and Hussain (2011) report that three out of 10 (30%) shelled almond samples from Pakistan were contaminated with with AFs. In Saudi Arabia, nine of 53 samples (17%) of almonds were contaminated with AFs (3.5 ± 3.8 µg/kg). On the other hand, almonds and walnuts are considered at lower AFs risk (Jelinek et al., 1989). Among the nuts analyzed in our study, peanut showed the highest levels and the highest mean contamination with AFs ranging from 0.34 to 25.82 µg/kg (mean 7.10 µg/kg). Peanuts are considered to be at high risk of contamination with AFs because they are frequently contaminated with *Aspergillus*, especially aflatoxigenic species. Recently, it has been reported that AFB1 was detected in 25% of raw peanut from China, ranging from 0.01 to 720 µg/kg (Ding et al., 2012). On the other hand, Juan et al. (2008) showed a weak contamination of the analyzed samples of peanut with AFs (5%). Mphande et al. (2004) reported that 78% of raw peanut from Botswana contained AFs at concentrations ranging from 12 to 329 µg/kg. Six out of 8 (75%) walnuts samples contained AFs ranging from 0.60 to 8.76 µg/kg. Literature available on the occurrence of AFs in walnut indicates variable levels of contamination. Deabes (2010) reported that concentration of AFs in walnut samples from Saudi Arabia ranged from 12 to 140 µg/kg of sample. From this country, El Tawila reported that 50% of walnut samples contained low amounts of AFs compared to groundnuts and pistachio. However, the contamination levels of AFs in walnut samples from Morocco ranged from 1.24 to 4320 µg/kg (mean 360 µg/kg).

Difference in climate conditions, methods of handling during harvesting, drying process and transferring leading to mechanical damages of nuts and inadequate drying after rewetting for dehulling are determinant for the final aflatoxins content. In conclusion, our results show high contamination by strains belonging to *Aspergillus* section *Flavi* and *Nigri.* Only two of 40 samples showed levels above "recognized" limits in Algeria, suggesting that, despite the extent of contamination, the risk is minimal. However, if storage conditions are more favorable for aflatoxigenic fungi, the aflatoxins levels may be more important. Finding a practical strategy to reduce the risk of aflatoxin contamination of food and feed is vital.

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Full Length Research Paper

Production of oleoresin from ginger (*Zingiber officinale*) peels and evaluation of its antimicrobial and antioxidative properties

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Dried ginger (ginger peel, peeled and unpeeled ginger) extracts were produced using acetone, and subjected to antimicrobial and anti-oxidative properties. In the antimicrobial assay using agar well diffusion technique the extracts were inoculated with five different organisms (*Escherichia coli*, *Staphylococcus aureus, Salmonella typhimurium, Aspergillus niger* and *Bacillus subtilis*) at different concentrations of 0.5, 1.0 and 1.5 ml, respectively, which were compared with the zero (0.00 ml) concentration of the extracts (control). The results showed that the three extracts (ginger peel, peeled and unpeeled ginger extracts) were effective against *B. subtilis* and *A. niger* at the different concentrations but were ineffective against *E. coli, S. aureus*, and *S. typhimurium*. The antioxidant activities of the three ginger (peel, peeled and unpeeled) extracts were found to be 75.50±0.70%, 73.01±0.00% and 51.01±0.41% for ginger peel, unpeeled ginger and peeled ginger respectively with the peel extract having highest antioxidant activity. These were compared with that of the control-synthetic antioxidant- butylated hydroxytoluene (BHT) which had activity of 98.5±0 0.70%. This showed that ginger peel extract could be used as an antioxidant in the place of synthetic antioxidants in foods and related products.

Key words: Agar well diffusion, antioxidant, ginger extracts, ginger peels, oleoresin.

INTRODUCTION

Lipid oxidation remains a major concern in food processing, due to the formation of oxidation products such as fatty acid hydroperoxides and secondary degradation products (alkanes, aldehydes, alkenes) according to Dandlen et al. (2010). The later components are responsible for off -flavours and they arise from hydroperoxy radicals formed during autoxidation. The formation of these off-flavours, with characteristic rancid odours, is responsible for the decrease in both the nutritional quality and safety of foods (Donnelly and Robinson, 1995; Yanishlieva et al., 2006). Oxidation processes are also deleterious in human health, since they induce tissue damage responsible for several pathologies, including cancer, neurodegenerative and ischaemic heart diseases, malaria, arterio-sclerosis and other pathological conditions (Erdemoglu, 2006). The utilization of antioxidants can prevent food oxidation or cell damage. To prevent this degradation process of lipids, the food industry adds antioxidants of low cost and high stability, mostly synthetic ones, such as butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT), gallats and *tert*-butylhydroquinone (TBHQ), in well-defined

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concentrations.

On the other hand, antioxidants are micronutrients that have gained interest in recent years due to their ability to neutralize the actions of free radicals (Carenas and Packer, 1996). Free radicals are potentially harmful products generated during a number of natural processes in the body, and are associated with the ageing of cells and tissues. Failure to remove active oxygen compounds, over a long term, can lead to cardiovascular disease, cancer, diabetes, arthritis and various neurodegenerative disorders (Sies, 1996). The use of synthetic antioxidants is restricted in several countries, because of their possible undesirable effects on human health (Branen, 1975; Chen et al., 1992; Kahl and Kappus, 1993). This has set the stage for rising consumer demand for food products with fewer synthetic additives, increased safety, quality and shelf- life. Consequently, it is of great interest to manufacturers the idea of identification of natural preservatives for food processing that will reduce the use of artificial preservatives, and possibly replace them with alter-natives that consumers perceive as natural. This has resulted in a renewed search for preservatives from natural sources and in particular from plant extracts, including their essential oils and oleoresin, as well as the use of natural antimicrobials to preserve foods (Poojari et al., 2009). Though, there is wide range of potential antimicrobials available, only few are suitable for use. Over the years, researches carried out on ginger established that it is concentrated with active substances that have anti-bacterial, anti-flatulent, antimicrobial, anti-inflammatory, antiseptic, anti-spasm, anti-viral and antioxidative properties (Altman and Marcussen, 2001). Ginger rhizome constitutes an aromatic and pungent spice that has unique culinary, medicinal and commercial relevance to the economy of its growers in tropical and subtropical countries. The characteristic pungent odour of peeled ginger rhizome is due to its oleoresin (viscous-to-thick material) content, which is an oily liquid containing oxymethyl phenols like shagoal, zingerone and gingerol, that are probably responsible for its antioxidant property (Bode and Dong, 2004). Ginger's oleoresin is principally composed of ginger essential oil and 6-gingerol, the major pharmacologically active component, and a lesser amount of a structurally related vanilloid (Bode and Dong, 2004). Zingerone is also produced from gingerols when ginger is cooked or dried.

According to Bode and Dong (2004), a common mechanism has been offered to explain the actions and health benefits of ginger and other herbs and spices which is related to their antioxidant properties. Modern scientific research has revealed that ginger possesses numerous therapeutic properties including antioxidant effects, an ability to inhibit the formation of inflammatory compounds, and direct anti-inflammatory effects (Akoachere, 2002). The antioxidant powers of ginger have been proven in applications where ginger extract was added to meat products, and was further tested with fresh, frozen and pre-cooked pork patties. Lee et al. (1986) demonstrated that the shelf-life of some food products determined by thiobarbituric acid (TBA) value was improved by the inclusion of ginger extract. Ginger has also been shown to be effective against the growth of both Gram-negative and positive bacteria including *Escherichia coli*, *Proteus vulgaris*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Streptococccus viridans* (Thompson et al., 1973). Orji et al. (2003) reported that different chemical substances are often obtained in members of even the same species of plants in different areas. Hence, the interest in ginger peels extract. The main objective of this research was to produce and assess extracts from ginger peels (the proximate composition, antioxidative and antimicrobial properties of ginger peels extract on test organisms).

MATERIALS AND METHODS

Procurement of raw materials and test organisms

A known weight (10 kg) of fresh ginger rhizomes (Nigerian variety-*Tafin giwa*) were purchased from Ogige market in Nsukka, Enugu State, Nigeria. Test organisms and reagents used for the antimicrobial activity were obtained from Pharmaceutical Microbiology Laboratory, Faculty of Pharmacy, University of Nigeria, Nsukka, Enugu State (Table 1). All reagents used for the antioxidant activity screening of the samples were obtained from Crop Science Laboratory, University of Nigeria, Nsukka, Enugu State, Nigeria.

Sample preparation

Ten kilograms (10 kg) fresh ginger rhizomes thoroughly were washed in tap water, 4 kg were dried without peeling, while the remaining 6 kg were peeled. The peeled and unpeeled rhizomes as well as the peels were sun dried (35±2°C) separately. The drying of the peels took four (4) days while the drying of the peeled and unpeeled ginger rhizomes took about two weeks to attain constant weight. After drying, the three samples (peels, unpeeled and peeled rhizomes) were milled into powdered form using a hammer mill (Thomas Wiley Mill model ED-5).

Extraction of oleoresin from ginger

Solvent extraction was used to extract an initial weight of 35 g of the powdered samples (peel, unpeeled and peeled ginger rhizomes) separately in a 500 ml glass column with 100 ml acetone (solvent), which was also used for washing the samples after the first extraction (Meadows et al., 2005). The solvent was allowed to percolate the separate ginger samples for 48 h each before the first extracts were collected. The glass columns containing the samples were re-soaked with additional 100 ml of acetone for another 24 h before collecting the second set of the extracts. The process was stopped when a cotton wool soaked with the extract from the glass column was devoid of ginger aroma. The extracts from each sample were pooled together and the solvent was removed using Rotavapor RE 111 with Buchi 461 water bath (Buchi, Sweden).

Table 1. Test organisms used for antimicrobial testing.

S/N	Organism	Source
1	*Escherichia coli*	Pharmaceutical Microbiology laboratory, UNN
2	*Salmonella typhimurium*	Pharmaceutical Microbiology laboratory, UNN
3	*Staphylococcus aureus*	Pharmaceutical Microbiology laboratory, UNN
4	*Aspergillus niger*	Pharmaceutical Microbiology laboratory, UNN
5	*Bacillus subtilis*	Pharmaceutical Microbiology laboratory, UNN

Table 2. Proximate composition of ginger peel.

Parameters	Composition (%)
Moisture	7.31^{c} ±0.02
Crude ash	7.46^{c} ±0.34
Crude fat	9.21^{b} ±0.00
Crude protein	9.42^{b} ±0.03
Crude fibre	7.02^{c} ±0.01
Carbohydrate	59.58^{a}±0.00

Values are means ± standard deviation of duplicate readings.

Portions (300 ml) of each extract was poured in rotary evaporators flask and evaporated at 65°C till all the solvents were expelled. The concentrated extracts (oleoresin) were collected and treated as crude ginger peel, unpeeled and peeled ginger oleoresin extracts, respectively (Lewis et al., 1972). The difference between the empty flask and flask with the separate concentrated extracts was used in obtaining the oleoresin content yield (Onyenekwe, 2000).

Sample analysis

Chemical analysis

The moisture, ash, fibre and fat contents were determined by the Association of the Official Analytical Chemists [AOAC, 2010] methods, 14004, 14009 and 14006, respectively. The Kjeldahl (Kjeltec, Tecator, Sweden) method was used to determine the nitrogen content of the samples, while protein was estimated as 6.25 N (method 7015, [AOAC, 2010]). The carbohydrate content was obtained by subtracting the above values from hundred (Pearson, 1976). The vitamins E and C content as well as the peroxide value of the ginger (peel, unpeeled and peeled) extracts were determined using the method described by Pearson (1976).

Determination of the antioxidant and antimicrobial activities of the ginger extracts

The antioxidant activities of the ginger peel, unpeeled ginger and peeled ginger extracts was determined using the Ferric Thiocyanate Method (FTC) described by Osawa and Namiki (1981) while Agar Well Diffusion method described by Wan et al. (1998) was used to determine their antimicrobial properties. The measurements (in millimeters) of the zones of inhibitions of the extracts against the test organisms (Table 1) were taken and recorded. The tests were performed in duplicates for each microorganism.

Data analysis and experimental design

The antimicrobial testing was statistically analyzed by using a 3-factorial or split-split plot in completely randomized design (CRD). While the antioxidative testing was statistically analyzed by using a 2x3 factorial or 2x3 split plot in completely randomized design (CRD). The vitamin evaluation were subjected to one-way analysis of variance and the means were separated by Duncan's multiple range test using SPSS version 17 computer statistical package. Significant levels were accepted at $p>0.05$.

RESULTS

Proximate composition of ginger peels

The result in Table 2 shows the proximate composition (% dry basis) of ginger peels. The moisture content of the ginger peels was found to be 7.31±0.02%, while that of the crude protein content was 9.42±0.03%. The crude fat, crude ash, crude fibre and carbohydrate contents were 9.21±0.01, 7.46±0.34, 7.02±0.01 and 59.58±0.00%, respectively.

Antioxidative properties of ginger sample (peel, peeled and unpeeled) extracts

Table 3 shows the result of anti-oxidant activities of crude oleoresin extracts obtained from ginger peel (A), peeled ginger (B) and unpeeled ginger (C). It revealed that the ginger peel extract displayed strong antioxidant activity as compared to the other two samples, with the peeled ginger, having the lowest antioxidant activity in the order-Sample A > Sample C > Sample B. The results also indicated that the ginger extracts significantly ($p<0.05$) inhibited linoleic acid peroxidation as compared to the control, since a high inhibition percent indicates a high antioxidant activity. Hence, the ginger peel could be said to contain all the essential components responsible for antioxidant property of ginger.

Table 3. Antioxidant activity of ginger (peel, peeled and unpeeled) crude oleoresin extracts measured after 24 h of incubation.

Sample	Absorbance at 500 nm	Presence of inhibition [k](%)
A	0.010	75.50±0.70[b]
B	0.010	51.01±0.41[d]
C	0.020	73.01±0.00[c]
Control	0.002	98.50±0.70[a]

Value are means ±standard deviation of duplicate readings. Means with different superscripts in the same column are significantly different (P<0.05)
A = Ginger peel extract; B = peeled ginger extract; C = Unpeeled ginger extract; Control = butylated hydroxytoluene (BHT)[k] Percent of inhibition = capacity of extracts to inhibit the peroxide formation in linoleic acid (a high inhibition percent indicates a high anti-oxidant activity).

Table 4. Peroxide values of ginger (peel, peeled and unpeeled) extracts.

Sample	Peroxide value (millieq/kg)
A	10.0±0.00[d]
B	15.10±0.14[b]
C	13.0±0.35[c]
Control	22.0±0.12[a]

Values are means ±standard deviation of duplicate readings. Means with different superscripts in the same column are significantly different (p<0.05).
Sample A = ginger peel; Sample B = peeled ginger; Sample C = Unpeeled ginger; Control = blank titration.

Peroxide value of ginger oleoresins

The peroxide value of the ginger (peel, peeled and unpeeled) extracts presented in Table 4 shows that the ginger peel extract (A) had a better peroxide value of 10 millieq/kg, which was found to be within the peroxide value range of fresh oils. Samples B and C had higher peroxide value which could be as a result of long storage of the oils (crude oleoresin).

Vitamin C and E contents of the ginger oleoresin extracts

The vitamin C and E contents of the ginger (peel, peeled and unpeeled) extracts presented in Table 5 shows that samples A, B and C had a vitamin C content of 14.81,11.42 and 9.61 mg/100g respectively, while the control (standard powdered ascorbic acid) had vitamin C content of 0.61 mg/100g. Thus, the samples had significantly (p<0.05) higher vitamin C contents than the control. Samples A, B and C had 0.38, 0.61 and 0.50 mg/100 g vitamin E contents respectively, while the control (standard powdered ascorbic acid) had a vitamin E content of 0.01 mg/100g. The samples differed significantly (p<0.05) from the control, with sample A having the least value, while sample B had the highest vitamin E content.

Antimicrobial properties of ginger peels, peeled and unpeeled extracts (crude oleoresin)

The result in Table 6 shows the antimicrobial properties of ginger peels, peeled and unpeeled extracts. It revealed that at a volume of 1.5 ml of the different ginger extracts, the measured zones of inhibition were 11, 8 and 14 mm for *B. subtilis* (Figure 1), for extracts A, B and C and 8, 5 and 7 mm for *A. niger* (Figure 2), for extracts A, B and C respectively. Also, *S. aureus* (Figure 3), and *S.typhimurium* (Figure 4) and *E. coli* (Figure 5), were resistant to the three ginger extracts/crude oleoresin at different doses used for the antimicrobial testing. *A. niger* (Figure 2) and *B. subtilis* (Figure 5) were inhibited at dose of 0.5, 1.0 and 1.5 ml with significant difference (p<0.05) observed between the doses and the zones of inhibition at higher dose of the ginger (peel, peeled and unpeeled) crude oleoresin/extracts.

DISCUSSION

Proximate composition (%) of ginger peel on dry basis

The peels moisture content (7.31%) when compared with that of whole ginger which is 9.38% as reported by Remadevi et al. (2004) showing that whole ginger rhizome contains more moisture than the peels. The crude protein when compared with that of whole ginger which is 9.12% as reported by Remadevi et al. (2004),

Table 5. Vitamins C and E content of ginger (peel, peeled and unpeeled) extracts.

Sample	Vitamin C (mg/100 g)	Vitamin E (mg/100 g)
A	14.81±0.01[a]	0.38±0.00[c]
B	11.42±0.03[b]	0.61±0.01[a]
C	9.61±0.01[c]	0.50±0.00[b]
Control	0.61±0.01[d]	0.01±0.00[d]

Values are means ± standard deviation of duplicate readings. Means with different superscripts in the same column are significantly different ($p<0.05$). Sample A = ginger peel; Sample B = peeled ginger ; Sample C = unpeeled ginger Controls = powdered ascorbic acid (standard) and encapsulated commercial vitamin e (standard).

Table 6. Antimicrobial activity of ginger (peel, peeled and unpeeled) extracts (crude oleoresin) using the agar well diffusion technique after incubation for 24, 48 and 72 h.

Sample	Doses of oleoresin (ml)	Organism zones of inhibition (mm)					
		I	II	III	IV	V	VI
A	0.5	4.0±0.41[c]	+	+	+	3.0±0.41[c]	+
	1.0	6.0±0.00[b]	+	+	+	5.0±0.00[b]	+
	1.5	11.0±0.41[a]	+	+	+	8.0±0.00[a]	+
B	0.5	3.0±0.41[c]	+	+	+	0.00±0.00[c]	+
	1.0	5.0±0.00[b]	+	+	+	3.0±0.00[b]	+
	1.5	8.0±0.00[a]	+	+	+	5.0±0.00[a]	+
C	0.5	2.0±0.00[c]	+	+	+	2.0±0.00[c]	+
	1.0	8.0±0.00[b]	+	+	+	4.0±0.00[b]	+
	1.5	14.0±0.00[a]	+	+	+	7.0±0.00[a]	+

Values are means ± standard deviation of duplicate readings. Means with different superscripts in the same column are significantly different ($p<0.05$). Sample A = ginger peel; Sample B = peeled ginger; Sample C = unpeeled ginger; I = *Bacillus subtilis*; II = *Staphylococcus aureus*; III = *Salmonella typhimurium*; IV = *Escherichia coli;* V = *Aspergillus niger*; VI = Control; + = Profuse growth of organisms.

indicated that there was no difference in the protein content of the whole ginger and that of the peel. The crude fat compared with that of whole ginger (5.95%) of Remadevi et al. (2004), implies that ginger peels have higher fat deposits than whole ginger. However, the crude ash when compared with that of whole ginger given as 4.77% by Remadevi et al. (2004) revealed that the peels was 32% higher, while the peels crude fiber compared with that of whole ginger which is 7.17% by Remadevi et al. (2004) was found to be 3.8% higher.

Antioxidative properties and peroxide value of ginger (peel, peeled and unpeeled) extracts

The observation of the antioxidative properties could be attributed to the fact that the peeled ginger may not contain much of the essential constituents required like shogoal, zingerone and gingerol which are probably responsible for ginger's antioxidant property as reported by Fuhrman (2000). The peroxide values of the ginger (peel, peeled and unpeeled) extracts were compared with other researchers. The peroxide value of sample A was probably due to the fact that the rancid tastes of oils become noticeable when the peroxide value is higher than 10 millieq/kg of a sample (Pearson, 1976). Expectedly in sample B and C, this showed that the longer the oils stayed un-used, the lesser their degree of freshness. Therefore, a higher peroxide value indicates a higher susceptibility of fats or oils to rancidity. In general, the greater the degree of unsaturation, the greater is the liability of fats/oils to oxidative rancidity.

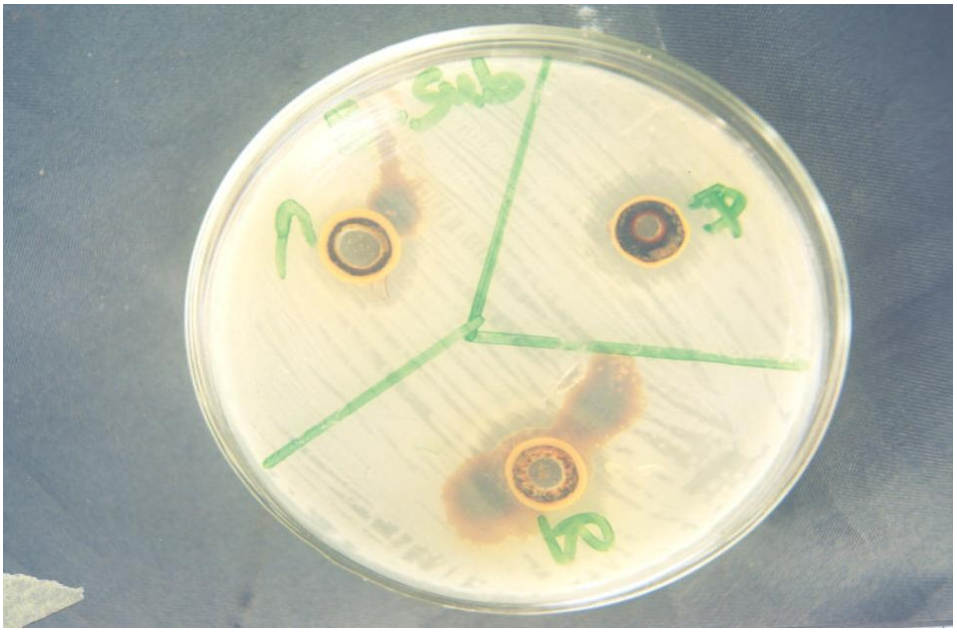

Figure 1. Petri dish showing zones of inhibition of *B. subtilis* with ginger peel, unpeeled ginger and peeled ginger crude oleoresin.

Figure 2. Petri dish showing zones of inhibition of *A. niger with* ginger peeled, unpeeled ginger and peeled ginger crude oleoresin.

Antimicrobial properties determination of ginger (peel, peeled, unpeeled) extracts

The zones of inhibition (mm) increased with increase in doses of oleoresin of the ginger samples used for the antimicrobial testing. This was in agreement with a report published on well diffusion assay protocols for phenolic compound and some plant extracts by Rauha et al. (1994), which showed that there was wide variation in a reported volume of antimicrobial used in well diffusion

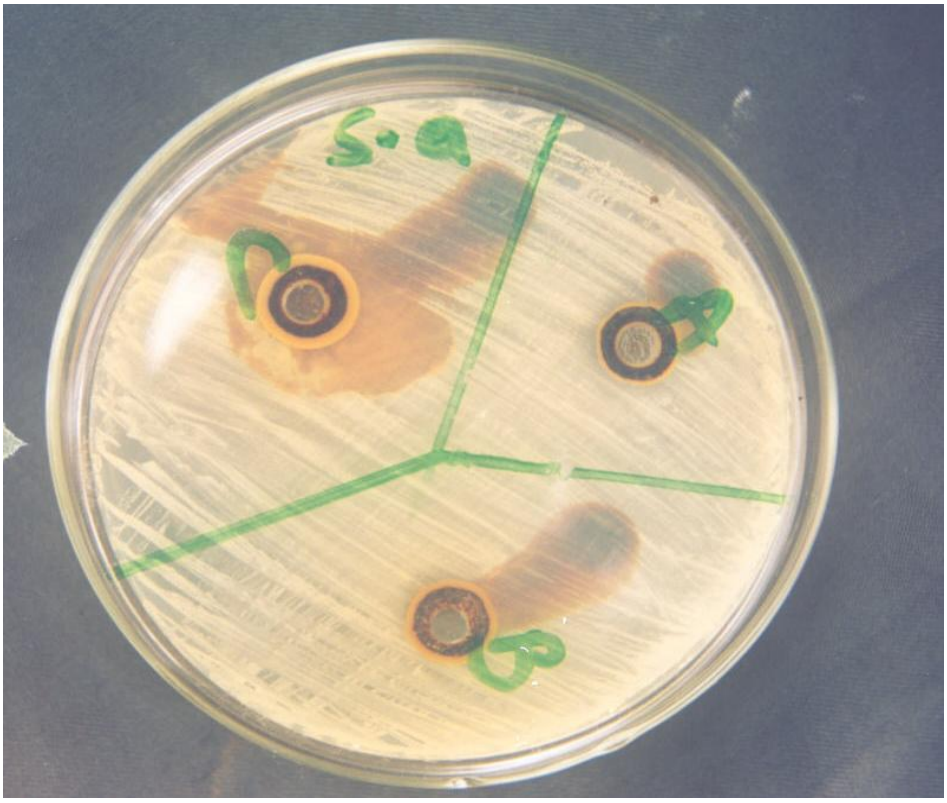

Figure 3. Petri dish showing zones of inhibition of *S. aureus* with ginger peel, unpeeled ginger and peeled ginger crude oleoresin.

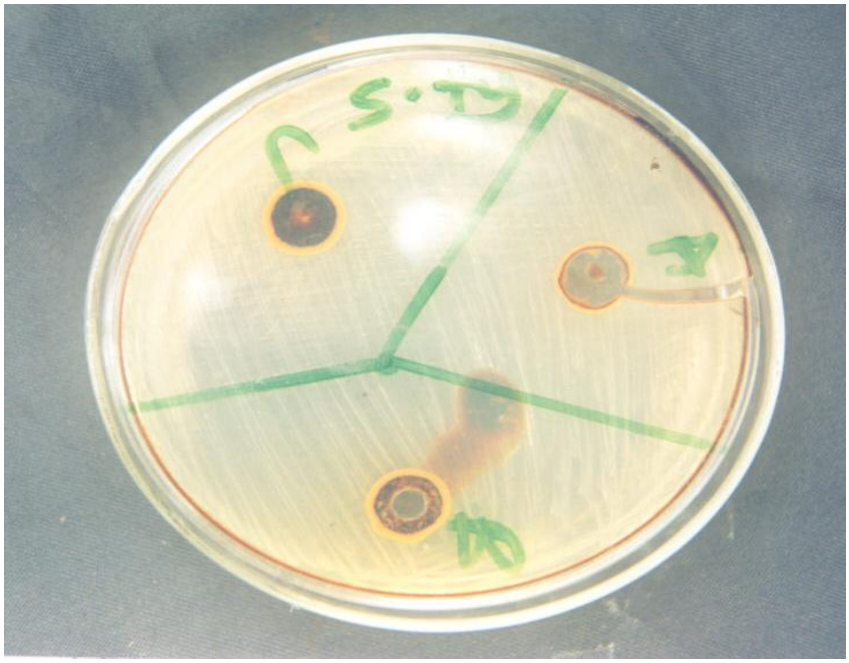

Figure 4. Petri dish showing zones of inhibition of *S. typhimurium* with ginger peel, unpeeled ginger and peeled ginger crude oleoresin.

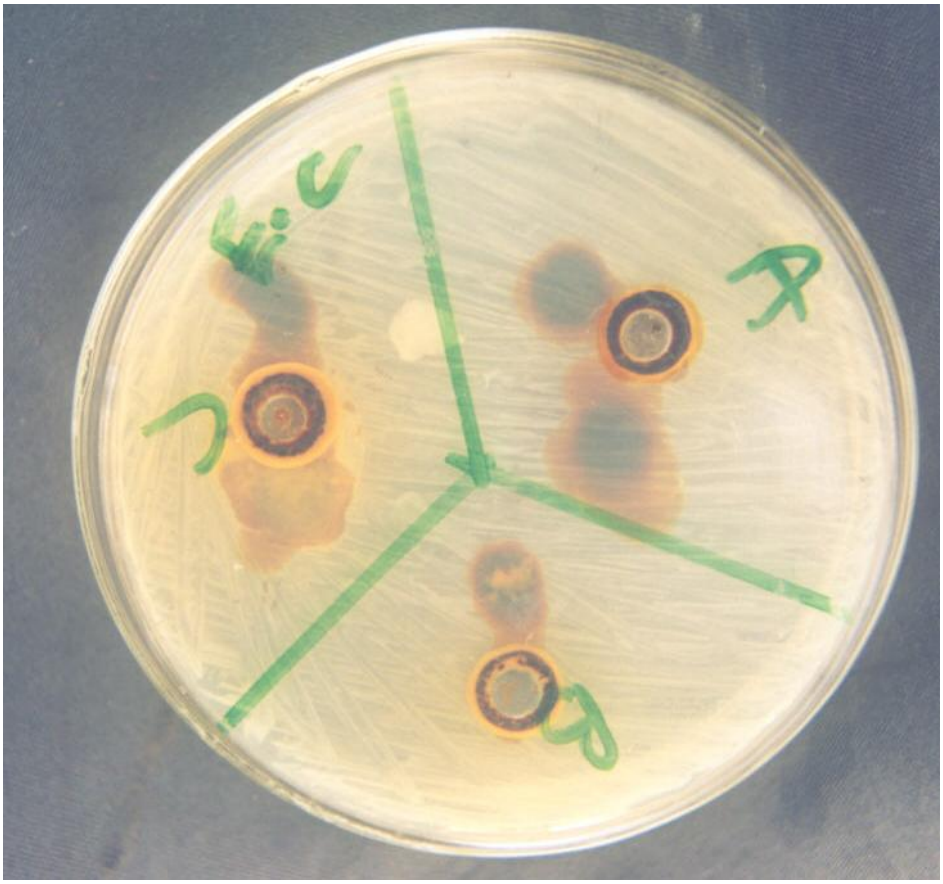

Figure 5. Petri dish showing zones of inhibition of *E.coli* with ginger peel, unpeeled ginger and peeled ginger crude oleoresin.

assay and in the well size used. There had been reports that ginger is effective against the growth of both Gram-positive and negative bacteria including *E. coli*, *S. typhimurium* and *S. aureus* (Lewis, 1972). However, in the present work, the ginger (peel, peeled and unpeeled) oleoresin/extracts were observed to be inactive at 0.5, 1.0 and 1.5ml doses against *E. coli, S. aureus* and *S. typhimurium* even after being incubated for 72 h.

This observation could be attributed to the un-even diffusion of the extracts through the agar medium because the extracts were not initially in direct contact with the organisms and must first diffuse into the agar to exert any antimicrobial effect or it could be due to low level of doses of the ginger extracts used (Yamada et al., 1992). The ginger (peel, peeled and unpeeled) crude oleoresin/extracts however, were effective against *B. subtilis* as reported by Yamada et al. (1992), who reported that *B. subtilis* and *S. aureus* are more susceptible to antimicrobial agents than *E. coli* bacteria. The ginger, extracts were also effective against *A. niger* as observed by Singh (2001) that the essential oil of ginger show antifungal activity against *Aspergillus* species. However, the two organisms that exhibited antimicrobial activity (*B. subtilis* and *A. niger*) did not show any visible activity after 24 and 48 h but after 72 h of incubation. However, lack of antimicrobial activities exhibited by all the extracts of *Cassia occidentalis* and generally at concentrations between 500 - 1000 mg on *P. multocida, S. typhi, S. typhimurium, S. pyogenes, S. pneumoniae and K. pneumoniae* is suggestive of limited antimicrobial activity of the plant. This was not pointed out by Gasquet (1993), Percez (1994) and Saraf (1994). Moreso, uniformity of antimicrobial activity exhibited by hexane, chloroform, methanol and aqueous extracts of *C. occidentalis* leaf on only *E. coli* may confirm the limited antibacterial activity of the plant even *in vivo*. Nonetheless, there is need to separate the chemical constituents of the plant leaf and then test each component on the microorganisms.

Conclusion

It is evident from this research work that ginger peels

possess both antioxidative and antimicrobial properties, an indication that no part of the ginger plant can actually be regarded as a waste. In this context, the antioxidant property of ginger peels merits special consideration since ginger is a widely used food spice that can be consumed by all. Ginger could also be said to have added advantage of being a natural antioxidant over standard oral synthetic antioxidant vitamin supplements such as vitamin E and C since it contains both of them as shown in this present work.

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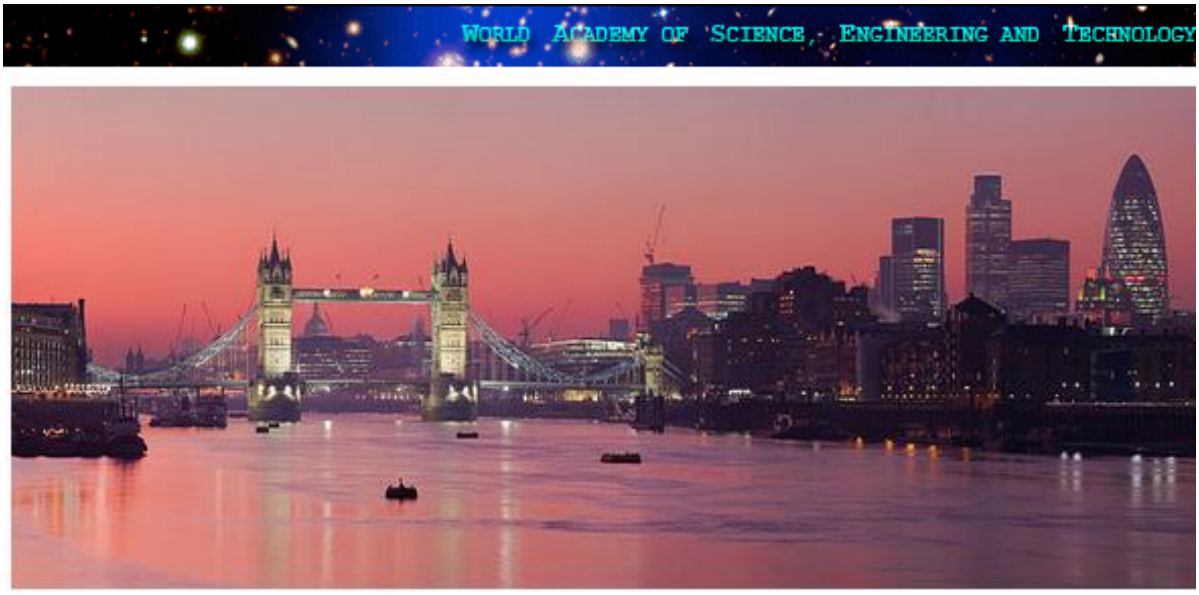

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