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African Journal of Microbiology Research

Full Length Research Paper

Discrimination of *Ralstonia solanacearum* isolates by genetic signatures produced by single-strand conformation polymorphism and low-stringency single specific primer PCR analysis

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Development of molecular biology based techniques have led to reliable characterization and determination of the genetic diversity among phytopathogens. Single-strand conformation polymorphism (SSCP) and Low-stringency single specific primer (LSSP)-PCR were assessed for genetic typing of *Ralstonia solanacearum* isolates from suspicious bacterial wilt fields. *R. solanacearum* isolates obtained were amplified by colony PCR analysis with *egl* specific primers which amplified a PCR product of 237 bp. These amplified products were denatured and separated in a polyacrylamide gel to develop PCR-SSCP fingerprints, which confirms *R. solanacearum* by producing similar four banding patterns. The amplified product of colony-PCR was subsequently used as a template for LSSP-PCR analysis. The individual genotyping of each *R. solanacearum* obtained by LSSP-PCR were able to discriminate solanaceae and ginger isolates into two different clusters along with pathogenic and non-pathogenic. The LSSP-PCR profile of *R. solanacearum* isolates were closely related and evolved by the genome of host origin and diverge in genomic stability which was further confirmed by sequence analysis. In conclusion, SSCP and LSSP-PCR techniques were most effective compared to biochemical and physiological assays for identification and genetic variability in *R. solanacearum,* which has high genetic divergence. The rapid identification of *R. solanacearum* plays a crucial role in prevention of bacterial wilt.

Key words: *Ralstonia solanacearum,* Bacterial wilt, SSCP-PCR, LSSP-PCR, Molecular detection.

INTRODUCTION

Ralstonia solanacearum (Smith) is a gram-negative, soil-borne bacterium belonging to the class β-proteobacteria, which causes a widespread disease known as bacterial wilt (Yabuuchi et al., 1994). Many economically important crops as well as weeds have been infected and they act as a carrier for this pathogen. The host range of *R.*

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solanacearum is exceedingly wide, including 450 plant species representing more than 50 families of botanical flora (Ramesh et al., 2009).

The pathogen comprises diverse species such as *Ralstonia syzygii* and *Ralstonia pickettii* and blood disease bacterium (BDB) which differ in host range, pathogenicity, geographical distribution and physiological properties. The pathogen survives for years in wet soil, water ponds, on plant debris or in asymptomatic weed hosts, which act as inoculum reservoirs. *R. solanacearum* is endemic and made as quarantine important in many developed and developing countries in tropical, subtropical, and warm temperate regions of the world (Mansfield et al., 2012; Ramesh et al., 2014).

Conventionally the pathogen was characterized by its host range and divided into five races. race 1 infect solanaceae family and many other hosts; race 2 infect primarily banana; race 3 infect potato, tomato and a few other hosts, usually in more temperate conditions; race 4 infect ginger and race 5 infect mulberry (Hayward, 1991). In past few decades another classification was adapted; *R. solanacearum* was classified into six biovars along with the race. Biovars classification is based on the ability to produce acid from carbohydrates. The only association between races and biovars is that race 3 corresponds to biovar 2 (Hayward, 1991). These methods of characterization are of limited utility because, the host range overlaps especially after artificial inoculation (Santana et al., 2012).

Molecular-based approaches have been developed to enhance understanding of genetic diversity in *R. solanacearum.* There are seven extracellular enzymes secreted by type II secretion system during infection of bacterial wilt such as, β-1, 4-endoglucanase (*Egl*) (Ramesh et al., 2014) an exoglucanase (CbhA), an endopolygalacturonase (PehA or PglA), two exo-polygalacturonases (PehB and PehC), a pectin methylesterase (Pme) which have been extensively studied (Kang et al., 2002). The genetic variablity among the strains of *Ralstonia* spp (*R. syzygii* and *R. pickettii* and BDB) is due to recombination within the *egl* and *hrpB* genes, rather due to mutations (Castillo et al., 2007). Several genes were targeted such as *hrpB, egl* and *speI* genes for specific identification of *R. solanacearum* (Umesha and Avinash, 2014).

Single-strand conformation polymorphism (SSCP) and low-stringency single specific primer (LSSP-PCR) are the key alternative techniques which differentiate and identify the plant pathogens into species and isolate level. There are several reports that SSCP-PCR technique can be used for detection of most infectious infected virus and bacterial pathogens along with oncogenes and allelic variations in the human genome (Gadiou et al., 2009; Chandrasekhar et al., 2012; Delamare et al., 2012).

Five major pathogens *Escherichia coli, Clostridium perfringens, Campylobacter jejuni, Salmonella enterica* and *Bacillus cereus* were confirmed by SSCP using 16S rRNA gene (Oh et al., 2008). In our previous studies we have developed the species–specific DNA fingerprints for species identification and diagnosis of phytobacterium pathogens and their diseases (Umesha et al., 2012).

Low-stringency single specific primer technique represents a simple repetition of PCR process with one of the two primers used in the initial amplification by creating low-stringency. Serovars of *Leptospira* were analyzed by G1 and G2 primers that led to differentiation of *Leptospira* species (Oliveira et al., 2003). Discrimination of serogroups of *Leptospira* from different animal reservoirs and clinical suspicion of leptospirosis were studied using LSSP-PCR (Bomfim and Koury, 2006).

Low-stringency single specific primer PCR has been efficiently used to ascertain genetic variability in phytopathogenic virus and protozoal parasites such as *Entamoeba histotlytica, Trypansoma cruzi* and *Trypanosoma* (Marquez et al., 2007; Oh et al., 2008). Recently, kDNA genetic correlations among human and canine isolates of *Lesishmania infantum* were examined by LSSP-PCR (Alvarenga et al., 2012). Differentiation of Plum pox virus isolates was achieved by the SSCP and LSSP-PCR techniques with the help of HC-Pro genomic region (Gadiou et al., 2009).

Management of bacterial wilt using chemicals has faced constraints because of genetic diversity in *R. solanacearum* (Ramadasappa et al., 2012; Ramesh et al., 2014; Naik et al., 2015). Diversity in *R. solanacearum* strains is a major problem in India and Andaman Islands even today (Sakthivel et al., 2016). SSCP and LSSP-PCR techniques were efficiently employed to ascertain genetic diversity of many pathogens. Hence in this present study, our aim is to develop SSCP-PCR and LSSP-PCR techniques to study genomic variations of *R. solanacearum* isolated from the different host reservoirs. To our knowledge, this will be the first report to employ SSCP and LSSP-PCR to understand the polymorphic variations and discrimination of pathogenic and non pathogenic isolates among *R. solanacearum* with significant focus on *egl* specific gene.

MATERIAL AND METHODS

Collection and screening of plant material and soil samples for *R. solanacearum*

Bacterial wilt suspected plant material and soil samples from different hosts viz., tomato, chilli, potato, eggplant, ginger and black pepper from agricultural fields (Karnataka, India) were collected from during 2012 to 2014 (Table 1).

The samples were brought into the laboratory and subjected to laboratory assays such as direct plating and liquid assay methods for isolation of *R. solanacearum* by following the standard procedures of ISTA (ISTA, 2005). The suspected plant materials were cut into small pieces (5 mm) and the surface was disinfected with sodium hypochlorite solution (3%; v/v) followed by five repeated washings with sterile distilled water.

Samples were directly plated on semi-selective medium [Kelman's

Table 1. *Ralstonia solanacearum* isolates used in this study: source and year of isolation; pathogenicity test and biovars classification and sequencing with corresponding sequence accession number in NCBI database.

Host	Isolate	Year of isolation	Pathogenicity	Biovars	Accession no.
Tomato	DOB RS T1	2012	+	3	KP711278
Tomato	DOB RS T2	2012	+	3	KP658425
Tomato	DOB RS T3	2012	+	1	KP658424
Tomato	DOB RS TS4	2012	+	3	NS
Tomato	DOB RS TS5	2012	+	3	NS
Tomato	DOB RS TS6	2012	+	1	NS
Tomato	DOB RS T7	2012	+	1	NS
Tomato	DOB RS T8	2012	+	1	NS
Tomato	DOB RS TS9	2012	+	1	NS
Tomato	DOB RS T10	2013	+	3	KP658426
Tomato	DOB RS T11	2013	+	3	KP711279
Tomato	DOB RS TS12	2013	+	3	NS
Tomato	DOB RS TS13	2013	+	3	NS
Tomato	DOB RS TS14	2013	+	3	NS
Tomato	DOB RS TS15	2013	+	3	NS
Tomato	DOB RS TS16	2013	+	1	NS
Tomato	DOB RS TS17	2013	+	1	NS
Tomato	DOB RS TS18	2013	+	1	NS
Tomato	DOB RS TS19	2013	+	1	NS
Tomato	DOB RS T20	2013	+	1	NS
Tomato	DOB RS T21	2014	+	3	KP711280
Tomato	DOB RS T22	2014	+	1	KP711281
Tomato	DOB RS T23	2014	+	3	KP711282
Tomato	DOB RS T24	2014	+	3	NS
Tomato	DOB RS T25	2014	+	3	NS
Tomato	DOB RS T26	2012	-	1	NS
Tomato	DOB RS T27	2012	-	3	NS
Tomato	DOB RS T28	2012	-	3	NS
Tomato	DOB RS T29	2013	-	3	NS
Tomato	DOB RS T30	2013	-	3	NS
Chilli	DOB RS C1	2012	+	3	KP658422
Chilli	DOB RS CS2	2012	+	3	NS
Chilli	DOB RS CS3	2012	+	3	NS
Chilli	DOB RS CS4	2012	+	3	NS
Chilli	DOB RS CS5	2012	+	3	NS
Chilli	DOB RS CS6	2012	+	3	NS
Chilli	DOB RS CS7	2012	+	3	NS
Chilli	DOB RS C8	2012	+	3	NS
Chilli	DOB RS C9	2012	+	3	NS
Chilli	DOBRS C10	2014	+	3	NS
Chilli	DOB RS C11	2014	+	3	NS
Chilli	DOBRS C12	2014	+	3	KP658423
Chilli	DOB RS C13	2014	+	3	NS
Chilli	DOBRS C14	2014	+	3	NS
Chilli	DOB RS CS15	2014	+	3	NS
Chilli	DOBRS CS16	2014	+	3	NS
Chilli	DOBRS CS17	2014	+	3	NS
Chilli	DOB RS CS18	2014	+	3	NS
Chilli	DOB RS CS19	2014	+	3	NS

Table 1. Contd.

Potato	DOB RS P1	2012	+	2	NS
Potato	DOB RS P2	2012	+	2	NS
Potato	DOB RS PS3	2012	+	2	NS
Potato	DOB RS PS4	2012	+	2	NS
Potato	DOB RS PS5	2012	+	2	NS
Potato	DOB RS PS6	2012	+	2	NS
Potato	DOB RS PS7	2012	+	2	NS
Potato	DOB RS PS8	2013	+	2	KP701010
Potato	DOB RS P9	2013	+	2	KP701011
Potato	DOB RS P10	2014	+	2	KP701012
Potato	DOB RS P11	2014	+	2	KP701013
Potato	DOB RS PS12	2014	+	2	NS
Potato	DOB RS PS13	2014	+	2	NS
Potato	DOB RS PS14	2014	+	2	NS
Potato	DOB RS PS15	2014	+	2	NS
Potato	DOB RS P16	2014	+	2	NS
Potato	DOB RS P17	2014	+	2	NS
Potato	DOB RS P18	2014	+	2	NS
Potato	DOB RS P19	2012	-	2	NS
Potato	DOB RS P20	2012	-	2	NS
Potato	DOB RS P21	2012	-	2	NS
Potato	DOB RS P22	2014	-	2	NS
Eggplant	DOB RS E1	2012	+	1	NS
Eggplant	DOB RS E2	2012	+	1	NS
Eggplant	DOB RS E3	2012	+	1	NS
Eggplant	DOB RS E4	2012	+	1	KP148262
Eggplant	DOB RS E5	2012	+	3	NA
Eggplant	DOB RS E6	2012	+	3	KP221801
Eggplant	DOB RS E7	2012	+	1	KP221802
Eggplant	DOB RS E8	2012	+	1	KP221803
Eggplant	DOB RS E9	2012	+	3	NS
Eggplant	DOB RS ES10	2012	+	1	NS
Eggplant	DOB RS ES11	2012	+	1	NS
Eggplant	DOB RS ES12	2012	+	1	NS
Eggplant	DOB RS ES13	2012	+	1	NS
Eggplant	DOB RS ES14	2012	+	1	NS
Eggplant	DOB RS ES15	2012	+	3	NS
Eggplant	DOB RS ES16	2012	+	3	NS
Eggplant	DOB RS ES17	2012	+	3	NS
Eggplant	DOB RS ES18	2013	+	1	KP221804
Eggplant	DOB RS ES19	2013	+	1	KP221805
Eggplant	DOB RS ES20	2013	+	1	NS
Eggplant	DOB RS ES21	2013	+	1	NS
Eggplant	DOB RS E22	2013	+	1	NS
Eggplant	DOB RS E23	2013	+	1	NS
Eggplant	DOB RS E24	2013	+	3	NS
Eggplant	DOB RS E25	2013	+	3	NS
Eggplant	DOB RS E26	2013	+	3	NS
Eggplant	DOB RS E27	2014	+	1	KP711283
Eggplant	DOB RS E28	2014	+	1	KP711284
Eggplant	DOB RS E29	2014	+	1	NS
Eggplant	DOB RS E30	2014	+	1	NS

Table 1. Contd.

Eggplant	DOB RS E31	2014	+	1	NS
Eggplant	DOB RS E32	2014	+	1	NS
Eggplant	DOB RS E33	2014	+	1	NS
Eggplant	DOB RS E34	2014	+	3	NS
Eggplant	DOB RS E35	2014	+	3	NS
Eggplant	DOB RS E36	2014	+	3	NS
Eggplant	DOB RS E37	2012	-	3	NS
Eggplant	DOB RS E38	2012	-	1	NS
Eggplant	DOB RS E39	2012	-	1	NS
Eggplant	DOB RS E40	2012	-	1	NS
Eggplant	DOB RS E41	2013	-	1	NS
Eggplant	DOB RS E38	2014	-	3	NS
Black pepper	DOB RS BPS1	2014	-	4	KP658429
Black pepper	DOB RS BPS2	2014	-	4	KP658430
Black pepper	DOB RS BPS3	2014	-	4	NS
Black pepper	DOB RS BPS4	2014	-	4	NS
Black pepper	DOB RS BPS5	2014	-	4	NS
Black pepper	DOB RS BPS6	2014	-	4	NS
Ginger	DOB RS GS1	2014	-	4	NS
Ginger	DOBRS GS2	2014	-	4	NS
Ginger	DOBRS GS3	2014	-	4	NS
Ginger	DOBRS GS4	2014	-	4	NS
Ginger	DOBRS GS5	2014	-	4	NS
Ginger	DOB RS GS6	2014	-	4	NS
Ginger	DOBRS GS7	2014	-	4	NS
Ginger	DOBRS GS8	2014	-	4	NS
Ginger	DOBRS GS9	2014	-	4	NS
Ginger	DOB RS GS10	2014	-	4	NS
Ginger	DOBRS GS11	2014	-	4	NS
Ginger	DOBRS GS12	2014	-	4	NS
Ginger	DOBRS GS13	2014	-	4	NS
Ginger	DOB RS GS14	2014	-	4	NS
Ginger	DOBRS GS15	2014	-	4	NS
Ginger	DOBRS GS16	2014	-	4	NS
Ginger	DOBRS GS17	2014	-	4	NS
Ginger	DOB RS G18	2014	-	4	KP658428
Ginger	DOBRS G19	2014	-	4	NS
Ginger	DOBRS GS20	2014	-	4	NS
Ginger	DOBRS GS21	2014	-	4	KP658427
Potato	DOBCPR 12	2009	+	2	KP658421
Soil	NCIM 5331	2009	-	3	GQ17488

Ralstonia solanacearum isolates obtained from different host and agricultural fields of Karnataka. SI-Serial number, '+' indicates positive for pathogenicity test, '-' indicates negative for pathogenicity test, NS-Not Submitted to data base due to sequence similarity while annotation.

triphenyl tetrazolium chloride TZC medium; (glucose, 10 g; peptone, 10 g; casein hydrolysate, 1 g; agar, 18 g; distilled water 1,000 ml, 5 ml of TZC solution filter sterilized was added to the autoclaved medium to give final concentration of 0.005%)] (Kelman, 1954). Liquid assay was performed by macerating the plant material using sterile mortar and pestle in 10 ml of sterile distilled water.

The supernatant (1 ml) was mixed with 9 ml of sterile distilled water to obtain a dilution of 10^{-1} and further serial dilutions were prepared up to 10^{-5}. Fifty microliters of each dilution were spread on semi-selective media. In addition, the collected soil samples were also subjected to serial dilution technique up to 10^{-5} dilutions, and aliquots of 50 µl of each dilution were spread on semi-selective media using Drigalski's spreaders in triplicates. All plates were incubated at 28±°C for 24 to 48 h.

The bacterial culture supplemented with 20% glycerol stock and stored in Department Stock Collection Centre (Departmental studies

in Biotechnology, University of Mysore, Mysore, India) at -80°C. Bacterial isolates collected from agricultural fields from soil, and plant material were subjected to biochemical/physiological as well as hypersensitivity tests as reported earlier (Umesha and Avinash, 2014).

Pathogenicity tests were conducted in five week-old-highly susceptible cultivars of tomato (cv. PKM-I) and eggplant (cv. Chaman 363) by inoculating each isolates. The density of cell suspension was adjusted to 0.45 OD at 600 nm to obtain final concentration 1 x 10^7 cfu/ml using spectrophotometer (Beckman Coulter, CA. USA) (Avinash and Umesha, 2014). This bacterial suspension was poured near the roots of plants under green house condition. A reference culture of *R. solanacearum* was procured from NCIM (5331), Pune and Central Potato Research Institute (DOBCPR 12), Shimla, India and they are used as a standard for all these biochemical/physiological and pathogenicity tests. All experiments were carried out in three replicates and repeated thrice.

Molecular characterization of *R. solanacearum*

PCR reaction was performed with RS-Egl-F (5'-GACACCACGACCCTGAAGAC-3') and RS-Egl-R (5'-AAGGTATGCCAGGTGGCGCA-3') primers. The specific primers derived from sequence of *egl* gene were designed from primer 3 software and custom synthesized from Sigma, India. Detection of *R. solanacearum* was performed using sub cultures grown at 28±2°C in TZC medium. Colony PCR was performed by following the procedure of Umesha et al. (2012). Briefly, a loop full of colonies from each bacterial isolate was suspended in 100 *µl* sterile distilled water and serially diluted up to 10^{-6}. The suspensions were boiled for 10 min and for each isolate, an aliquot of 1.25 µl was used as template for PCR amplification. For identification of *R. solanacearum,* PCR was performed in a final volume of 25 µl prepared in 0.2 ml PCR tubes. The PCR reaction mixture consists of 1 µl of 100 mM dNTPs, 2.5 µl of 10 x buffer, 1 U of *Taq* DNA polymerase, 2.0 µl of each forward and reverse primers of 25 pmol. The PCR tubes were placed in a thermo cycler (Labnet, Multigene gradient, CA. USA). Programmed thermal cycle as initial denaturation at 95°C for 5 min, followed 25 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 30 s, elongation step at 72°C for 1 min and final extension at 72°C for 5 min. The amplification of the primers was checked by diluting single colony bacterial isolates from 10^{-1} up to 10^{-6} in sterile distilled water without isolating DNA. The PCR amplicons were stained with ethidium bromide and gels were documented using Geldoc 1000 System-PC (Bio-Rad, Gurgaon, India). Immediately, the amplified DNA products were excised from the agarose gel with a sterile scalpel and the gel was purified with a QIAquick® Gel Extraction Kit Quiaquick gel extraction Kit (Qiagen, Hilden, Germany) following manufacturer's instruction. DNA was further quantified with NanoDrop 2000, 10 µl eluted samples (20 ng/µl) were used as template for SSCP.

SSCP-PCR analysis

Ten µl of individual eluted (20 ng/µl) PCR products were mixed with 25 µl of denaturing buffer [95% formamide, 20 mM EDTA and 0.05% bromophenol blue]. The mixtures were heated at 96°C for 10 min and immediately chilled with ice (Chandrasekhar et al., 2012; Umesha et al., 2012). Denatured PCR products were loaded on to 8% acrylamide–bisacrylamide non-denaturing gel, containing 8 ml of acrylamide/bis (40%) and 4 ml of 10x TBE, 40 µl of tetramethylethylenediamine, 10% ammonium per sulfate and 28 ml water. Thirty five µl of each mixture was loaded and electrophoresed in pre-chilled 1x TBE buffer at 200 V for 2 h at room temperature. An aliquot of ssDNA ladder was also loaded into gel to facilitate comparison of SSCP fingerprinting patterns which were further stained with silver staining and gels were documented.

LSSP-PCR analysis

The LSSP-PCR analysis was carried out in 20 µl reaction mixture containing 3 µl of amplified DNA template 20 ng/µl, 100 mM dNTPs, 5 µl of 10 x buffer, 2 U of *Taq* DNA, and 50 pmol of RS-Egl-F primer. After denaturation step at 95°C for 10 min, annealing at 35°C for 1 min and extension at 72°C for 1 min, the same experimental conditions were repeated with respect to RS-Egl-R. LSSP-PCR profiles were visualized on silver staining in 8% polyacramide gels after electrophoresis in pre-chilled 1x TBE buffer at 200 V for 2 h at room temperature.

Genetic profiling of 126 *R. solanacearum* isolates which are confirmed by pathogenic test, along with 15 non-pathogenic was screened for inter-specific variability. The LSSP-PCR experiments were conducted using other phytopathogenic bacteria such as *Xanthomonas perforans* and *X. oryzae* pv. *oryzae* were analyzed to verify nonspecific banding patterns and all these experiments were repeated thrice for negative controls. Bands ranging from 100 to 600 bp were selected for phenotypic analysis. LSSP-PCR genetic profiles were scored based on the presence (1) or absence (0) of each amplified banding patterns and bands were compared using matching coefficient of similarity to determine the proportion of mismatched bands among isolates. Cluster analysis was carried out based on similarity of genetic profile from LSSP fingerprints. To assure the reproducibility and stability of genetic signature of LSSP-PCR in these *egl* primers, the experiments were conducted in triplicates with the same specific PCR template for two times. The most identical LSSP-PCR signatures were analyzed with the dendrogram. The obtained clusters from the dendrogram analyzed based on resemblance to original distance using Unweighted Pair Group Method with Arithmetic mean (UPGMA) software associated with arithmetic averages clustering algorithm and the randomization procedure as implemented in Tools for Population Genetic Analyses (TFPGA) (Bomfim and Koury, 2006).

Sequence analysis

To determine the nucleotide sequences of *R. solanacearum*, colony PCR amplified products (237 bp) (Figure 1) were sequenced (Eurofins, Bangalore, India). Prominent LSSP bands (Figure 3 and 4) were selected and used for excision and nucleotide sequence determination by a ''crush and soak'' method (Bharathkumar et al., 2008) and further sequenced. The alignment of nucleotide sequences from the isolates along with the reference isolate was analyzed in CLUSTALW software (http://www.ebi.ac.Uk/ Tools/msa/ clustalw2/). The homologies of the sequences were analyzed using BLAST-N (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analyses of sequences from different isolates were conducted using Mega 6.0 software. Phylogenetic tree was constructed using the neighbour-joining and maximum likelihood (p-distance) with bootstrap test of 1000 repetitions. Nucleotide sequences obtained from the current study have been deposited in the GenBank and obtained their accession numbers (Table 1).

RESULTS AND DISCUSSION

Collection and screening of plant material and soil samples for *R. solanacearum*

In the present study, we have isolated *R. solanacearum*

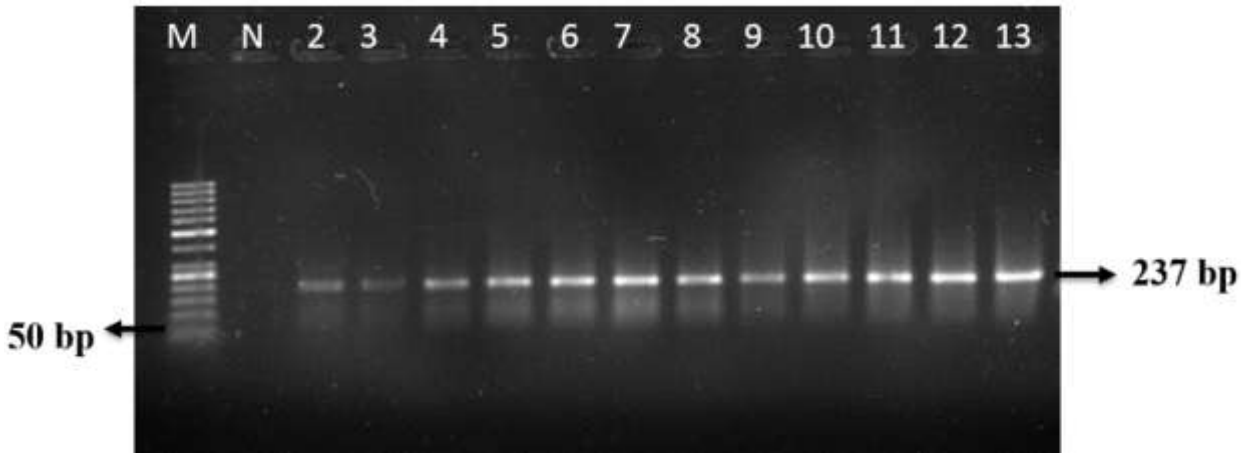

Figure 1. Colony PCR amplification of *Ralstonia solanacearum* with negative control. 1.5% Agarose gel electrophoresis of PCR products. Lane 2 to 7 indicates tenfold serial dilutions of *Ralstonia solanacearum* colony from 10^1 to 10^6. Lane 8-13 amplification of different isolates of *Ralstonia solanacearum*, N-Negative, M: 50-bp DNA ladder.

from agricultural fields and demonstrated the application of SSCP-PCR and LSSP-PCR techniques for molecular characterization. The present study indicated the presence of *R. solanacearum* in soil and plant material from the agricultural fields of India. The morphological identification of *R. solanacearum* showed typical creamy mucoid colony with pink centers which was observed in Lewis et al. (2007) and Avinash and Umesha (2014).

A total of 160 plant samples and 90 soil samples were collected during the field survey from 300 different suspected agricultural fields. Among these, 156 bacterial isolates showed the typical mucoid creamy colony with pink centers on TZC medium. Further *R. solanacearum* isolates were subjected to biochemical/physiological tests which exhibited typical characteristics of *R. solanacearum*. One hundred and fourty one out of 156 bacterial isolates proved to be *R. solanacearum* by biochemical as well as physiological characterization tests by differentiating into 4 different biovars biovras (1, 2, 3, 4) by utilization of carbohydrates (Table 1).

R. solanacearum isolates were preserved in 20% of glycerol stored at -80°C for long term preservation. Pathogenicity test conducted in highly susceptible cultivars of eggplant (cv. Chaman 363) and tomato (cv. PKM 1), exhibited typical wilt symptoms among 76% of isolates. Interestingly, *R. solanacearum* isolates which were isolated from ginger and black pepper did not induce any bacterial wilt symptoms either on tomato or eggplant. Control plants did not show any disease symptoms of bacterial wilt. The greenhouse experiments were performed in four replicates (16 plants each) and derived from three independent experiments with similar results.

Generally, tomato is being used as a biological indicator plant for conducting pathogenicity test of *R. solanacearum* (Hayward, 1991) and recently eggplant (cv. Agassaim) was used to assay pathogenicity of *R. solanacearum*. The present study has an evident that, eggplant cultivar (cv. Chaman 363) can be used for pathogenicity test along with tomato which confirm the earlier results of Ramesh et al. (2014).

R. solanacearum isolates from solanaceae species induce bacterial wilt diseases on eggplant and tomato cultivars, whereas the isolates obtained from the ginger and black pepper were non-pathogenic to these cultivars. Similar observations were reported earlier confirming that *R. solanacearum* isolates from ginger were non-pathogenic to solanaceous vegetables (Mondal et al., 2011).

Colony PCR and purification of amplified product

R. solanacearum subjected to specific PCR assay with developed primers RS-Egl-F and RS-Egl-R revealed specific amplification of a 237 bp product (Figure 1), thus confirming the pathogen as *R. solanacearum*. There are several reports with respect to *egl* specific region from different hosts, for instance ICMP 8229 from ginger, CIP 65 from chilli and E 152 from eggplant, Banana was identified in different geographical regions viz., Philippines, Cost Rica, Malaysia (Lewis et al., 2007).

Similarly, reports were also found with regard to highly virulent *R. solanacearum* strains UW120 and UW276 which are found in Mexico, USA and Kenya in solanaceous plant (Wicker et al., 2007). The results obtained from the present studies correlates previous reports in amplifying *R. solanacearum* isolates from Indian origin using endoglucanase region and confirms the presence of *R. solanacearum* in India (Ramesh et al., 2014; Sagar et al., 2014).

Diversity within the *R. solanacearum* isolates has

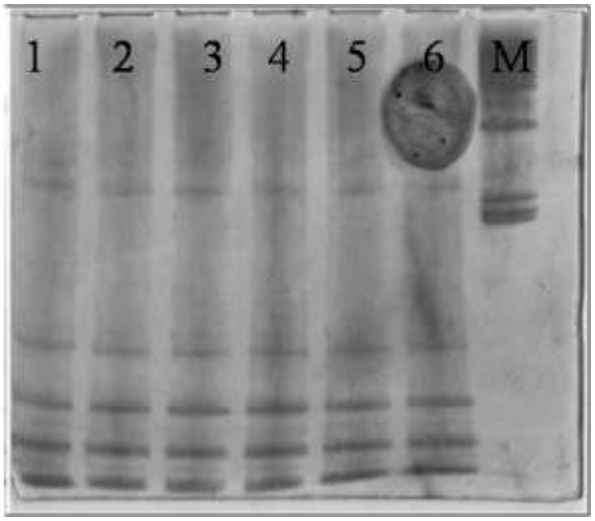

Figure 2. SSCP-PCR profile of *egl* region in *Ralstonia solanacearum* isolates. The purified DNA was denatured and SSCP banding patterns were visualized on non-denaturing polyacrylamide gels. Lane 1-6 indicates isolates of *Ralstonia solanacearum* from different hosts. ssDNA marker (M).

been studied from decades using techniques such as 16S rRNA, AFLP, RFLP, BOX-PCR, rep-PCR and multiplex PCR (Wicker et al., 2007; Parkinson et al., 2013). Even though these techniques are specific, sensitive and rapid but not free from difficulties such as additional enzymes, instrumentation and high cost, are difficult to adopt in developing countries when screening large number of isolates. However the problem can be overcome by simple repetition of PCR and denaturation of the amplified product by SSCP and LSSP-PCR.

SSCP-PCR analysis

In the present study, the SSCP was performed for 141 isolates according to the procedure by Orita et al. (1989) with slight modification as represented in Chandrasekhar et al., (2012). The samples were denaturated to get single stranded DNA followed by 3-dimentional folding of single strands. The motilities of the ssDNA of all tested isolates exhibited similar species–specific banding patterns.

Electrophoresis of denatured 237 bp purified products exhibited four major bands for *R. solanacearum* with respect to *egl* gene genome (Figure 2). There was no variation in the banding patterns of *R. solanacearum* among pathogenic and non-pathogenic isolates from different agricultural fields and variable hosts. These results indicated that the mobility of the molecules during SSCP analysis was similar for all isolates of *R. solanacearum,* along with reference isolate NCIM 5331 and DOBCPR12 (Figure 2). Whereas, SSCP-PCR profile of *R. syzygii*, *R. pickettii* and BDB were distinguish by variation of two to three bands (Supplementary 1).

Due to its robustness, SSCP-PCR can be used for rapid specific identification and differentiation of *R. solanacearum, R. syzygii*, *R. pickettii* and BDB up to species level. The developed technique was unable to show any genetic diversity among the isolates, but the SSCP-PCR profile has great advantage to distinguish the pathogen up to species level, viz., *R. solanacearum, R. syzygii*, BDB, and *R. pickettii*. Similarly, in our previous reports, 40 isolates of *R. solanacearum* were confirmed by 16S rRNA primers, which exhibited four major bands. The other phytopathogenic bacteria *Xanthomonas perforans* and *Xanthomonas oryzae* pv. *oryzae* exhibited two major bands differentiates phytopathogenic bacteria by SSCP-PCR (Chandrasekhar et al., 2012). Colony SSCP-PCR was developed for identification of phytopathogenic bacteria which exhibited SSCP banding patterns of *X. perforans* and *R. solanacearum* (Umesha et al., 2012).

LSSP- PCR analysis

Genetic profiling of *R. solanacearum* from different hosts and geographic origin was analyzed through LSSP-PCR, which showed variations in the genetic profiles with RS-Egl-F.*R.*

Solanacearum exhibited an interspecific variability, comprised of DNA fragments varying from 100 to 600 bp. However, LSSP-PCR profile was able to share similar DNA fragments of 200 and 300 bp in all 141 isolates of *R.*

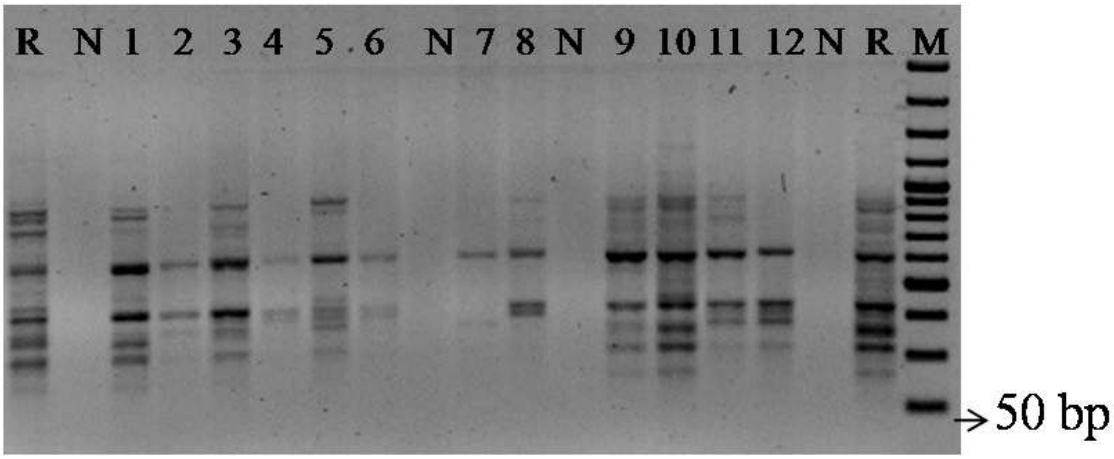

Figure 3. LSSP Signature of *Ralstonia solanacearum* isolated from different hosts in FR/F. Lane 1-6 indicates pathogenic isolated from solanaceae hosts, Lane 7-8 non-pathogenic isolated from solanaceae hosts. Lane 9-10 from ginger, 11-12 isolates are from black pepper, N-Negative control, R-NCIM 5331 reference strain and M-Marker.

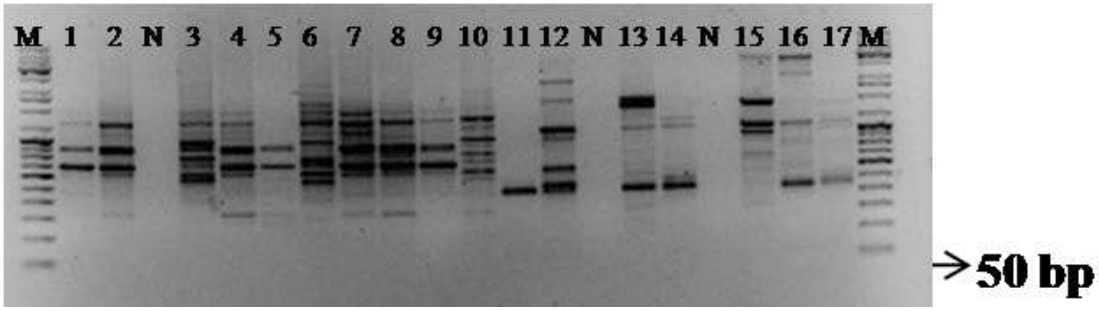

Figure 4. LSSP Signature of *Ralstonia solanacearum* isolated from different hosts in FR/R. Lane 1-2 indicates NCIM 5331 and DOBCPR 12 reference strains, N-Negative control; Lane 3-12 indicates isolates of solanaceous hosts, lane 13-14 from black pepper, 15-17 isolates are from ginger and M-Marker.

solanacearum. Similarly, the variation in the banding patterns at 100, 237, 290, 350, 430 and 500 bp and absence of some banding patterns also exhibited variations with different host range (Figure 3).

LSSP-PCR patterns exhibited four to six bands among *R. solanacearum* isolates which are pathogenic to solanaceous hosts. Whereas, LSSP patterns in 15 non-pathogenic isolates from solanaceous crops exhibited (one-four) decreased in the banding pattern (Supplementary 2) compared to pathogenic isolates. By comparing the size and number of DNA fragments, it was possible to determine the similarity existing among LSSP-PCR patterns obtained from *R. solanacearum* within solanaceous isolates. The developed technique is able to differentiate pathogenic and non-pathogenic isolates of *R. solanacearum* among solanaceous crops. Interestingly, the LSSP-PCR of *R. solanacearum* isolates from ginger and black pepper exhibited high level DNA polymorphism of seven to eight banding patterns (Figure 3). Out of 141 *R. solanacearum* isolates, all isolates showed variable genetic profile when examined by LSSP-PCR using *egl* forward (F) and *egl* reverse (R) primers. The LSSP-PCR analysis of RS-Egl-R primer in *R. solanacearum* isolates revealed diversified banding patterns compared to RS-Egl-F. The genetic profile of the obtained fragments varied with reference to different hosts. Genetic signature determined by Image3 software were made up of fragments ranging from 150 to 800 bp, exhibited an interspecific genetic variability. The similarities in the banding patterns were also observed with respect to reverse primer, amplified at 300, 400 and 600 bp in all 141 *R. solanacearum* isolates. Most similar genetic profiling of *R. solanacearum* was exhibited within the solanaceous host. The *R. solanacearum* isolated from ginger and black pepper exhibited different genetic profiles. There were no amplification from colony/template DNA of pathogens viz., *X. perforans* and *X. oryzae* pv. *oryzae* with respect to forward and reverse primers in LSSP-PCR analysis (Figure 4). The constitutive bands of DNA fragments of *R. solanacearum* were analyzed based

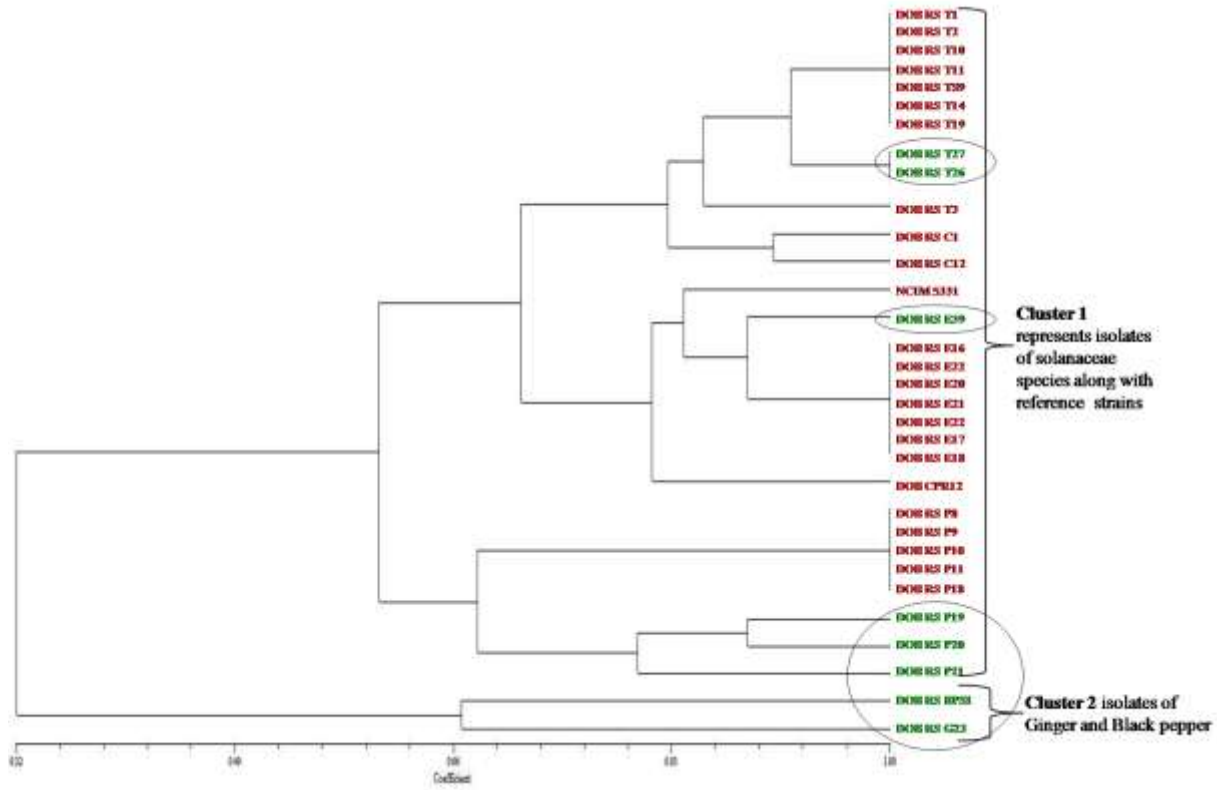

Figure 5. Dendrogram obtained by analysis of LSSP-PCR profile in 32 isolates of *Ralstonia solanacearum* from different hosts. Genetic distance obtained by LSSP-PCR banding profile in selected pathogenic (Red) and non pathogenic (Green, circular) isolates were used to build the phylogenetic tree among 141 isolates. Isolates belonged to the similar genotype of host specific indicated at right. Cluster 1 represents isolates of solanaceae, Cluster 2 represents isolates from ginger and black pepper.

on the data matrix represented by presence or absence of specific fragments using dendrogram. The isolates from distinct hosts were grouped in two main clusters with reference to fragments obtained by LSSP-PCR banding patterns. The *R. solanacearum* isolates obtained from the solanaceae species were grouped in the cluster I, and those obtained from the ginger grouped in the lower cluster in cluster II (Figure 5). The fragment distribution among solanaceae species were diversified among pathogenic and non-pathogenic isolates (Figure 5) when analyzed with UPGMA software along with their significant statistics (0.32-1.00). The polymorphism presumed that the heterogeneity of genetic profile obtained by LSSP-PCR analysis of *R. solanacearum* not only exhibited a frequent genetic diversity, but also exhibited the high interspecific variability existing among the *R. solanacearum* isolates infecting different hosts and pathogenicity. However, the genetic profile and the information of RFLP and AFLP mainly depend on the number of restriction enzymes employed.

In the present study, the *R. solanacearum* signatures obtained were informative and composed of genetic variations in a single LSSP-PCR without addition of multiple restriction enzymes and probes. Dendrogram obtained by the UPGMA cluster algorithm with FR/F and FR/R the distance allows the clustering in two main clusters which differentiates *R. solanacearum* infects solanaceous species and ginger. The polymorphism presumed that the heterogenecity of genetic profile obtained by LSSP-PCR analysis of *R. solanacearum* not only exhibited a frequent genetic diversity, but also showed the high inter-specific variability existing among the *R. solanacearum* infecting different hosts (Figure 5). Whereas similar results, dendrogram of UPGMA cluster analysis of *Leptospira* isolates differentiates the strains from urine and clinical samples of cattle. Similarly, dendrogram obtained by the analysis of LSSP-PCR profile of 40 isolates of *Leishmania infantum* differentiates human and canine samples correlate the present reports (Alvarenga et al., 2012).

Sequence analysis

The alignment of *R. solanacearum* sequence from different hosts of solanaceae members and ginger

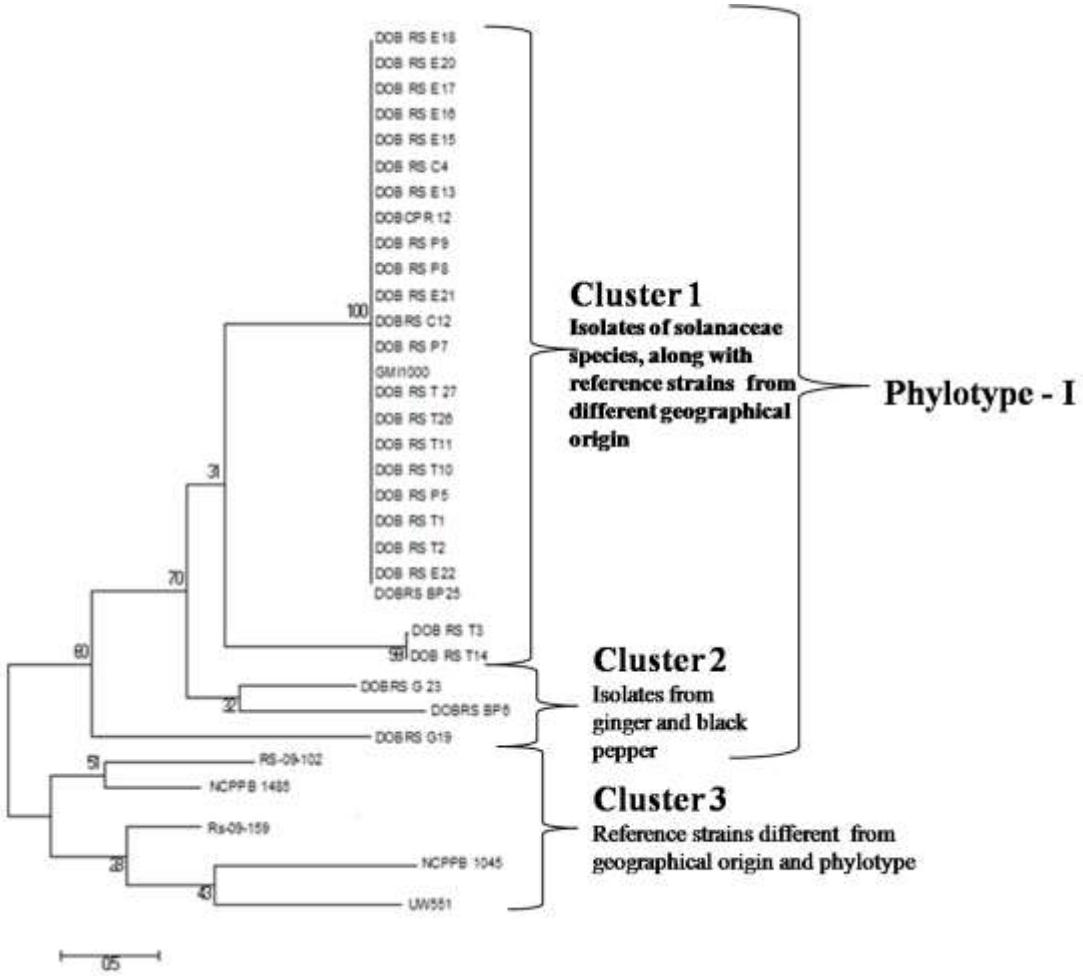

Figure 6. Phylogenetic analysis of *egl* sequences among *Ralstonia solanacearum* isolates. Sequences from NCBI of different isolates were analyzed in Mega 6.0. Phylogenetic tree was constructed using the neighbor-joining maximum likelihood (P-distance). Bootstrap test was for 1000 repetitions. Cluster 1 represents isolates of solanaceae species, Cluster 2 represents isolates from ginger and black pepper. Cluster 3 represents Reference strains different from geographical origin and phylotype.

revealed the presence of some nucleotide substitutions, which were shared within the geographical origin and host range. It can be hypothesised that, the distinct LSSP genetic profile of *R. solanacearum* obtained in this study is due to the presence of polymorphism in the target gene sequences. The sequenced data confirms solanaceae species in infecting *R. solanacearum* isolates which exhibited divergence from ginger isolates as reported earlier (Ramesh et al., 2014; Sagar et al*.,* 2014).

Nucleotide data base showed the presence of four phylotypes, subdivided into sequevars based on *egl* sequence similarities in which Indian isolates belongs to phylotype I and subdivided into two subgroups (Ramesh et al., 2014). Phylogenetic grouping by *egl* sequences of *R. solanacearum* isolates represented as phylotype I, whereas phylotype II, III and IV (Figure 6) were not recorded among isolates of Karnataka. Sequence information and diversity analysis of Indian isolates infecting solanaceous vegetables provides valuable information about the existence of major phylogenetic group, divergence with phylotype I by dividing two clusters (Ramesh et al., 2014; Sagar et al., 2014) which confirm in genetic profiles of LSSP-PCR among our isolates.

Conclusion

The study has demonstrated the application of SSCP and LSSP-PCR for the differentiation of *R. solanacearum* isolated from distinct hosts. The developed Egl-F and Egl-R primers are specific to identify *R. solanacearum* with direct colony PCR which avoids the extra step of DNA extraction. SSCP allowed specific patterns to

distinguish pathogen up to species level. The individual genetic profiles of *R. solanacearum* isolates were obtained by LSSP-PCR; the polymorphic variation discriminates pathogenic and non-pathogenic isolates by LSSP polymorphic banding patterns within and diverged host. The genetic variation of LSSP was confirmed by sequence analysis of isolated strains. The results correlated with the genetic variability of *R. solanacearum* discriminate from diverge host and represented as phylotype I, included strains originated primarily from Asia. Due to, low cost, sensitivity, specificity, simplicity of execution and high reproducible genetic profile, the use of LSSP-PCR technique could be extended to other similar plant pathogenic bacteria identification and can be considered as a valuable microbiological tool to study genetic diversity of quarantine pathogens along with their epidemiology in developing countries.

Conflicts of interest

The authors have not declared any conflicts of interest

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Full Length Research Paper

Lactic acid bacteria from traditionally processed corn beer and palm wine against selected food-borne pathogens isolated in south west region of Cameroon

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The present study was undertaken to assess the inhibitory potential of lactic acid bacteria isolated from traditionally processed corn-beer and palm-wine on *Escherichia coli, Salmonella typhi* and *Staphylococcus aureus.* Lactic acid bacteria were isolated on MRS agar using pour plate method. The catalase negative and Gram positive isolates were selected as presumptive lactic acid bacteria and were biochemically characterized using the API 50 CHL BioMerieux kit to identify them at species level. The LAB isolates were then assessed for antimicrobial activity potentials against food-borne pathogens. Thirteen LAB isolates which constituted nine different species namely: *Lactobacillus fermentum*, *Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus acidophilus, Lactobacillus bulgaricus, Leuconostoc mesenteroides, Leuconostoc carnosum, Pedioccocus acidilactici* and *Lactobacillus pentosus* were isolated from these two beverages. The entire LAB isolates demonstrated great potentials to inhibit the test pathogens. *P. acidilactici* from corn-beer exhibited the overall highest inhibitory activity with zones of inhibition of 19, 20 and 16 mm on *E. coli, S. typhi* and *S. aureus,* respectively; while the isolate from palm-wine, *L. pentosus* exerted the highest antimicrobial action on the test pathogens. It was observed that most of the LAB isolates inhibited the indicator pathogens mainly by bacteriocin production. *S. typhi* was the most susceptible food-borne bacterial pathogen to the inhibitory activity of the LAB isolates, followed by *E. coli*.

Key words: Lactic acid bacteria, food-borne pathogens, antimicrobial activity, probiotics, bacteriocins.

INTRODUCTION

The occurrence of foodborne microbial pathogens and the increased foodborne toxi-infections remain a critical issue in many countries around the world (Newell et al., 2010). The development of foodborne diseases is a

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major health problem in underdeveloped and developing countries. Sub-Saharan Africa is one of the most affected regions (Quilan, 2013). This is associated with poverty and poor hygiene conditions. Salmonellosis, occupy an important place. A recent survey carried out by Nguendo (2014) reveals that 70% of food borne illness cases in Cameroon is associated with the consumption of street foods sold in major cities such as Douala and Yaoundé. Methods used for the treatment of these diseases involve mainly the use of antibiotics. However, the prolonged use of antibiotics in these regions has been indexed as the main cause of the occurrence of bacterial strains resistant to antibiotics. The emergence of antibiotic resistant strains is a major public health problem (Tatsinkou et al., 2016). The exploitation of the inhibitory activity of the lactic bacteria appeared in recent years as an alternative treatment and even prevention of some foodborne toxi-infection (Mezaini et al., 2009; Obi et al., 2015; Khalid, 2011). Indeed the lactic acid bacteria are known to be non-pathogenic and Generally Regarded as Safe, and are increasingly used because of their probiotic properties (Zacharof and Lovitt, 2012; Mariam et al., 2014).

Probiotics are microbial strains and administration in adequate amount is beneficial in humans or animals. Many recent studies have demonstrated the potential of LAB to inhibit the growth of food borne pathogens by various mechanisms, including the production of antimicrobial proteins called bacteriocins (O'Shea et al., 2013).

The traditional processed corn and palm wines are widely consumed in Africa and particularly in Cameroon. They have recently been described as potential niches of lactic acid bacteria. Which are partly involved in the process of fermentation and the flavor of these native drinks. To the authors' knowledge, very few studies have been conducted on the probiotic potential of lactic acid bacteria strains isolated from traditional processed corn beer and palm wine; hence, the need to conduct a study on the probiotic potential of these strains, mainly the inhibitory activity against food pathogens.

MATERIALS AND METHODS

Study site and sample collection

This study was conducted in Fako Division of the South West Region of Cameroon. The specimens were obtained from Buea, Limbe, Tiko and Muyuka, these being the four major towns in Fako division, South west Region of Cameroon. The samples were processed in the laboratory of the School of Assistant Laboratory Technicians of Limbe and the Biotechnology unit of the University of Buea.

Three samples of each of the two specimens (corn-beer and Palm wine) were collected from the four towns in Fako Division to give a sample size of 24. About 50 ml of each fresh sample was poured in sterile wide-mouth collection cups and labelled and the temperature was taken at the spot. Samples obtained in towns further from Limbe were transported in cool packs. Upon arrival at the laboratory, the pH of each sample was measured by using calibrated Universal Litmus pH test strips (LabRat Supplies). Then each sample was separated in two cups. All the samples were continuously labelled as they were being separated. The pH was measured so as to ascertain whether the antimicrobial potentials of the lactic acid bacteria had any relationship with acidity of the samples.

Media preparation and sample processing

All media were prepared following the manufacturer's instruction. The media used were de Man Rogosa and Sharpe (MRS) (Oxoid) agar for isolation of LAB, MRS Broth (Oxoid) for the production of antimicrobials, Nutrient Agar (Liofilchem s.r.i Bacteriology products) for total microbial count, and Muller Hinton Agar (Oxoid) for antimicrobial sensitivity testing. Aseptic techniques were observed throughout the media preparation process. A 1:10 dilution of each sample was made prior to culturing. This was done by diluting 1 ml of the sample within 9 ml of physiological saline (0.85% NaCl). Further, ten-fold serial dilutions ranging from 10^{-1} to 10^{-5} were prepared. The 10^{-5} diluted samples were used for culture on MRS Agar and Nutrient agar.

Isolation and phenotypic identification of LAB

Samples for the isolation of lactic acid bacteria were cultured on MRS Agar. The pour plate method was employed; about 1 ml of each of the 10^{-5} diluted sample was pipette into separate sterile plates and about 15 ml of the prepared molten MRS agar was poured on it. The plates were then gently rotated clockwise and anti-clockwise so as to allow for a homogeneous distribution of the agar and the diluted sample. The agar was allowed to solidify, then inverted and incubated at 30°C for 48 h.

At the end of the incubation period, the MRS plates were observed for colony formation. Colonies which were different from each other in their morphology and phenotypic appearance were picked up using a sterile inoculating loop and were purified on MRS Agar by re-streaking on plates until only a single type of colony was present. The different pure cultures so obtained were characterized for their colony morphology and subjected to Gram staining and catalase test. Colonies found Gram positive, non-motile, rod shaped bacteria that demonstrated a catalase negative result were selected as presumptive lactic acid bacteria. They were then preserved on MRS Agar slants and store at 4°C for further investigations.

The identification of lactic acid bacteria at species level was done by biochemical characterization using the API 50CH kit (BioMerieux, France). The API 50 CH is a standardized system that associates the fermentation of 50 carbohydrates to bacteria species. It is used for the identification of *Lactobacillus* and related genera.

A positive test corresponds to the acidification revealed by the bromocresol purple indicator contained in the medium changing to yellow. For the esculin test (tube no. 25), a change in colour from purple to black was interpreted as positive (+). The biochemical profiles obtained for the LAB strains was analyzed using the API identification software database (API LAB PLUS), Version 5.

Determination of antimicrobial activity

Preparation of the sample filtrate

Each presumed LAB isolate was inoculated from slants into 5 ml of MRS broth and incubated at 37°C for 24 h. The culture broth of each isolate was then centrifuged at 6,000 rpm for 10 min. The cell free supernatant (CFS) was then collected and passed through a 0.2µm sterile syringe filter and stored at -20°C prior to use. The CFS was thus used for susceptibility testing, determination of

minimum inhibition concentration (MIC) and determination of the antimicrobial substances.

Sources of food-borne pathogens

The food borne bacteria pathogens that were used for this study were provided by the Laboratory of Microbiology of the Buea Regional Hospital Annex and Limbe Regional hospitals. They were isolated from stools of patients with symptoms of food borne diseases and characterized and identified by API 20 A, 20 E, 20 Staph Kits (BioMerieux, France). We were given pure cultures that were store on slant and preserved at 4°C.

Susceptibility testing by the agar well diffusion method

The Agar well diffusion methods suggested by Tagg and McGiven (1971), was modified and used to test for the antimicrobial activity of the presumed LAB isolates against selected food borne pathogens. The pure cultures of the selected foodborne pathogens were inoculated from slants to about 5 ml Muller Hinton broths and incubated for 24 h at 30°C. About 1% of the 24 h culture of the pathogen was suspended in 9 ml of normal saline and adjusted to 0.5 McFarland standards. A lawn of the indicator strains were then made by spreading the cell suspension over the surface of Muller Hinton agar plates with a sterile cotton swab. The plates were allowed to dry and a sterile cork borer of diameter 5 mm was used to bore uniform wells in the agar. Each well was then filled with 100 µl of the different concentrations of the cell-free supernatant (LAB culture filtrate). After incubation at 37°C for 48 h, the plates were observed for a zone of inhibition around the well. The antimicrobial activity was expressed as the diameter of the inhibition zones around the wells. Results were considered positive if the diameter of the zones of inhibition were greater than or equal to 7 mm. The negative control used was 100 µL of distilled water while the positive control used were 100 µl of diluted Ciprofloxacin and Azithromycin.

Determination of minimum inhibition concentration

The MIC was performed using the broth dilution method, by following the modified version of the procedure described in the BSAC Guide to Sensitivity Testing (1991). In this method, 4 to 5 isolated colonies of the test pathogens were obtained and cultured overnight at 37°C on Muller Hinton agar. The cultures were standardized using standard microbiological techniques to have a concentration of very near 1 million cells per milliliter (10^6 cfu/mL). After overnight incubation of the test pathogen, the CFS (antimicrobial substance) of LAB isolates were then diluted using Muller Hinton Broth, beginning with 1/2 dilution up to 1/12 serial dilutions. The different test pathogens were then inoculated into each of the diluted tests tubes to a final density of 5×10^5 cfu/ml. The tubes were then incubated for 18 h at 37°C. After the incubation time, the tubes were inspected for growth of the test pathogens. Meanwhile, aliquots of growth control (with no antimicrobial substance) were plated to verify cfu/ml counts of viable bacteria. These were equally incubated under the same conditions and colonies were counted. The MIC was recorded as the lowest concentration of the antimicrobial substance that prevents the appearance of visible turbidity.

Determination of inhibitory substance

The Cell free supernatant (CFS) of each LAB Isolate was prepared as stated above. Each sample filtrate was then separated into 3 different sterile test tubes for the determination of antimicrobial substance due to bacteriocin, acid and hydrogen peroxide, respectively. The process was as follow:

1. The CFS in the first tube was used to determine whether the antimicrobial activity is due to bacteriocin. The CFS in this tube was thus adjusted to pH 7.0 by the addition of sterile 1 N sodium hydroxide (NaOH), to eliminate any effect of acidity; while inhibitory activity due to hydrogen peroxide was eliminated by the addition of a catalase enzyme (5 mg/dl), Amplex Red Catalase Assay Kit (A22180). The filtrate was then used for susceptibility studies.
2. The CFS in the second tube was used to determine whether the inhibitory substance is an acid. Thus, catalase enzyme (5 mg/dl) was added to this tube to eliminate any inhibitory activity due to hydrogen peroxide. The tube was then heated in boiling water (100°C) to denature proteins and thus eliminate any inhibitory activity due to the presence bacteriocin in the CFS.
3. The CFS in the third tube was used to determine whether the inhibitory substance is hydrogen peroxide (H_2O_2). Here, the CFS was adjusted to pH 7.0 by the addition of sterile 1 N NaOH to eliminate inhibitory effect due to acid.

The catalase enzyme (5 mg/dl) was added to this tube to eliminate any inhibitory activity due to hydrogen peroxide. The pure cultures of the selected food borne pathogens were inoculated from slants to about 5 ml MRS broths and incubated for 24 h at 30°C. About 1% of the 24 h culture of the pathogen was suspended in 9 mL of normal saline and adjusted to 0.5 McFarland standards. A lawn of the indicator strains were then made by spreading the cell suspension over the surface of Muller Hinton agar plates with a sterile cotton swab. The plates were allowed to dry and a sterile glass rod of diameter 3 mm was used to bore uniform wells in the agar. Each well was then filled with 10 µL of the different cell-free supernatant (LAB culture filtrate). After incubation at 37°C for 24 h, the plates were observed for a zone of inhibition around the well. The antimicrobial activity was expressed as the diameter of the inhibition zones around the wells. Results were considered positive if the diameter of the zones of inhibition were greater than or equal to 4 mm. The negative control used was sterile distilled water while the positive control used were 100 IU of diluted Ciprofloxacin and Azithromycin.

RESULTS AND DISCUSSION

Characteristics of samples

The measurement of the pH shows that corn-beer is more acidic in nature with a pH range of 6.18 at collection time to 4.32 after 48 h fermentation; against 6.51 to 4.66 for palm-wine. However, palm wine taste more sour than corn-beer. This may be because corn-beer is richer in moulds and yeasts than palm wine (Ogbonnaya and Bernice, 2012). These microorganisms may produce organic acid which might have an influence on the souring taste of the corn-beer.

The lactic acid bacteria population in corn-beer ranges from 1.2×10^7 to 6.7×10^7 CFU/mL as against 2.5×10^7 to 7.5×10^7 CFU/ml for palm-wine. Thus, palm-wine is richer in LAB population than corn-beer. The sludge of corn-beer and the sap of the palm tree have been shown to be a rich medium capable of supporting the growth of various types of microorganisms. The dominant populations of microorganisms include aerobic meso-philic bacteria, yeasts, moulds and lactic acid bacteria (Chandrasekhar et al., 2012; Parveens and Hafiz, 2003).

Table 1. Distribution of lactic acid bacteria in palm-wine and corn beer samples.

Isolate	Palm wine	Corn beer
	Frequency (%)	Frequency (%)
L. fermentum	0 (0.0)	2 (10.5)
L. plantarum	3 (21.4)	3 (15.8)
L. mesenteroides	3 (21.4)	3 (15.8)
L. brevis	3 (21.4)	3 (15.8)
L. carnosum	3 (21.4)	1 (5.3)
L. acidophilus	0 (0.0)	2 (10.5)
L. bulgaricus	0 (0.0)	1 (5.3)
P. acidilactici	0 (0.0)	4 (21.1)
L. pentosus	2 (14.3)	0 (0.0)
Total	14 (100)	19 (100)

Frequency = number of the strain/number of isolates x100.

Isolation, characterization and identification of LAB

Following culture on MRS agar, a total of thirty-three isolates Gram positive and catalase negative rods, cocci or tetrads were obtained from palm-wine and corn-beer, as presumptive lactic acid bacteria. Of this number, fourteen were from palm-wine and nineteen from corn-beer. The isolates from corn-beer were coded Cb1, Cb2, Cb3, Cb4, Cb5, Cb6, Cb7, Cb8, Cb9, Cb10, Cb11, Cb12, Cb13, Cb14, Cb15, Cb16, Cb17, Cb18 and Cb19; while those from palm wine were coded Pw1, Pw2, Pw3, Pw4, Pw5, Pw6, Pw7, Pw8, Pw9, Pw10, Pw11, Pw12, Pw13 and Pw14. These isolates were further biochemically characterized and identified using the API 50 CHL Kit (BioMerieux, France).

The nineteen catalase negative isolates obtained from corn beer were identified using API 50 CHL BioMerieux Kit. They were identified as *Lactobacillus plantarum* (Cb1*,* Cb2, Cb3, Cb8), *Lactobacillus brevis* (Cb5, Cb12, Cb19)*, Leuconostoc mesenteroides* (Cb4, Cb6, CB15) and *Leuconostoc carnosum* (Cb7); *Pediococcus acidilactici* (Cb9, Cb11, Cb13, Cb17), *Lactobacillus fermentum* (Cb10), *Lactobacillus acidophilus* (Cb14, Cb16), *Lactobacillus bulgaricus* (C18) while the fourteen (14) LAB isolates from corn-beer, were found to consist of mainly 5 different species namely*, L. plantarum* (Pw2, Pw5, Pw11) , *L. brevis* (Pw1, Pw12, Pw14)*, L. mesenteroides* (Pw3, Pw9, Pw13)*, L. carnosum* (Pw4, Pw7, Pw10)*, L. pentosus* (Pw6, Pw8)*.* The distribution of the LAB isolates is given in Table 1.

Nineteen presumptive LAB isolates were obtained from corn-beer samples from which 8 different LAB species (*L. fermentum, L. plantarum, L. mesenteroides, L. brevis, L. Carnosum, P. acidilactici, L. acidophilus* and *L. bulgaricus*) were identified (Table 1). Meanwhile fourteen (14) presumptive LAB isolates were obtained from palm-wine samples from which 5 different LAB species (*L. brevis, L. plantarum, L. mesenteroides, L. carnosum* and *L. pentosus*) were identified (Table 1). This is somehow contradictory to the fact that palm-wine is richer in LAB population. However, it can be concluded that corn-beer is richer in the content of LAB isolates than palm wine while palm wine is richer in LAB population than corn beer. Four LAB species namely: *L. plantarum, L. mesenteroides, L. brevis* and *L. carnosum* were commonly isolated from both corn-beer and palm-wine samples. However, four other LAB species (*P. acidilactici, L. acidophilus and L. bulgaricus*) were isolated only from corn-beer, while just one LAB species (*L. pentosus*) was isolated only in palm-wine samples. Thus, there is some basic similarity in the content of LAB species found in both beverages.

It can be observed from Tables 1 and 2 that lactobacilli and leuconostocs were the sole lactic acid bacteria isolated from palm wine samples, with lactobacilli being the predominant LAB (57.1%). Lactobacilli are also the predominant LAB in corn-beer (57.9%). This result is similar to the finding of Nwachukwu et al. (2010). They have isolated similar species in traditional weaning food called “Ogi” in Nigeria

Determination of antimicrobial activity

Figure 1 shows the plate assays for inhibitory activity of some LAB isolates against indicator food borne pathogens. All the LAB isolates from corn-beer inhibited the indicator food-borne bacterial pathogens to some degree. The inhibitory activity and the MICs are summarized in Figures 2 and 3, respectively. *Pediococcus acidilactici* manifested a very remarkable antimicrobial effect on the tested food-borne pathogens with mean zones of inhibition of 19, 20 and 16 mm; and MICs of 64, 64 and 32 mg/ml (Figure 3), on *E. coli, S. typhi* and *S. aureus,* respectively. These LAB species were obtained from samples of all the towns in Fako Division with no significant difference in their zone of inhibition. These results were similar to those obtained by Lee et al. (2013). These authors reported the ability of LAB such as *L. rhamnosus* and *L. lactis* to inhibit *Clostridium difficile*.

On the other hand, all the LAB isolates from palm-wine equally inhibited all the food-borne bacterial pathogens tested, by very significant degrees (Figure 2). *L. pentosus* manifested the most remarkable antimicrobial effect from these palm wine samples with mean zones of inhibition of 15, 17 and 15 mm (Figure 2); and MICs of 32, 64 and 32 mg/ml (Figure 3), on *E. coli*, *S. typhi* and *S. aureus,* respectively. These LAB species were obtained only from palm-wine samples from Tiko and Buea and there was no significance difference in their zones of inhibition on the indicator pathogens.

Meanwhile, *L. carnosum* from corn-beer samples manifested the least antimicrobial inhibitory potential on the tests organism, while *L. brevis* from palm-wine manifested the least. These two LAB species are found in

Table 2. Inhibitory substances of the LAB isolates from corn-beer by zones of inhibition.

Bacteria	Inhibition zone on the indicator food borne pathogen (mm)								
	E. coli			*S. typhi*			*S. aureus*		
	B	H	A	B	H	A	B	H	A
L. fermentum	7	8	-	5	6	4	8	6	5
L. plantarum	9	6	-	6	4	-	8	6	-
Lc. mesenteroides	-	8	5	4	14	-	10	4	-
L. brevis	8	6	-	7	5	4	7	4	-
L. carnosum	11	-	-	7	4	-	-	4	6
P. acidilactici	12	-	6	4	-	15	4	-	4
L. acidophilus	4	4	5	10	5	-	5	-	4
L. bulgaricus	8	-	4	6	7	-	8	7	-
Total	59	32	20	49	45	23	50	31	19
Percentage	50.86	27.59	17.24	40.83	37.50	19.17	49.50	30.70	18.81
Missing (%)		4.31			2.5			0.99	

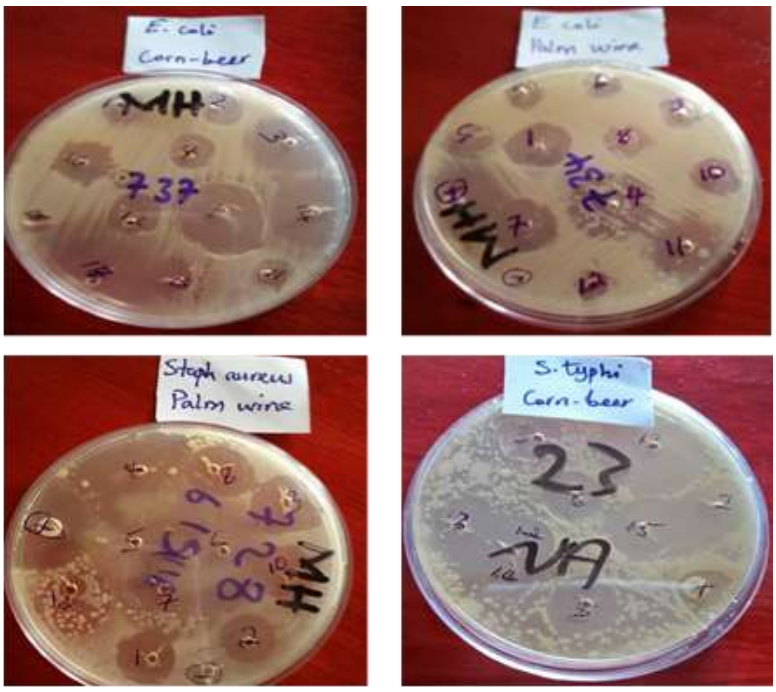

Figure 1. Plate assays for inhibitory activity of lactic acid bacteria on potential foodborne pathogens determined by well diffusion methods.

born corn-beer and palm-wine.

Generally, LABs from corn-beer have more antimicrobial inhibitory potential on food-borne bacterial pathogens than LABs from palm-wine. However, the least inhibitory potential on food borne pathogens was observed from corn-beer and was impacted by *L. acidophilus* (07 mm) and *L. carnosum* (9 mm) on *S. aureus*.

The most susceptible food-borne pathogen to LAB antimicrobial from corn-beer and palm-wine was *S. typhi*. This is interesting because *Salmonella* sp. has been reported as one of the leading cause of illnesses due to food-borne pathogens, and the pathogen has become very resistant to many antibiotics in Cameroon (Akoachere et al., 2009). Therefore, antimicrobials produced by lactic acid bacteria from corn-beer and palm-wine can be exploited for the treatment and prevention of *S. typhi* infections. On the other hand, the least susceptible food-borne pathogen is *S. aureus*. This is of little worrying because, since the emergence of

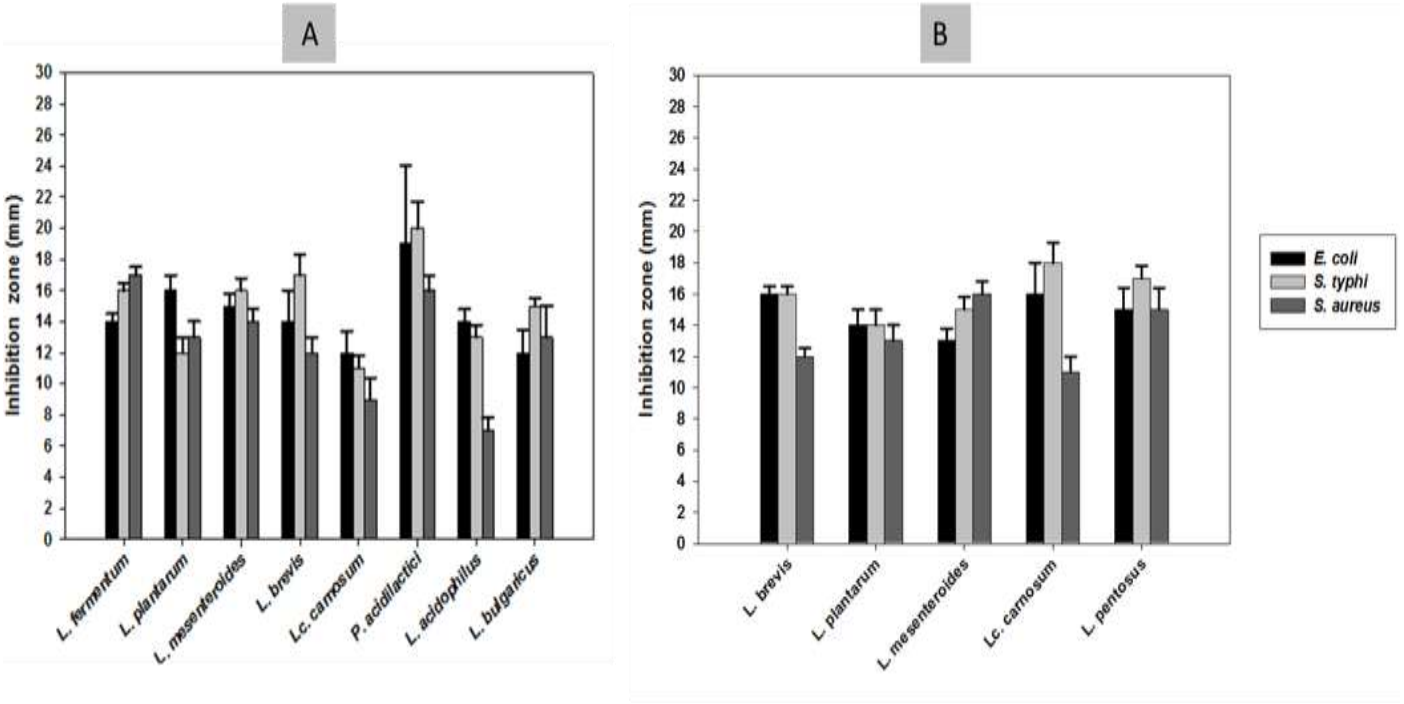

Figure 2. Inhibitory activity of LAB against food borne pathogens (A) corn beer isolates, (B) palm wine isolates. The data shown are averages of triplicate assays with SD within 10% of mean value.

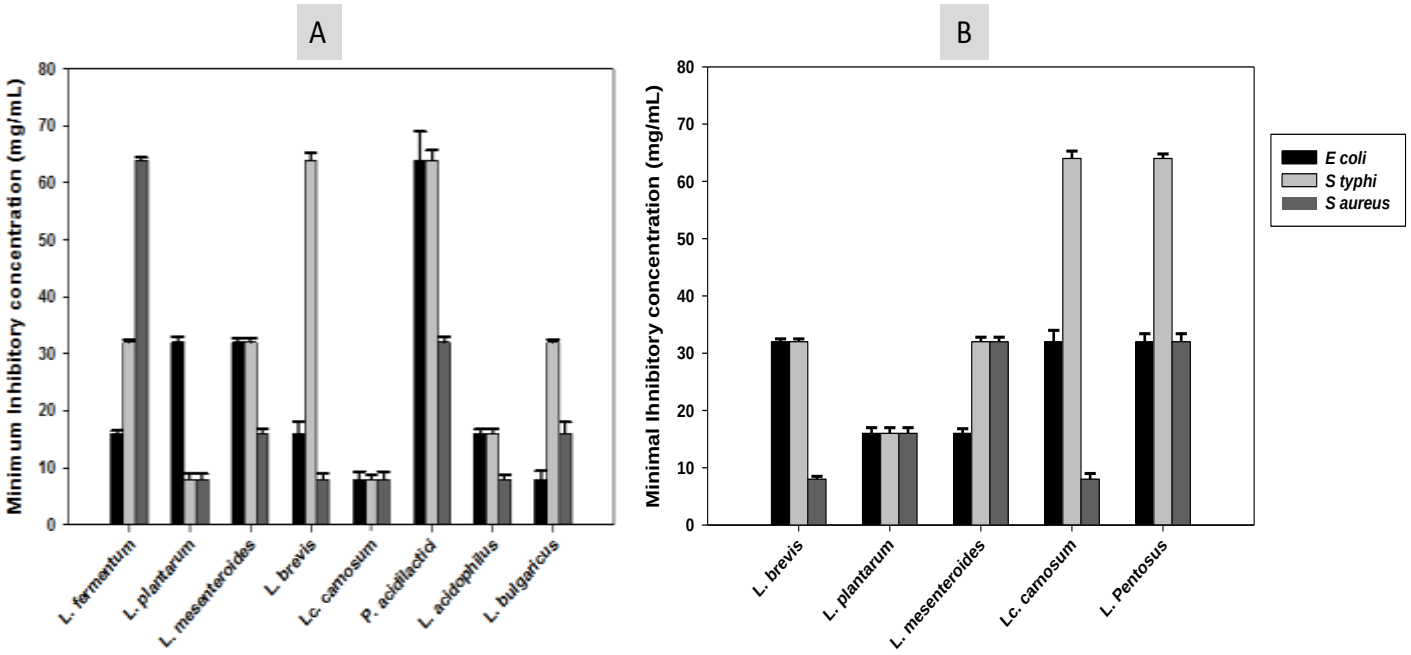

Figure 3. Minimal Inhibitory concentration of supernatant of lactic acid bacteria cultured in MRS broth: (A) Corn beer isolates, (B) palm wine isolates. The data shown are averages of triplicate assays with SD within 10% of mean value.

methicillin resistant *S. aureus* (MRSA), *Staphylococcus* has become resistant to many antibiotics. The inhibitory properties of LAB antimicrobials would have been a reliable candidate to look up in an attempt to cub

Table 3. Inhibitory substances of the LAB isolates from palm-wine.

Bacteria	Inhibition zone on the indicator food borne pathogen (mm)								
	E. coli			*S. typhi*			*S. aureus*		
	B	H	A	B	H	A	B	H	A
L. brevis	9	7	-	4	6	4	6	7	-
L. plantarum	8	4	-	8	6	-	7	5	-
Lc. mesenteroides	-	9	7	6	8	-	10	4	-
L. carnosum	14	-	-	10	7	-	-	-	9
L. pentosus	4	7	4	6	8	6	4	6	8
Total	35	27	11	34	35	10	27	22	17
Percentage (%)	47.30	36.49	14.90	42.5	43.75	12.5	40.30	32.84	25.37
Missing (%)	1.31	1.25	1.49						

Staphylococcal infections but this does not look very promising. However, *S. aureus* was greatly inhibited by antimicrobial from *L. fermentum* (17 mm).

Determination of the inhibitory substance of the LAB isolates

All the LAB isolates inhibited the indicator food borne pathogens tested to some degree by one or two or all of the tested antimicrobial substances namely: Bacteriocins (B), hydrogen peroxide (H) and organic acids (A) (Tables 2 and 3). However, the LAB isolates from corn-beer inhibited the majority of the pathogens by mostly their bacteriocins (Table 2), while the LAB isolates from palm-wine inhibited the indicator pathogens by mostly their bacteriocins and hydrogen peroxide (Table 3). Acid exerted the least inhibitory action on the test pathogens. Since LAB samples showed a high acidity, the real effect of the acid on the indicator pathogens might have been lost in the initial medium in which the LABs were contained. The missing percentages suggest that other factors may contribute to inhibition of the food-borne pathogens, rather than just bacteriocin, hydrogen peroxide and acid. Example of such factors may be bacteriophages. The missing value may also be due to the loss in the synergetic effect of the inhibitory substances by the study methodology.

Following the profound antimicrobial effect exerted by LAB isolates on indicator pathogens, and the determined antimicrobial substances to that effect, the purification of antimicrobial substance produced by LAB from corn-beer and palm-wine can be of great medicinal value to the fight against food-borne pathogens. It is worth noting that bacteriocins from LAB are Generally Regarded as Safe (GRAS) (O'Shea et al., 2013).

Conclusion

The findings obtained in this study showed that traditionally processed corn-beer and palm-wine, are particularly rich in their content of lactic acid bacteria whose antimicrobial properties can be exploited in the fight against human diseases especially food-borne pathogenic bacteria. Bacteriocins were the most common inhibitory substance utilized by the LAB isolates from corn-beer and palm-wine to inhibit food borne pathogens. *S. typhi* appeared to be the most susceptible food-borne bacterial pathogen to the LAB. Therefore, corn-beer and palm-wine obtained from Fako division of Cameroon can display great potentials for the development of antimicrobial against bacterial food borne pathogens.

Conflicts of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Detection of microbial surface contamination and antibiotic resistant *Escherichia coli* on beef carcasses in Arusha, Tanzania

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Food serves as an important fomite for transmission of disease-causing and antibiotic resistant bacteria to humans. Because this may be an especially challenging problem in low-income countries, the level of microbial surface contamination and abundance of antibiotic resistant *Escherichia coli* on beef carcasses were estimated. Out of 125 surface swab samples (plated on MacConkey agar), 70 to 100% were positive to Gram-negative bacteria and *E. coli*, respectively. More than 50% of individual carcasses had bacterial loads below the maximum threshold recommended by the FAO. For carcasses in small- and medium-scale facilities, the average load of bacteria ranged between 0.8 and 1.5 log cfu/cm^2, while carcasses in the large slaughter facility had an average loads of between 1.77 and 1.42 log cfu/cm^2. Of the 1,272 *E. coli* isolates tested, 49.4% were resistant to at least one antibiotic. Isolates were frequently resistant to tetracycline (21.7%) and ampicillin (19.2%) while the frequency of resistance to the remaining nine antibiotics was <3%. In addition, 5.3% of isolates were multidrug resistant with 18 different phenotypes. The combination of resistance to ampicillin and tetracycline was the most common. Although, poor sanitation practices were observed, results reflect lower bacterial counts and limited prevalence of antibiotic resistant *E. coli* relative to other reports in the literature.

Key words: *Escherichia coli*, antibiotic resistance, slaughterhouse hygiene, meat contamination, public health.

INTRODUCTION

There is a clear correlation between the use of unhygienic slaughterhouse practices and the incidence of meat-borne disease outbreaks (Sousa, 2008; Ali et al., 2010) in part, because the nutrient composition and water activity of meat is attractive to a broad spectrum of microorganisms (De Filippis et al., 2013). High microbial surface contamination of beef carcasses constitutes a significant risk to meat handlers and consumers

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Table 1. The acceptable level of bacterial load in red meat (USDA – FAO, 2013). All values are log-transformed colony forming units per square centimeter of surface sampled. Samples were collected after carcass is dressed.

Organisms	Acceptable	Critical/Marginal	Not acceptable
Total bacteria	<3.5	3.5-5.0	> 5.0
Total coliforms	<1.5	1.5-2.5	> 2.5
E. coli	≤ 0.8	> 0.8 and ≤ 1.8	> 1.8

especially during processing and/or eating contaminated meat or meat products (Heiman et al., 2015). In an effort to combat these challenges, the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) have established maximum microbiological surface contamination guidelines for bacterial load on the surface of meat carcasses (Table 1). In many instances, carcass surfaces have been contaminated with enteric bacteria during evisceration and/or from the contaminated tools and surfaces of slaughterhouses. Therefore, the microbial quality of carcass surfaces should be correlated with the standard operating protocols and hygiene practices of abattoirs (Wheatley et al., 2010).

Enteric bacteria (e.g. Gram-negative, particularly *Escherichia coli*) from beef cattle comprise the primary microbial contaminants of carcass surfaces (Ali et al., 2010). These bacteria can be pathogenic or non-pathogenic and can harbor antibiotic resistance traits that could be spread to the microbiota of people and other animals (Nys et al., 2004). *E. coli* is typically part of the fecal bacterial community in cattle and can acquire antibiotic-resistance traits quickly through horizontal gene transfer (Silva et al., 2014). It is also relatively simple to identify by using selective media. Thus, *E. coli* is often selected as a representative organism to evaluate microbial contamination because its susceptibility patterns reflect the diversity of resistance in a bacterial population (Medeiros et al., 2011).

From a broader perspective, antibiotic resistance is considered the 3rd major public health challenge of the 21st century (Spellberg et al., 2008). Food, including beef, can play an important role as a fomite for transmission of antibiotic resistant bacteria to people (Christopher et al., 2013). Studies in Tanzania have shown that among the 9% of children's death caused annually by pathogenic *E. coli*, at least 1/3rd of the pathogens were resistant to antibiotics (Huynh et al., 2015). Despite disease outbreaks caused by multidrug resistant (MDR) *E. coli* in children and surgical patients (Manyahi et al., 2014), little is known about the prevalence of antibiotic resistant enteric bacteria from food animal sources in Tanzania.

Therefore, we evaluated the microbial quality and prevalence of antibiotic resistant *E. coli* on beef carcass' surfaces. We also studied the impact of the size of the slaughter facilities and the carcass handling protocol in the prevalence of bacteria and antibiotic resistance on the surface of beef carcass in the Arusha region of Tanzania.

MATERIALS AND METHODS

MacConkey agar, Hi-Chrome agar and Luria-Bertani (LB) broth (Becton, Dickson and Company, Sparks, MD, USA) were used for bacterial enumeration, isolation and culture. Unless otherwise specified, incubation was overnight (16 – 18 h) at 37°C for all procedures. Antibiotic susceptibility patterns of all *E. coli* isolates were determined by agar dilution method with the minimum inhibitory concentration (MIC) breakpoints informed by Clinical and Laboratory Standards Institute guidelines (CLSI, 2014). The stock solutions of antimicrobials were prepared by mixing antibiotic powder with water or with dimethyl sulfoxide (DMSO, Becton, Dickson and Company, Sparks, MD, USA) and stored at 20°C.

Sampling strategy

To examine the association between microbial surface contamination and sanitation procedures in abattoirs, the presence/absence of basic slaughter house facilities including slaughtering and carcass cleaning protocols were noted as recommended by Small Slaughterhouses and Meat Hygiene for Developing Countries (WHO publication VPH 83.56). The carcass surface swab samples were collected to determine the load of cultivatable Gram-negative bacteria including *E. coli.* Observations and samples were collected from five cattle slaughter facilities involving five sampling events between May and July, 2015 in Arusha region, Tanzania. The number of animals slaughtered at these facilities varied between 2 and >100 per day. Based on the number of animals slaughtered per day, these facilities were considered small- (2-10, sites A and B), medium- (18-25, sites C and D) and large- (>100, site E) scale facilities (Table 2). All facilities slaughtered animals between 3 am and 6 am. Carcasses were selected for sampling based on their availability. For this project, it is surmised that large and modernized slaughter facilities would maintain preferable slaughter and sanitary procedures resulting is less surface contamination to the meat.

Sample collection

Sterile, pre-moistened (with sterile water) 35 cm^2 gauze pads were used to swab approximately 100 cm^2 of surface area on two sides of each carcass immediately after slaughtering (small and medium scale) and in large-scale facilities immediately before carcasses were moved to the chilling facility (all other slaughter procedures were complete at this time). The moistened pads were prepared in a sterile 50 ml conical tube containing 15 ml of sterile water for Gram-negative bacteria (*E. coli*) as transport medium. After rubbing, the gauze pads were placed into the same sterile tube and transported (within 2 h of collection) to the laboratory for further processing in ice-cold boxes.

Table 2. Distribution of Gram-negative and *E. coli* positive meat samples and the Log_{10} colony forming units of total Gram-negative count and *E. coli* of the beef carcass' samples collected from five slaughter houses located in the vicinity of Arusha, Tanzania.

Abattoir	N	Positive samples (%)		Microbial load (Log_{10} CFU/cm^2) Average (%) ±SE (95% confidence interval)	
		Gram-negative bacteria[1]	*E. coli*	Gram-negative bacteria[1]	*E. coli*
Small (2-10)[#]					
A	20	100	90	1.42 ± 0.26 (1.16-1.67)	1.05 ±0.32(0.73-1.36)
B	20	100	85	1.36 ± 0.21 (1.15 -1.57)	0.94 ± 0.25(0.69-1.19)
Average		100	87.5	1.39	0.99
Medium (18-25)[#]					
C	25	100	76	1.53 ± 0.16 (1.37 - 1.69)	0.70 ± 0.21(0.49-0.91)
D	20	95	70	1.02 ± 0.26 (0.76-1.28)	0.62 ± 0.26(0.37-0.88)
Average		97.5	73	1.29	0.65
P-value		0.5	0.069	0.66	0.0082
Large (30 – 100)[#]E	40	100	95	1.77 ± 0.16 (1.61 - 1.92)	1.42 ± 0.16(1.25-1.58)

[#]Number of animals slaughtered per day. [1]Counts of all the bacteria grew on Mac Conkeyagar plates. SE– Standard error; CFU – colony forming units. N- number of swabs.

Enumeration and isolation of *Escherichia coli*

Swabs were mixed well in the transport media (sterile water) and approximately 300 µl of suspension was plated onto MacConkey agar plates with the help of sterile glass beads. The plates were air-dried briefly and incubated overnight. After incubation, plates were examined for the presence of bacterial colonies that were then enumerated as the total number of Gram-negative bacteria. Colonies with morphology similar to *E. coli* (pink to dark rose lactose fermenting colonies) were enumerated separately and up to 12 isolates were picked using sterile tooth-picks and inoculated into 150 µl of Luria-Bertani broth within individual wells of 96-well assay plates. These presumptive *E. coli* isolates were confirmed for their identity by using Hi-Chrome agar. Colonies that exhibited morphology similar to *E. coli* on Hi-chrome agar were added with glycerol (15% final concentration vol/vol) and stored at -80°C for further characterization. Colonies that did not meet these selection criteria were not included in the analysis of *E. coli* isolates (Mwanyika et al. In press). *E. coli* counts were reported as colony forming units per cm^2 (cfu/cm^2) of carcass surface (100 cm^2 equal to 15 mL of swab wash).

Determining antibiotic susceptibility

To determine the prevalence of antibiotic resistance up to 12 *E. coli* isolates from each sample were used with a breakpoint assay (Galland et al. 2001). Briefly, each isolate was tested against 11 antibiotics with their corresponding break point concentration listed by Clinical Laboratory Standards Institute (Tadesse et al., 2012). These were: Ampicillin (Amp, 32 µg/ml, VWR International LLC, Sanborn, NY, USA), amoxicillin (Amx, 32 µg/ml, MP BiomedicalsLLC, Solon, OH), ceftazdime (Ceft, 8 µg/ml, SIGMA-ALDRICH Co., St.Louis , MO), cefotaxime (Ctx, 8 µg/ml, Chem-Impex International INC, Wood Dale, IL), ciprofloxacin (Cip, 4 µg/ml, Enzo Life Sciences Inc, Farmingdale, NY), chloramphenicol (Chlo, 32 µg/ml, Medaitech Inc., Manassas, VA), gentamycin (Gen, 32 µg/ml, Mediatech Inc.), streptomycin (Str, 16 µg/ml, AmrescoInc., Solon, OH), sulfamethoxazole (Sul, 512 µg/ml, MP Biomedicals), tetracycline (Tet, 16 µg/ml, MP Biomedicals) and trimethoprim (Tri, 8 µg/ml, MP Biomedicals).

MacConkey agar plates (150 mm diameter) were prepared with each antibiotic using the final concentrations described above. *E. coli* isolates from the 96-well plates were transferred onto the agar plates containing antibiotics by using a sterile 96-pin replicator. One susceptible (*E. coli* K-12) and two antibiotic resistant (*E. coli* NM-1 and NM-2, water isolates collected from Tanzania) strains were used as negative and positive controls, respectively for antibiotic susceptibility profiling of *E. coli* isolates. For this study, isolates resistant to ≥ 2 antibiotics were considered MDR.

Statistical analysis

Bacterial counts per sample and per slaughterhouse were reported as the mean log_{10} cfu/cm^2 with 95% confidence intervals (CI). Binary coding was used to record the susceptible (0) or resistant (1) phenotypes for each isolate X antibiotic test. Data was processed by using MS Excel 2000 (Microsoft Corporation, Redmond, WA) and descriptive metrics were computed by using MS Access. Data was subsequently analyzed using R-statistical package (version 3.2.1) to determine (1) the differences in bacterial counts between abattoirs and (2) the differences in the prevalence of resistance between abattoirs and antimicrobials. One-way analysis of variance (ANOVA), Turkey HSD post-hoc multiple comparison test ($\alpha = 0.05$) and Student t-tests were used to compare the differences between small and medium scale facilities. Due to the lack of replicates, the large-scale abattoir was not compared with other abattoir types.

RESULTS

The largest abattoir ("E") was well equipped including hand-wash stations with hot running water and stations to sanitize knives. Animals were delivered from auctions by truck and were typically held in pens for 24 h before slaughter. The slaughter process at this facility was unidirectional, which has the advantage of limiting contact

Table 3. Prevalence of antibiotic resistant (ABR) *E. coli* isolated from the surface of beef carcasses in five cattle abattoirs located in Arusha district, Tanzania.

Abattoir	Number of *E. coli*[a] collected	Average(%) ±SE;(95%CI) of ABR
Small (2- 10)[#]	432	
A	216	21 ± 0.09 (0.11 - 0.30)
B	216	19 ± 0.07 (0.13 - 0.27)
Average		21
Medium (18-25)[#]	396	
C	228	32 ± 0.12 (0.20 - 0.43)
D	168	28 ± 0.12 (0.16 - 0.40)
Average		31
P value		0.07
Large (30 – 100)[#]E	444	27 ± 0.06 (0.21 - 0.33)

[a]Up to 12 isolates from each sample. *P*-value for the difference in average ABR between medium and small-scale abattoirs.

between initial slaughter waste and the finished products. Prior to storage in a cold room, carcasses were washed with hot water (no additives) and obvious contaminated areas were trimmed off. Routine inspections were conducted before carcasses were sent to a cold room. The inspection protocol was based on physical inspection for lesions typical of foot and mouth disease, bovine tuberculosis and cysticercosis with attention to the liver, heart, kidneys and lymph nodes.

Abattoirs A, B, C and D (small and medium) were located in areas with lower human population density. Most of the animals were from farmers in the immediate area and occasionally from auctions. Qualitatively, these facilities appeared to employ less stringent hygiene practices and lacked separate working areas, potable running water, and drainage systems; cold rooms were not available for storage. Two facilities (A and B) used small rivers as a source of water and workers cleaned themselves at the river after slaughtering animals. All slaughter processes at these facilities were performed on the floor in the same area where the finished product and initial slaughter wastes were in close physical proximity. Importantly, when the number of animals to be slaughtered exceeded the abattoir's handling capacity, especially on market days, cattle were slaughtered outside in open areas near the slaughterhouses. A sponge dipped in cold water was used to clean carcasses of visible contaminants before the products were removed from the abattoir. Only abattoir C in this category had meat inspectors to examine the carcasses on a regular basis.

Prevalence and load of bacteria

A total of 125 surface swab samples collected from five different slaughter houses (40 each from small and large and 45 from medium; Table 2) were processed to estimate the prevalence and load of cultivable Gram-negative bacteria including *E. coli*. From 125 samples, 1272 *E. coli* isolates were recovered (Table 3). Almost all (99.2%) of the carcass samples were positive for Gram-negative bacteria and 84.8% of the samples were positive for *E. coli* (Table 2). Detection of total Gram-negative bacteria and *E. coli* positive samples did not differ significantly between small- and medium-scale slaughterhouses (Table 2). Nevertheless, beef carcasses from both small- and medium-scale slaughter facilities harbored bacterial counts that were lower than the maximum recommended by FAO (Table 1) for total bacteria (<1.5 log cfu/cm^2) and *E. coli* (<0.8 log cfu/cm^2) (Table 2). Beef carcasses from the large slaughterhouse harbored more total bacteria (1.79 log cfu) and *E. coli* (1.44 log cfu) on their surfaces (Tables 1 and 2). With respect to FAO standards (Table 1), the *E. coli* load was satisfactory for 35.2% of individual carcasses while the remaining were considered excessively contaminated. If we assume that total bacterial counts from MacConkey agar (total Gram-negative bacteria) are the equivalent to the FAO standard for total bacteria (Table 1), then 47.2% of carcasses were considered safe for human consumption.

Prevalence of antibiotic resistant *E. coli*

On average, antibiotic-resistant isolates of *E. coli* were more prevalent in medium (31%± 0.08, mean ± 95% CI) as compared to small facilities (21%± 0.06) (P = 0.07; Table 3). The large slaughterhouse also harbored an equal or higher percentage of resistant isolates as compared to medium facilities (Table 3). Among the *E. coli* isolates, Tet resistance was predominant in small (31%) and large (27%) houses followed by Amp resistance (42% and 11%; Table 4); in medium-scale facilities Amp resistance was predominant (25%) followed by Tet (11%) resistance. Resistance to Amx, Cip, Str, Sul

Table 4. Average prevalence (%) of antibiotic resistant *E. coli* collected from the surface of beef carcasses obtained from five different abattoirs in Arusha district, Tanzania. The mean ± standard error was listed for small and medium slaughterhouses.

Slaughter size	Amp	Amx	Chl	Cip	Ctx	Gen	Str	Sul	Tet	Tri
Small	42 ± 6.5	0	0	0	6.5 ± 1.4	0	0	0	31 ± 1.3	0
A	13	0	0	0	0.4	0	0	0	37	0
B	71	0	0	0	12.5	0	0	0	25	0
Medium	25± 4	0	0	0	2 ± 0.4	1± 0.2	0	0	11 ± 0.9	0
C	41	0	0	0	4	2	0	0	15	0
D	8.3	0	0	0	0	0	0	0	7	0
Large E	11	1	2.7	3	8	0	1	4	27	1

and Tri was not found in *E. coli* isolated from small and medium facilities and resistance to Chl, Ctx and Gen was limited or nonexistent in those facilities. Except Chl and Gen resistance, *E. coli* isolated from the large facility harbored bacteria that were positive for all other tested resistance phenotypes (Table 4). *E. coli* isolates resistant for Cfz were not found in any of the samples tested. All of the multidrug resistant *E. coli* isolated in this study were resistant to 2-3 antibiotics. Among the 19 unique combinations of resistance traits, isolates from small houses harbored four (AmpTet) and medium-scale isolates harbored five phenotypes while the large slaughterhouse harbored 15 phenotypes (AmpTet-2% followed by ChlSul-1.3% and others) (Table 5).

DISCUSSION

For many countries Hazard Analysis Critical Control Point (HACCP) systems have become mandatory as a hygiene control strategy in meat processing plants (Çalicioğlu et al., 2010). An example of the benefit for adopting HACCP comes from South Africa, which has documented a significant increase in microbiological quality of beef carcasses following HACCP implementation with a commensurate economic benefit through expansion of meat markets (Govender et al., 2013). In Tanzania, food inspection still relies on a "see, smell and touch" protocol that does not detect microbiological and chemical contaminants (Ruteri, 2009). For this reason Tanzanian meat is regarded as unsafe despite having the third largest livestock production system in Africa (Ologhobo et al., 2010). Recently, the Tanzanian Food and Drug Authority shutdown several abattoirs in Arusha region after failed inspections. Presumably, the level of production is stretching the capacity of local abattoirs and increasing the likelihood that non-compliant practices are being used.

If we assume that total Gram-negative bacterial counts are equivalent to FAO standards for total bacteria on beef carcasses, then more than 50% of beef carcasses from the current study could be accepted for human consumption. The cool weather in Arusha might have affected bacterial count because abattoirs used generally poor manufacturing practices. The remaining >45% of beef carcasses were excessively contaminated. While we cannot statistically evaluate the correlation between facility size and microbiological quality of meat, it is notable that the one large facility included in the current study had higher loads of bacteria and a greater diversity of antibiotic resistant *E. coli*. This was the case despite what appeared to be application of more effective sanitation control procedures. Consequently, the total number of animals being handled, or the pace by which they are processed, may be a more important risk factor for carcass contamination. Consistent with this hypothesis, a study from Alberta, Canada, found that high volume abattoirs had significantly higher (P<0.01) mean counts for coliforms and *E. coli* than low volume abattoirs (Bohaychuk et al., 2009).

Transportation and lairage effects could be another factor. Transportation can stress animals and increase defecation rates with correspondingly greater possibilities of hide contamination. Also cattle in the large abattoir were mixed with small ruminants in the holding pen and this could facilitate transmission of bacteria between animals. For example, one study found that detection of *Salmonella* increased from 18.5 to 47.7% with a 66 h increase in time that stock spent in lairage (Morgan et al., 1987). Others have reported that there is an increase in carcass bacterial counts associated with transportation (Engineer et al., 2008).

The higher prevalence of antibiotic resistant bacteria at the large facility could also be affected if their suppliers use more antibiotics (Nyenje and Ndip, 2013). For example, because their stock comes from larger suppliers, high-density husbandry increases the chances of disease transmission and presumably increases the demand or need for antimicrobials to sustain their herd health.

The medium volume facilities examined in this study routinely hung carcasses soon after slaughtering, which could explain a reduced bacterial load. Carcass hanging was not observed at the two small-scale abattoirs. In Nigeria, meat from small abattoirs had mean *E. coli* counts of 4.2 log cfu/g (Iroha et al., 2011). In Brazil,

Table 5. Average prevalence (%) of antibiotic resistant phenotypes among the *E. coli* isolates obtained from small, medium and large-sized slaughterhouses in the Arusha region.

AMR phenotypes[a]	Small	Medium	Large
None	65.17 ± 11.5	36.25 ± 15.4	54.7
Amp	26.25 ± 15.4	20.46 ± 14.2	8.33
Amx	0	0	0.23
Cfd	0	0.21 ± 0.21	0
Chm	0	0	0.43
Cip	0.21 ± 0.21	0.83 ± 0.83	0
Ctx	4.38 ± 3.96	0.5 ± 0.5	6.62
Gen	0	0.7 ± 0.5	0
Sul	0	0	0.64
Tet	17.29 ± 17.29	8.29 ± 4.62	22.22
AmpCtx	2.08 ± 2.08	1.33 ± 1.33	0
AmpGen	0	0.17 ± 0.17	0
AmpTet	2.17 ± 0.5	11.46 ± 9.38	1.92
AmxChm	0	0	1.92
AmxTet	0	0	1.92
ChmSul	0	0	1.28
CtxTet	0	0	0.85
SulTet	0	0	0.43
AmpGenTet	0	0.21 ± 0.21	0
AmxChmTet	0	0	0.21
ChmSulTet	0	0	0.21
SulTetTri	0	0	0.21
AmpAmxStrSul	0	0	0.21
AmpChmStrTri	0	0	0.21
AmpChmSulTet	0	0	0.21
AmpStrSulTri	0	0	0.21
AmpSulTetTri	0	0	0.21
AmpStrSulTetTri	0	0	0.21

[a]Amp = ampicillin; Amx = amoxicillin; Cfd = ceftazidime; Chm = chloramphenicol; Cip = ciprofloxacin; Ctx = cefotaxime; Gen = gentamycin; Str = streptomycin; Sul = sulfamethoxazole; Tet = tetracycline; Tri = trimethoprim.

Brazil, carcass surfaces in a small abattoir had a total viable bacterial count ranging between 1.78 and 2.78 $\log_{10}$ cfu/cm^2 (Caselani et al., 2013). Importantly, abattoir layout can have a significant impact via the distribution of air-borne contaminants (Prendergast et al., 2004).

Resistance to ampicillin and tetracycline were the most common phenotypes found in our study. Another study reported that tetracycline resistance was frequently detected among *E. coli* in South African cattle, swine and people (Ateba and Bezuidenhout, 2008). Tetracycline resistance was evident for *Salmonella, E. coli and Campylobacter* found in meat from Canada (Cook et al., 2009). The wide distribution of these resistance traits is favored by the location of genes on mobile genetic elements such as plasmids and transposons (Schnabel and Jones, 1999). Use of tetracycline antibiotics in livestock farming is relatively common as compared to other classes of antibiotics and a recent study in Tanzania found that 70% of meat was positive for tetracycline residues (Darwish et al., 2013). Consequently, use of antibiotics in livestock may drive the higher prevalence of these resistance traits in enteric bacteria.

Importantly, very limited resistance to third-generation cephalosporines (Ctx, Cfz), was detected considered drugs of last resort for the treatment of food-borne pathogens like *Salmonella* and *Shigella* (Xia et al., 2011). Resistance to chloramphenicol was also very limited (1%). We conducted an informal survey of veterinary drug vendors in the Arusha area and mostly found oxytetracycline, penicillin-streptomycin and tylosin products (data not shown). There was no evidence that vendors carried either cephalosporins or chloramphenicol products for use in food animals. Low levels of resistance to chloramphenicol have also been reported in other studies (Tadesse et al., 2012; Kibret and Tadesse, 2013).

Importantly, several countries have banned the use of chloramphenicol in food animal production, these include the USA, Canada and South Africa (Berendsen et al., 2010; Ting et al., 2001).

In Ifakara Tanzania (Vila et al., 1999), multidrug resistant *E. coli* was associated with 38% of diarrhea in children from a rural community. Another study showed that the rate of bacteremia in children was 13.9% and one third of these children presumably died due to failure of treatment associated with multidrug resistant bacteria of which Gram-negative bacteria were the leading cause (43.5%) (Blomberg et al., 2007). Interestingly, beef may not be a major source of these isolates given that for the current study only 4.6% of *E. coli* were resistant to ≥2 antibiotics. In contrast, a high prevalence of resistant bacteria has been reported from raw milk from cattle and from other food animals including broiler chickens (Nonga et al., 2010; Lubote et al., 2014).

It is important to note that beef demand in Tanzania is increasing with the increase in human population, but it is questionable if the production system is equipped to deal with a rapid pace of change. Diseases like “Ndigana and Kizunguzungu” (anaplasmosis and ehrlichiosis, respectively) and East Coast Fever are significant challenge to farmers in northern Tanzania (Halliday et al., 2015). Their prevention and treatment promote the reliance on antimicrobials (including oxytetracycline) and this may contribute to selection for antibiotic resistant organisms.

Clearly, less microbial surface contamination is possible and should be the goal for the industry in Tanzania. For example, whereas the vast majority of carcasses in the current study were contaminated with *E. coli*, this was only true for 7.6% beef carcasses in Irish abattoirs (McEvoy et al., 2003) while only 3% of retail meat samples were positive for *Salmonella* in Washington, D.C (White et al., 2001). Beef carcasses from the Arusha area have limited contamination from multidrug-resistant *E. coli*. This study provides a baseline to consider how this prevalence may change with population growth in this region.

Conflict of Interests

The authors have not declared any conflict of interests.

Abbreviations

ABR, Antibiotic resistance; **FAO,** Food and Agriculture Organization; **HACCP,** Hazard Analysis Critical Control Point; **MDR,** multi-drug resistance; **WHO,** World Health Organization.

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Full Length Research Paper

Characterization of *Fusarium oxysporum* isolates from tomato plants in Algeria

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Fusarium oxysporum* is an ubiquitous soil-borne fungus, having a high genetic and ecological diversity with the potential to cause diseases of many crop species of economic interest. Indeed, some strains of *F. oxysporum* known as pathogens generate common diseases such as wilting, root and crown rot on host plants. Two formae speciales are confined to the tomato: *F. oxysporum* f.sp. *lycopersici* (FOL) causing *Fusarium* wilt, while *F. oxysporum* f.sp. *radicis-lycopersici* (FORL) causes *Fusarium* crown and root rot. The study include 27 strains isolated from the stems, crown and roots of infected tomato plants; to confirm the identity of the fungus, the isolates were identified using analysis based on morphological criteria and sequencing of the translation elongation factor 1-alpha (TEF) gene using ef1 and ef2 primers. Twenty three strains belonged to *F. oxysporum*, three strains to *Fusarium solani*, and one strain to *Fusarium redolens*. Tomato seedlings were tested to confirm the pathogenicity of the isolates tested. Pathogenicity test confirmed that twenty two *F. oxysporum* isolates were pathogenic on tomato and produced crown and root rot typical of *F. oxysporum* f.sp. *radicis-lycopersici forma specialis*, while one strain initially identified as *F. oxysporum* did not induce disease symptoms and is considered as non-pathogenic. Additionally, no symptoms of *Fusarium* wilt were observed at all; therefore no strains can be affiliated to *F. oxysporum* f.sp. *lycopersici forma specialis.

Key words: *Fusarium oxysporum*, tomato, molecular identification, pathogenicity.

INTRODUCTION

In Algeria, the cultivation of tomato (*Solanum lycopersicum* L.) occupies a privileged place in the socio-economic sector. It is in fact, regarded as a priority crop with a total area of 22497 ha (FAO, 2013). Although estimated at 975075 tonnes and 43.34 t/ha (FAO, 2013), the production remains low compared to other

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Mediterranean countries, this is partly due to some fungal diseases, such as *Fusarium* wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* (Fol) (Snyder and Hansen, 1940). This pathogen invades the root epidermis and extends into the vascular tissue. It colonizes the xylem vessels producing mycelium and conidia. The characteristic wilt symptoms appear as a result of severe water stress due to vessel clogging (Beckman, 1987) or *Fusarium* crown and root rot caused by one of the most destructive pathogens; *F. oxysporum* f.sp. *radicis lycopersici* (Forl), the latter persists in soil (Benhamou et al., 1997) and produce a brown discoloration of roots, limited up to 10 to 30 cm above the ground.

The tap root of infected plants often rots partially or completely, and brown cankers which appear in the soil line. Infected plants can be stunted and withered. The infection can be spread by wounds and natural holes, contaminated seeds, microconidia from the air and infested soil or compost (Jarvis, 1988; Di Primo et al., 2001; Steinkellner et al., 2005; Koike et al., 2006). Actually, Fusarium crown and root rot was first discovered in Japan on 1969 (Menzies and Jarvis, 1994) and has spread worldwide since 1970 (Yamamoto et al., 1974) and recently appeared in Algeria.

This disease still causes massive damages in greenhouses and open fields, which incite us to characterize it in order to better understand its development and be able to control it. The aim of this study was to isolate *F. oxysporum*, to identify it using both morphological characteristics and DNA sequencing of the translation elongation factor 1-alpha gene (TEF), which appears to be consistently a single copy in *Fusarium* and shows a high level of sequence polymorphism among closely related species. For these reasons, TEF has become the marker of choice as a single-locus identification tool in *Fusarium* (Geiser et al., 2004; Kristensen et al., 2005). Additionally, the pathogenicity was also accessed.

MATERIALS AND METHODS

Isolation of fungi

Strains were isolated from tomato plants showing symptoms of wilt, crown and root rot. Samples were collected from the most productive regions of Algeria; from the West (Aïn Temouchent, Oran, Mostaganem, Mascara and Chlef), the East (Skikda, Guelma, Annaba and El Taref) and the South (El Oued). The roots, crowns and stems were first cut into small pieces, rinsed with distilled water, disinfected with sodium hypochlorite (5%) for 5 min and rinsed again with sterile distilled water to remove traces of bleach water and then dried using sterile filter papers.

The fragments were then cut lengthwise and placed in Petri dishes containing Potato Dextrose Agar (PDA) prepared as follows: potato infusion (made by boiling 200 g of sliced potatoes in 1 L of distilled water for 30 min) was thoroughly mixed with 20 g of Dextrose and 20 g of Agar powder. The pH was adjusted to 5.6 and the volume was completed to 1 L before autoclaving for 15 min at 121°C. The medium was mixed with 200 mg/L of Streptomycin to prevent bacterial proliferation (Davet and Rouxel, 1997; Rapilly, 1968). The plates were incubated at 25°C for 4 to 5 days. Strain purification was carried out by single spores culture according to Booth (1971). A drop of sterile distilled water was placed on a sterile slide under the dissecting microscope. An accumulation of spores is obtained on the wet tip of a needle and the point of the needle introduced into the drop of water on slide. The spores can be observed to flow from the tip of the needle into the water. When the suspension is adequate the needle can be withdrawn. Experience of the correct dilution can easily be obtained and is approximately the point when the spores are clearly distinguishable in the water and are not obscured by overlapping. The spore suspension on the slide is then picked up by a sterile loop and streaked across a 2% water agar plate. After 24 h of incubation at 25°C, the obtained germinating spores were then cultured on PDA.

Morphological identification

The morphological identification of strains was performed using Carnation Leaf Agar (CLA) medium based on the characteristics of microconidia, macroconidia, phialides and chlamydospores. Although this initial identification using morphological characters is important to sort the species into smaller groups. This is highly recommended to perform more advanced methods such as molecular identification (Leslie and Summerell, 2006).

Molecular identification

Differences in DNA sequences of genes have been used to support morphological identification of *Fusarium* sp. species (Harrow et al., 2010; Yli-Matilla et al., 2002). Therefore, strains were identified at the species level based on sequencing of the translation elongation factor 1-alpha gene using ef1 and ef2 primers (Geiser et al., 2004). Mycelium from our pure isolates and from the pathogenic strain: *F. oxysporum* f.sp. *radicis lycopersici* NH48 (Genbank accession: JN222908) (Edel-Hermann et al., 2011) were harvested in order to extract DNA using E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-Tek, Norcross, USA), according to the manufacturer protocol. The amplification of the translation elongation factor 1-alpha gene was performed using the following primers: ef1 (5'-ATG GGT AAG GA(A/G) GAC AAG AC-3') and ef2 (5'-GGA (G/A)GT ACC AGT (G/C)AT CAT GTT-3') (O'Donnell et al., 1998). The polymerase chain reaction (PCR) reaction mixture (25 µL) contained: 17.2 µL of H_2O, 1.5 µL of 150 µM dNTPs mix, 2.5 µL Taq polymerase buffer + $MgCl_2$ (10X), 0.2 µL (3 U) Taq DNA polymerase (Omega Bio-Tek, Norcross, USA), 1.3 µL (0.5 mM) each of the primers, and 1 µL of genomic DNA. A GeneAmp 9700 thermocycler (Applied Biosystems, Waltham, USA) was used for PCR amplifications with the following amplification cycles: initial denaturation at 94°C for 85 s followed by 35 cycles of denaturation at 95°C for 35 s, annealing at 59°C for 55 s and extension at 72°C for 90 s, followed by final extension for 10 min at 72°C. PCR products were detected by electrophoresis in a 1.5% agarose gel in TBE buffer (Edel-Hermann et al., 2011).

The PCR products were purified with the PureLink Quick PCR Purification Kit (Invitrogen, Carlsbad, USA) and sequenced using an Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems, Waltham, USA). Sequence editing was performed with Molecular Evolutionary Genetics Analysis MEGA5 software (Kumar et al., 2008; Tamura et al., 2013). Sequences were identified via comparison with the GenBank (NCBI) database using the Blastn algorithm (Altschul et al., 1997; Mc Ginnis and Madden, 2004) and blast search against Fusarium-ID database (Geiser et al., 2004).

Pathogenicity test

To highlight the pathogenicity and to determine the *forma specialis*

Table 1. General data of collected isolates.

Strains	Location	Isolation organs	Year of isolation
IB19501	Annaba	Stems	2009
IB19502	Annaba	Stems	2009
IB19503	Annaba	Stems	2009
IB19504	Mostaganem	Stems	2009
IB19505	Oran	Root	2009
IB19506	Oran	Crown	2009
IB19507	Oran	Stems	2009
IB19508	AïnTemouchent	Root	2009
IB19509	Mascara	Crown	2009
IB19510	Chlef	Root	2009
IB19511	Mostaganem	Root	2011
IB19512	Mostaganem	Crown	2011
IB19513	Mostaganem	Crown	2011
IB19514	Mostaganem	Crown	2011
IB19515	Mostaganem	Stems	2011
IB19516	Chlef	Root	2011
IB19517	Oran	Root	2011
IB19518	Oran	Root	2011
IB19519	Oran	Crown	2011
IB19520	Annaba	Root	2013
IB19521	Annaba	Crown	2013
IB19522	Annaba	Root	2013
IB19523	El Taref	Root	2013
IB19524	Skikda	Root	2013
IB19525	Guelma	Root	2013
IB19526	Guelma	Root	2013
IB19527	El Oued	Root	2013

of strains, a pathogenicity test was performed on tomato seedlings, the variety Monfavet H63-5 F1 hybrid sensitive to the three races of *F. oxysporum* f.sp. *lycopersici* was used for this test. Isolates were grown in labeled tubes containing an inclined PDA medium. After 8 days of incubation, 2 mL of liquid minimal medium (LMM) were added to each tube. Thereafter, 1 mL of the suspension was transferred into a new tube containing 9 mL of LMM after agitation then, spore suspensions were filtered in labeled hemolysis tubes. The concentration of the conidial suspension of each isolate was adjusted to approximately 10^6 spores/mL.

The roots from two weeks old tomato seedlings (stage of emergence of the first true leaf) were cut to the 7 mm inferior edge and then soaked for 30 min in the conidial suspensions (03 seedlings per conidial suspension prepared from each isolate).

Controls consisted of seedling soaked in conidial suspension, prepared from the pathogenic strain NH48 as the positive control was soaked in sterile distilled water instead of conidial suspension as the negative control. The seedlings were then transplanted into pots containing sand previously autoclaved and grown in an experimental greenhouse at 25°C at a photoperiod of 12 h (Edel-Hermann et al., 2011). After 3 weeks, plants were uprooted. The lower stem and taproot were longitudinally sectioned for examination of internal tissues (Kim et al., 2001). Symptom evaluation was made based on a rating scale of symptoms proposed by Vakalounakis and Fragkiadakis (1999). 0 = No symptoms; 1 = Light yellowing of leaves, light or moderate rot on taproot and secondary roots and crown rot; 2 = Moderate or severe yellowing of leaves with or without wilting, stunting, severe rot on taproot and secondary roots, crown rot with or without hypocotyls rot and vascular discoloration in the stem; 3 = Dead of seedlings. Disease incidence percentage was determined using the following formula (Song et al., 2004):

$$\text{Disease incidence (\%)} = \frac{\sum(\text{scale} \times \text{number of plants infected})}{\text{highest scale} \times \text{total number of plants}} \times 100$$

RESULTS AND DISCUSSION

A total of 27 strains were obtained from the Western, Eastern and Southern parts of Algeria. Their origins, the year and the part of the plant from which isolation was performed are shown in Table 1.

Gerlach and Nirenberg (1982) and Nelson et al. (1983) described the colony appearance of *F. oxysporum* on PDA as highly variable. Indeed, peach and violet colony colors, observed in the majority of isolates were consistent with the description of Booth (1971). Morphological studies showed Microconidia with zero to one septate; oval or cylindrical (Figure 1A), produced on

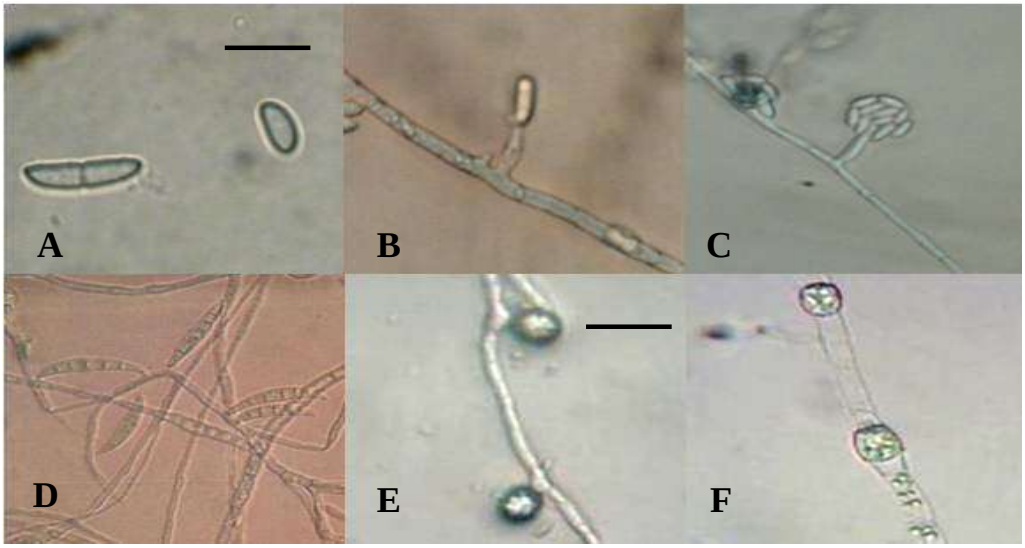

Figure 1. Different types of structures produced (Strain: IB19504). A: Microconidia, B: Microconidia in short monophialide, C: Microconidia in false heads, D: Macroconidia, E: Terminal chlamydospore, F: Intercalar chlamydospore, A-B-C-D: scale bar = 25 µm, E-F: scale bar = 50 µm.

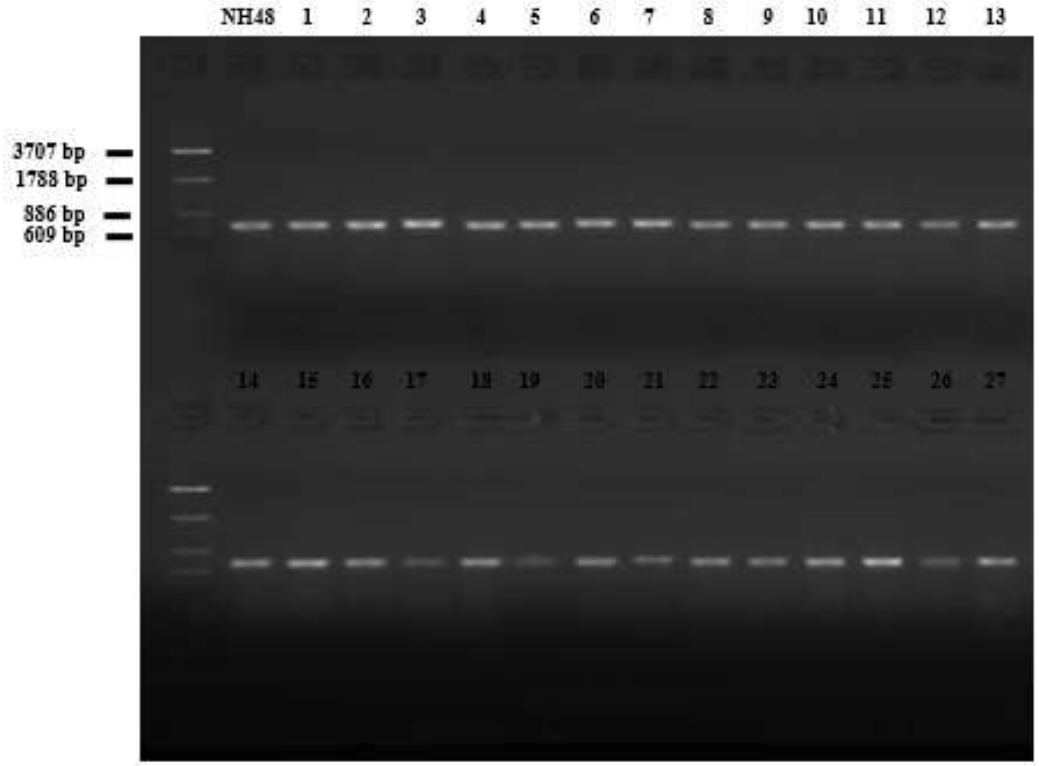

Figure 2. Electrophoretic separation of PCR products using the ef1/ ef2 primers. **NH48:** positive control, **Lane 1-27**: isolated strains.

short monophialides (Figure 1B) or in false heads (Figure 1C). Macroconidia were short to medium, falcate to straight and usually with three to five septate (Figure 1D). Chlamydospores were produced abundantly in

Table 2. Identity of isolated strains based on TEF sequences.

Wells	Strains	Location	Isolation organs	% similarity with TEF GenBank database	% similarity with Fusarium-ID database
01	IB19501	Annaba	*Fusarium oxysporum*	99	99.21
02	IB19502	Annaba	*Fusarium oxysporum*	99	99.85
03	IB19503	Annaba	*Fusarium redolens*	99	99.68
04	IB19504	Mostaganem	*Fusarium oxysporum*	99	99.83
05	IB19505	Oran	*Fusarium solani*	99	99.7
06	IB19506	Oran	*Fusarium solani*	99	99.84
07	IB19507	Oran	*Fusarium oxysporum*	99	99.83
08	IB19508	AïnTemouchent	*Fusarium oxysporum*	99	99.85
09	IB19509	Mascara	*Fusarium oxysporum*	99	99.85
10	IB19510	Chlef	*Fusarium oxysporum*	99	99.38
11	IB19511	Mostaganem	*Fusarium oxysporum*	100	100
12	IB19512	Mostaganem	*Fusarium oxysporum*	99	100
13	IB19513	Mostaganem	*Fusarium oxysporum*	99	99.7
14	IB19514	Mostaganem	*Fusarium oxysporum*	99	99.37
15	IB19515	Mostaganem	*Fusarium oxysporum*	99	99.7
16	IB19516	Chlef	*Fusarium oxysporum*	99	100
17	IB19517	Oran	*Fusarium oxysporum*	99	100
18	IB19518	Oran	*Fusarium oxysporum*	99	99.56
19	IB19519	Oran	*Fusarium oxysporum*	100	100
20	IB19520	Annaba	*Fusarium oxysporum*	100	100
21	IB19521	Annaba	*Fusarium solani*	100	100
22	IB19522	Annaba	*Fusarium oxysporum*	100	100
23	IB19523	El Taref	*Fusarium oxysporum*	99	100
24	IB19524	Skikda	*Fusarium oxysporum*	100	100
25	IB19525	Guelma	*Fusarium oxysporum*	99	100
26	IB19526	Guelma	*Fusarium oxysporum*	100	100
27	IB19527	El Oued	*Fusarium oxysporum*	99	99.85

terminal (Figure 1E) and intercalary chains (Figure 1F) in 3 to 6 week-old cultures. All these correspond to the critical morphological features of *F. oxysporum* species cited by Leslie and Summerell (2006).

The (TEF) PCR products generated using the ef1 and ef2 primers were analyzed on agarose electrophoresis gel (Figure 2), an amplicon of an approximately 700 bp was produced with all strains. The Blastn search for similarities in GenBank database indicated between 99 and 100% identity while the blast against Fusarium-ID database indicated between 99.85 and 100% identity for all isolates. Therefore, the species name assigned was according to the closest match with known species. The combination of these two tools gave similar results and allowed us to confirm the identity of our isolates, as follow: twenty three strains identified as *F. oxysporum*, three strains as *Fusarium solani* and one strain as *Fusarium redolens* (Table 2).

The pathogenicity test conducted on 15 days old plants with 3 replicates per strain showed that twenty two strains identified by their translation elongation factor 1-alpha gene as *F. oxysporum* were pathogenic and have produced severe crown and root rot as the positive control NH48 (Figure 3B 1-2). The longitudinal section of stems showed brown discoloration of the vascular system extending no more than 10 to 15 cm above the soil as a consequence of the pathogen invasion (Figure 3C). Infected plants also showed a proliferation of adventitious roots on the first 10 cm of stem above the soil line (Figure 3B3). These symptoms are typical of *F. oxysporum* f.sp. *radicis-lycopersici* (FORL) as described by Jarvis and Shoemaker (1978), and different, both in varietal reaction and symptomatology from the *Fusarium* wilt caused by *F. oxysporum* f.sp. *lycopersici* (FOL); for the latter, above ground symptoms are commonly apparent, plants are severely stunted and wilted and some of them collapsed and died within 3-4 weeks. Plants showed severe vascular discoloration extending well up the stem until the petiole of the lower leaves, this contrasted with the discoloration in crown and root rot which was limited to

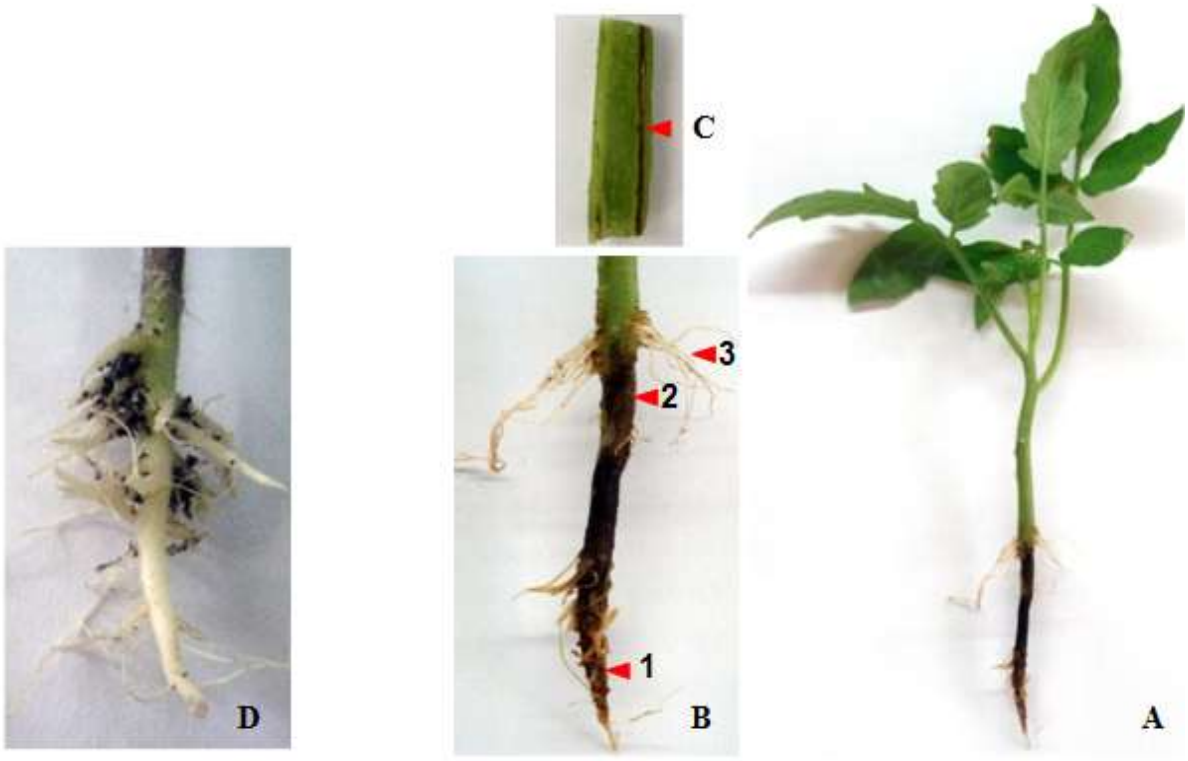

Figure 3. Symptoms of Fusarium crown and root rot caused by the strain: IB19504. A: overview, B1: Severe taproot rot, B2: Severe crown rot, B3: proliferation of adventitious roots, C: brown discoloration of the vascular system, D: healthy roots (negative control).

the lower stem near the soil line. Indeed, since then Yamamoto et al. (1974) described the *Fusarium* crown and root rot of tomato in Japan and named the pathogen as a new race of *F. oxysporum* f.sp. *lycopersici*, there has been some disagreement with this designation because the symptoms are not those typical of Fusarium wilt but of the Fusarium crown and root rot (Jarvis et al., 1977; Rowe et al., 1977; Nutter et al., 1978). These results suggest that the twenty two *F. oxyporum* strains belong to the *forma specialis F. oxysporum* f. sp. *radicis-lycopersici* with 66.67% as the highest disease incidence and 44.44% for the lowest one (Table 3).

However, the strains IB19505, IB19506 and IB19521 identified as *F. solani* and IB19503 identified as *F. redolens* have produced a slight browning of taproots and showed between 22.22 and 33.33% of disease incidence (Table 3). The strain IB19508, initially identified as *F. oxysporum* did not cause any symptoms and is therefore considered as non-pathogenic. The re-isolation of pathogens from stems, crowns and roots of each plant showing symptoms, confirmed that it is the same starting strains that were used to prepare initial inoculums and allowed us to establish a causative relationship between isolates and the disease. This, confirm the Koch's postulates.

Conclusion

The Mediterranean countries of the Maghreb, mainly Algeria where cultivation of tomatoes is very important, are facing an increase in *Fusarium* wilt and Fusarium crown and root rot. This scourge is favored by climate changes like rising temperatures throughout the year and increasing humidity which promote fungal growth. As a part of our study, the identity of the fungus was confirmed using the translation elongation factor 1-alpha gene and the pathogenicity of the isolates was revealed when tomato plants were tested. The obtained results allowed us to distinguish strains belonging to *F. redolens* and *F. solani* species in addition to strains belonging to *F. oxysporum* which produced typical symptoms of *Fusarium* crown and root rot and a non-pathogenic *F. oxysporum* strain, that could be used as a potential biological control agent to protect tomato crops from these pathogens and also to limit the use of pesticides, which fits perfectly with a respectful environment approach.

Conflict of Interests

The authors have not declared any conflict of interests.

Table 3. Pathogenicity test results.

Strains	Identity	Symptoms	*Forma specialis*	Disease incidence (%)
IB19501	*Fusarium oxysporum*	Crown and root rot	*radicis-lycopersici*	66.67
IB19502	*Fusarium oxysporum*	Crown and root rot	*radicis-lycopersici*	66.67
IB19503	*Fusarium redolens*	Slight browning of taproots	-	33.33
IB19504	*Fusarium oxysporum*	Crown and root rot	*radicis-lycopersici*	66.67
IB19505	*Fusarium solani*	Slight browning of taproots	-	33.33
IB19506	*Fusarium solani*	Slight browning of taproots	-	33.33
IB19507	*Fusarium oxysporum*	*Fusariumoxysporum*	*radicis-lycopersici*	66.67
IB19508	*Fusarium oxysporum*	No symptoms	Non-pathogenic	0
IB19509	*Fusarium oxysporum*	Crown and root rot	*radicis-lycopersici*	66.67
IB19510	*Fusarium oxysporum*	Crown and root rot	*radicis-lycopersici*	66.67
IB19511	*Fusarium oxysporum*	Crown and root rot	*radicis-lycopersici*	44.44
IB19512	*Fusarium oxysporum*	Crown and root rot	*radicis-lycopersici*	66.67
IB19513	*Fusarium oxysporum*	Crown and root rot	*radicis-lycopersici*	66.67
IB19514	*Fusarium oxysporum*	Crown and root rot	*radicis-lycopersici*	66.67
IB19515	*Fusarium oxysporum*	Crown and root rot	*radicis-lycopersici*	66.67
IB19516	*Fusarium oxysporum*	Crown and root rot	*radicis-lycopersici*	66.67
IB19517	*Fusarium oxysporum*	Crown and root rot	*radicis-lycopersici*	66.67
IB19518	*Fusarium oxysporum*	Crown and root rot	*radicis-lycopersici*	66.67
IB19519	*Fusarium oxysporum*	Crown and root rot	*radicis-lycopersici*	66.67
IB19520	*Fusarium oxysporum*	Crown and root rot	*radicis-lycopersici*	66.67
IB19521	*Fusarium solani*	Slight browning of taproots	-	22.22
IB19522	*Fusarium oxysporum*	Crown and root rot	*radicis-lycopersici*	66.67
IB19523	*Fusarium oxysporum*	Crown and root rot	*radicis-lycopersici*	66.67
IB19524	*Fusarium oxysporum*	Crown and root rot	*radicis-lycopersici*	66.67
IB19525	*Fusarium oxysporum*	Crown and root rot	*radicis-lycopersici*	44.44
IB19526	*Fusarium oxysporum*	Crown and root rot	*radicis-lycopersici*	66.67
IB19527	*Fusarium oxysporum*	Crown and root rot	*radicis-lycopersici*	66.67

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Full Length Research Paper

Growth inhibition of the stored fish (*Ethmalosa fimbriata*) fungus *Aspergillus flavus*, exposed to extracted essential oils from *Callistemon citrinus* and *Ocimum canum*

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The aim of this study was to evaluate the antifungal effects of essential oils from *Callistemon citrinus* and *Ocimum canum* against *Aspergillus flavus*. Major components in the oil of *C. citrinus* were 1,8-cineole (60.6%), α-pinene (18.5%), limonene (5.0%) and α-terpineol (5.0%). The oil of *O. canum* was mainly composed of 1.8-cineole (20.8%), linalol (14.3%), eugenol (11.9%), terpinen-4-ol (7.4%) and germacrene D (4.9%). Inhibition of the mycelia growth of *A flavus* increased significantly ($p < 0.05$) with the essential oils concentrations. Positives correlations were observed between inhibition percentages and the concentration of *C. citrinus* ($p < 0.001$; $r = 0.873$) and *O. canum* oils ($p< 0.001$; $r = 0.768$). *O. canum* oil was fungicide at 325 ppm while *C. citrinus* was fungistatic at all the tested concentrations with the highest inhibition percentage means of 89.74 %. Antifungal activity of the essential oil of *O. canum* was significantly ($p < 0.001$) higher than that of *C. citrinus*. Antioxidant activity of *O. canum* (49.3 mol/l) was significantly ($p < 0.001$) higher than that from *C. citrinus* (5.0 mol/l). These observations suggest the possible exploitation of the oils from *O.canum* and *C.citrinus* as potential approach for smoked *Ethmalosa fimbriata* preservation against *A. flavus.*

Key words: Antifungal effects, *Aspergillus favus*, *Ethmalosa fimbriata,* essential oils.

INTRODUCTION

Ethmalosa fimbriata, a fish commonly known as "Bonga", belongs to the family Clupeidae. In Cameroon, the farming of this fish is a common occupation of people living in the coastal areas and along major river banks. It is one of the best sources of proteins, vitamins, essential fatty acids and minerals. It contains essential nutrients required for supplementing both infant and adult diets (Stansby, 1987; Abdullahi et al., 2001). Various brands of

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oils are also extracted from the flesh and the fluids of this fish. Due to its high susceptibility to microbial spoilage, autolysis, oxidation and hydrolysis of fats, fish is a highly perishable product (Frazier and Westhoff, 1978). Care is therefore required in handling as well as its preservation for food. The commonly used post-harvest method of preserving *E. fimbrata* in Cameroon is smoke drying. This process can increases the shelf life of fish by eliminating microorganisms or prevent their growth (Falioye et al., 2002; Ali et al., 2011). However, despite this treatment, smoke dried fish is frequently altered by many fungi such as members of the genus *Aspergillus* especially when stored in an unsuitable environment (Falioye et al., 2002; Gram, 2010). Our preliminary survey in some local markets in Cameroon showed that *Aspergillus flavus* was the most commonly occurring fungus on stored *E. fimbriata*. This fungus is well known to decrease the commercial and nutritive value of the fish and produce a number of toxic metabolites including aflatoxin (Edema and Agbon, 2010). Additional control methods of this spoiling mold are essential. In this respect, natural products could be developed for reducing losses during storage. Selected plants and their essential oils have been evaluated as natural sources of compounds for food preservatives due to their antimicrobial and antioxidant effects (Tasadjieu et al., 2009; Prakash et al., 2015).Their main components show antioxidant activities and antimicrobial activity against a wide range of microorganisms including filamentous fungi (Hyldgaard et al., 2012; Sameza et al., 2014). Variable results have been observed depending on the origin of biological substances; testing conditions and target microorganisms (Delaquis et al., 2002; Hyldgaard et al., 2012). Cameroon flora is very rich with aromatic plants which have various biological activities (Amvam et al., 1998). Among these, *Callistemon citrinus* and *Ocimum canum* are used as spice, condiment, ornamental plants and African medicine. Previous works showed that, essential oils extracted from *Ocimum sp* and *Callistemon sp.* had many biological activies including antifungal and antioxidant. These activities were related to the main component 1,8 cineole (Kumar et al., 2011; Shukla et al., 2012; Alves Silva et al., 2013). In certain households in Cameroon, leaves of these plants are used to protect stored food products. The aim of this study was to evaluate the antioxidant potential and antifungal activities of essential oils from *C. citrinus* and *O*. *canum* against *Aspergillus flavus*, one of the most occurring spoiling fungi of stored smoke dried *E. fimbriata* in Cameroon.

MATERIALS AND METHODS

Plant material and essential oil extraction

C. citrinus and *O. canum* plants were harvested in March 2011 in Douala, Cameroon and dried during 3 days at room temperature (28 ± 2°C). Herbarium/plants were identified at the National Herbarium of Cameroon. Leaves of *C. citrinus* and *O. canum* were steam-distilled for 4 h using a Clevenger apparatus. Each oil recovered was dried over anhydrous Na_2SO_4, stored in an amber-colored flask and kept at 4°C until use.

Essential oils analysis

Essential oils obtained were analyzed by Gas chromatography (GC). It was performed on a Varian-HP 5890 with flame ionization detector fitted with a fused silica capillary column (30 m x 0.25 mm coated with DB-1, film thickness 0.25 mm). The temperature program was set to 60°C-246°C at 3°C/min, with injector temperature set at 200°C, detector temperature set at 200°C carrier with N_2 gas set to 1 ml/min. The linear retention indices of the components were determined relatively to the retention times of series of n-alkanes and the percentage composition were obtained from electronic integration measurements without taking into account relative response factors. Gas chromatography coupled with mass spectrometry analyses were performed using a Hewlett-Packard GC 5890 equipped with an HP-1 (cross-linked methyl siloxane) fused column (30 m x 0.25 mm, film thickness 0.25 µm) and interfaced with a quadrupole detector (model 5970); temperature programmed at 70°-200°C (10°C/min); injector temperature, 220°C; temperature of connection parts, 180°C; carrier gas, helium at a flow rate of 0.6 ml/min; injection type, split, 1:10 (1µl of a 10:100 pentane solution); ionization voltage, 70 eV; electron multiplier, 1400 eV; mass range, 35-300; scan rate, 2.96 scan/seconde. The identification of the constituents was assigned based on comparison of their retention indices and their mass spectra with those published by Adams (2007).

Aspergillus flavus

The *Aspergillus flavus* used in this study was isolated from fresh smoked fish samples of *E. fimbriata* purchased from a local market (Douala Cameroon). Fish tissue segments (2-4 mm) from flesh and gills were cut using a sterile scalpel and seeded aseptically on *A. flavus* selective medium (Griffin and Garren, 1974). After 3-4 days of incubation at 30°C, the mycelia emerging from the tissues were transferred to fresh medium. Colonies of *A. flavus* developing from tissue segments were transferred to Czapez-Dox Agar for confirmation of identity according to the criteria of Raper and Fennell (1965). The cultures were stored at 4°C on Sabouraud Dextrose Agar (SDA)-chloranphenicol slants and sub-cultured once a month.

Antifungal assay

The agar incorporation method (Lahlou, 2004) was used to evaluate the antifungal activity of the essential oils. The test was carried out in 90 mm Petri dishes containing SDA-chloramphenicol medium. The oils were first diluted with Di Methyl Sulphur Oxide (DMSO) (ratio1: 9). These essential oils were added aseptically into the medium at an appropriate volume to produce various concentrations ranging from 100 to 800 ppm. SDA-chloramphenicol medium supplemented only with DMSO was used as negative control. After solidification, the media were inoculated with 5 mm discs obtained from the edge of 3-days old mycelia culture of *A. flavus*. Each treatment consisted of triplicate plates incubated at 30°C in the dark. Mycelia growth was monitored by measuring the growth diameter following two perpendicular lines going through the centre of the dish. These measurements were made daily for 7 days. The inhibition percentage of mycelia growth was calculated by comparing them with those in the blank dish without essential oil using the formula below: %I = (Dc - Dt) / Dc, where Dc is the diameter of microbial colony in the control and Dt the diameter of

the colony in the treated plate. The fungicidal or fungistatic activity was determined by transferring the discs from the Petri dishes with no apparent growth into non-supplemented medium.

Antioxidant activity

The antioxidant potential of the essential oils was determined using the FRAP (Ferric Reducing Antioxidant Power) method adapted from Benzie and Strain (1996). FRAP reagent contained 4 ml of 10 mM TPTZ (tripyridyltriazine) in 40 mM HCl plus 4 ml of 10 mM $FeCl_3$,$6H_2O$ and 40 ml of 300 mM acetate buffer (pH 3.6). Tenµl of essential oil was diluted in methanol at different concentrations then 75 µl of essential oil-methanol were mixed with 2 ml of FRAP reagent daily prepared. The absorbance was recorded after 12 min incubation at the room temperature with a spectrophotometer UV V-1100 at 593 ηm. FRAP values were obtained by comparing with standard curves created by ascorbic acid solutions concentrations ranging from 50-800 µM. The Equivalent Concentration (EC) was defined as the concentration of antioxidant having a ferric-TPTZ reducing ability equivalent to that of 1 mM $FeCl_3$, $6H_2O$. EC was calculated as the concentration of antioxidant giving an increase in the FRAP assay equivalent to the theoretical absorbance value of a 1 mM of ascorbic acid solution, determined by using the corresponding regression equation.

Statistical analysis

Data were analyzed using Sigmastat version 2.03 (Synstat Sofware Inc). Results are presented in term of means ± standard deviation. Multiple comparisons of mean values were set up using one-way parametric ANOVA when the normality and equal variance test passed. When these conditions were not matched, we used the non-parametric Krustcall-Wallis test.

RESULTS AND DISCUSSION

Yields and chemical composition of the essential oils

The extraction yields of the essential oils from *C. citrinus* and *O. canum* were 0.8 and 0.3%, respectively (W/W). The yield obtained from *C. citrinus* is lower than that obtained by Oyedeji et al. (2009) (1.2%) and Silva et al. (2010) (1.1%) from South Africa and Brazil, respectively. Similarly value from the *O. canum* was less than the result of Hassane et al. (2011) who analyzed *O. canum* from two different regions of Reunion Islands and obtained 2.04 and 1.4%. Representative GC map of the oils are presented in Figures 1 and 2. Table 1 lists the components identified in the essential oils with their percentage composition and relative retention indices. Twenty-nine constituents were identified and classified in the oil of *C. citrinus,* representing 98.8% of the total oil. The major components were 1.8-cineole (60.6%), α-pinene (18.5%), limonene (5.0%) and α-terpineol (5.0%).The abundance of 1,8-cineole in the essential oil of *C. citrinus* makes it similar to those obtained in all the previous studies from Hymalayas and Reunion Island samples (Srivastava et al., 2001; Mya et al., 2002). However, a key difference in the oils lies in the relative quantities of α-pinene and α-terpineol. In the oil of *O. canum*, fifty components (96.7%) were identified and quantified (Table 1). The major compounds were 1.8-cineole (20.8%), linalol (14.3%), eugenol (11.9%), terpinen-4-ol (7.4%) and germakrene D (4.9%). The results on the analysis of the essential oils of *O. canum* growing in two regions of Comores Island also showed abundant of 1,8-cineole (Hassane et al., 2011). However quantitative and qualitative differences between our results and these oils were noticed. The Maweni-Dimani region from Comores essential oil had 1,8-cineole (48.88%), camphor (14.98%), α-pinene (5.71%), β-pinene (4.66%) and γ-elemene (3.91%) as predominate constituents while the Ivoini-Mitsamihouli sample was mainly composed by 1,8-cineole (34.22%), camphor (13.69%), isopropyl propanoate (9.13%), γ-elemene (5.43%) and α-pinene (3.83%). The differences in yield and constituents of the oils could be attributed to difference in genetic of the plants and geographical/environmental conditions (Amvam et al., 1998; Bakkali et al., 2008).

Effects of essential oils on fungal growth

Results showed that, the inhibition of the mycelia growth of the fungus increased significantly ($p < 0.05$) with the essential oils concentrations (Table 2). Total inhibition occurred at 325 ppm with the essential oil from *O. canum*. For *C. citrinus* the highest inhibition percentage means was 89.74%. *O. canum* oil was fungicide at 325 ppm while *C. citrinus* was fungistatic at all the tested concentrations. Results showed that from 200 ppm, the activity of the essential oil of *O. canum* was significantly ($p < 0.001$) higher than that of *C. citrinus*. There was a positive correlation between inhibition growth of *A. flavus* and the concentration of *C. citrinus* oil ($p < 0.001$; $r = 0.873$) and *O. canum* oil ($p < 0.001$; $r = 0.768$). Essentials oils from various sources exhibit broad-spectrum antimicrobial activity and their biological properties have been related to their chemical composition (Nguefack et al., 2012; Djenane et al., 2013). Indeed, compounds such as 1.8-cineole, pinene, limonene, terpineol and eugenol are present in both essential oils analyzed and have been shown to exert various biological activities including antifungal (Isman, 2000; Dayan et al., 2009). It has been demonstrated that essential oils inhibit postharvest pathogens mainly due to their direct effect on the mycelia growth by affecting the cellular metabolism of the pathogen (Serrano et al., 2005; Regnier et al., 2010). The hydrophobicity of the oils and their components allows them to partition in the lipid layer of the fungal cell membranes and result in disruption in membrane structure and cell membrane integrity (Beckman 2000). In addition, the inhibiting activities of these essential oils may not only be attributable to their major components but, to a synergistic effect of individual minor and/or major compounds (Nguefack et al., 2012; Sivakumar and

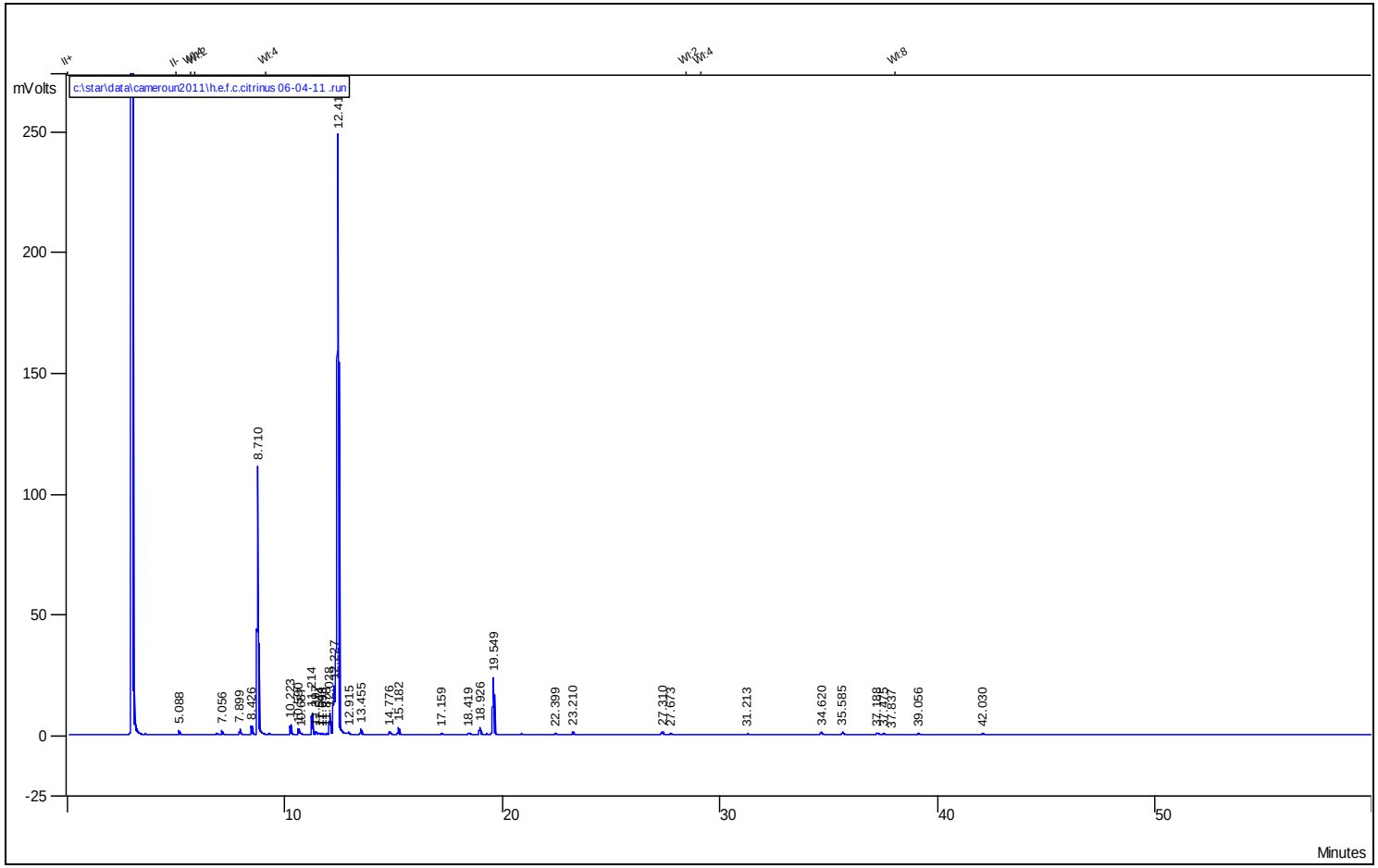

Figure 1. Chromatogram of chemical analysis of *C. Citrinus* essential oil.

Bautista-Banos, 2014).

Antioxidant activities of the essential oils

Mean values of equivalent concentration of antioxidant activities showed that the antioxidant potential of *O. canum* and *C. citrinus* essential oils were 49.3 and 5.0 mol/l respectively (Table 3). It was shown that, the activity of the oil from *O. canum* is significantly ($p < 0.001$) higher than that from *C. citrinus.* Previous studies of Prakash et al. (2011) reported that the essential oils of *Ocimum* sp. had antioxidant activities. Antioxidants retard oxidation and are sometimes added to meat and poultry products to prevent or slow oxidative degradation of fats. Antioxidant agents are effective due to different mechanisms such as free radical scavenging, chelating of pro-oxidant metal ions or quenching singlet-oxygen formation (Lopez-Luzt et al., 2008). These activities could

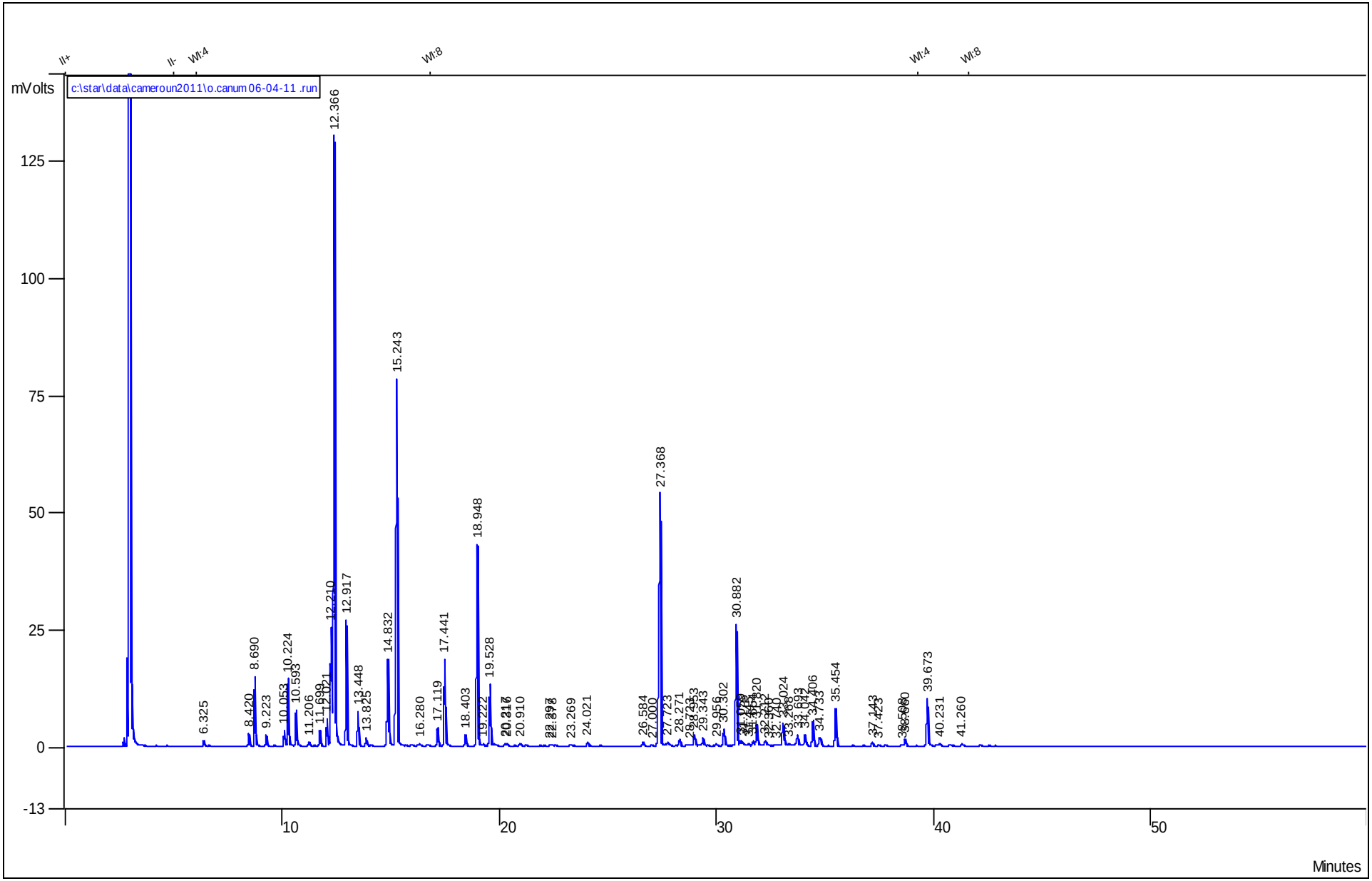

Figure 2. Chromatogram of chemical analysis of *O. canum* essential oil.

Table 1. Yields and chemical composition of essential oils of *Callistemon citrinus* and *Ocimum canum* from Cameroon.

Components	Percentage of constituents		
	RI on DB-1	*C. citrinus*	*O. canum*
Linear aliphatic compounds		**0.7**	**0.2**
Isovaleric acid	828	0.2	-
Hexan-1-ol	865	-	0.2
5-Hydroxylpentanal	886	0.2	-
Isobutyl d'isobutyrate	910	0.3	-
Monoterpenes		**97.3**	**82.2**
Monoterpene hydrocarbons		**29.8**	**20.7**
α-Thujene	926	0.5	0.3
α-Pinene	934	18.5	1.9
Camphene	948	-	0.3
Sabinene	972	-	0.5
β-Pinene	977	0.7	2.1
Myrcene	988	0.4	1.1
α-Phellandrene	1005	1.7	0.2
δ-3-Carene	1009	0.3	-
p-Cymene	1016	Tr	0.5
β-Phellandrene	1024	2.0	0.9
Limonene	1028	5.0	4.3
(E)-β- Ocimene	1045	0.1	3.9
γ-Terpinene	1057	0.4	1.1
Terpinolene	1088	0.2	3.6
Oxygen-containing monoterpenes		**67.5**	**61.5**
1.8-Cineole	1033	60.6	20.8
Hydrate cis-sabinene	1066	-	0.3
Linalol	1099	0.6	14.3
trans-Pinocarveol	1140	0.1	0.6
Camphre	1146	-	3.2
Bornéol	1168	0.1	0.4
Terpinèn-4-ol	1179	0.7	7.4
α-Terpineol	1192	5.0	2.2
cis-Carveol	1221	-	0.1
Hydrate sabinene acetate	1253	0.1	Tr
Thymol	1287	-	0.2
Eugenol	1359	0.2	11.9

Table 1. Contd.

Components	Percentage of constituents		
	RI on DB-1	***C. citrinus***	***O. canum***
Carvyle cis- acetate	1366	0.1	0.1
Sesquiterpenes		**0.8**	**14.2**
Sesquiterpenes hydrocarbons		**0.3**	**8.2**
α-Cubebene	1342	-	0.1
α-Copaene	1379	-	0.2
α-Bergamotene	1407	-	0.3
(Z)-β- Farnesene	1437	-	0.1
α-Humulene	1455	-	0.7
Germacrene D	1484	-	4.9
α-Zingiberene	1492	-	0.1
γ-Cadinene	1518	-	0.1
δ-Cadinene	1525	0.3	0.9
α-Cadinene	1541	-	0.3
Germacrene B	1601	Tr	0.5
Oxygen- containing sesquiterpenes		**0.5**	**6.0**
Elemol	1548	0.2	Tr
Nerolidol	1563	-	0.1
Germacrene D-4-ol	1575	-	1.3
Caryophyllene oxide	1585	0.2	Tr
Viridiflorol	1591	0.1	-
Humulene II epoxide	1610	-	0.5
β-Eudesmol	1643	-	1.5
α-Bisabolol	1684	-	0.1
(2Z, 6E)- Farnesol	1721	-	0.3
(2E, 6E)-Farnesol	1746	-	1.9
(2 E, 6 E)- Methyl farnesoate	1786	-	0.1
Aromatic constituent		**0.0**	**0.1**
1, 3, 5-Trimethylbenzene	1122	-	0.1
Total		**98.8**	**96.7**
Yield of oils (%)		**0.8**	**0.3**

Table 2. Range and mean values of the percentages of growth inhibition of *A. flavus* at different concentrations of essential oils.

Conc. (ppm)	n	Min.	Max.	Mean ± SD	Conc. (ppm)	n	Min.	Max.	Mean ± SD	Comparison : Student t test
Callistemon citrinus $F_{(6,140)}$ = 83.516 ; p < 0.001***					***Ocimum canum*** $F_{(8,180)}$ = 303.146 ; p < 0.001***					
100	21	0.00	11.11	1.60 ± 3.31	100	21	0.00	26.09	3.40 ±7.00	t = -0.854 ; ddl = 40 ; p= 0.398^{ns}
200	21	0.00	22.22	4.06 ± 3.30	200	21	0.00	32.73	55.95 ± 20.09	t = 11.125 ; ddl = 40 ; p< 0.001
300	21	0.00	37.04	15.62 ± 15.63	300	21	0.00	66.97	86.98 ± 15.46	t = 14.924 ; ddl = 40; p< 0.001
-	-		-	-	325	21	100.00	100.00	100.00 ± 0.00	-
-	-		-	-	350	21	100.00	100.00	100.00 ± 0.00	-
-	-		-	-	375	21	100.00	100.00	100.00 ± 0.00	-
400	21	0.00	100.00	32.53 ± 32.70	400	21	100.00	100.00	100.00 ± 0.00	t = -9.456 ; ddl = 40 ; p< 0.001
500	21	22.58	100.00	57.07 ± 22.39	500	21	100.00	100.00	100.00 ± 0.00	t = -8.788 ; ddl = 40 ; p< 0.001
-	-		-	-	600	21	100.00	100.00	100.00 ± 0.00	-
700	21	60.22	100.00	81.28 ± 17.02						-
800	21	73.68	100.00	89.74 ± 12.16						-

Comparison of the activities of the two oils at different concentrations				
Concentration (ppm)	100	200	300	400
O. canum vs C. citrinus	q=0.741ns	q=16.039***	q=20.917***	q=15.187***

*** = Highly significant difference (p <0.001); ns = no significant difference.

Table 3. Variation of equivalent concentration (mol/l) of antioxidant of *Callistemon citrinus* and *Ocimum canum* essential oils.

Essential oil	N	Min.	Max.	Means ± SD	Essential oil	n	Min.	Max.	Means ± SD	Student t-test Comparaison :
C. citrinus	15	4.0	6 .5	5.0 ± 0.8	***O. canum***	9	42 .0	57.4	49.3 ± 6.8	t =-25.418 ; ddl = 22 ; p < 0.001

be related to the presence of compounds such as eugenol, thymol and 1-8 cineole known for their antioxidant properties (Mishra et al., 2013). We also identified these compounds in *O. canum* and *C. citinus* oil in noticeable amount (Table 1).

The essential oils from *O. canum* and *C. citrinus* exhibited antifungal activity since they were able to kill or inhibit the mycelia growth of *A. flavus.* They also had antioxidant activities. Through these properties, they could be used as an approach solution to preserve stored smoked *E. fimbriata* and other foodstuff. Nevertheless, detailed studies are required to study the preservative effect of these oils directly on the dried fish.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Characterization of *Bacillus cereus* spores isolated from Algerian processed cheese

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The main purpose of this study is to characterize the isolated *Bacillus cereus* spores from processed cheese manufactured and marketed in Algeria. Sixty samples of four brands of processed cheese were analyzed. The panC gene sequencing identified four *B. cereus* spores belonging to Group III, according to the classification of Guinebretiere et al. (2010). In nutrient broth, values of D for *B. cereus* spores (LMBCF001, LMBCF002, LMBCF003, LMBCF004) vary between 4.76 min at 120°C and 93.75 min at 110°C and the values of ZT vary from 7.75 to 21.34°C. In processed cheese, D values obtained for the isolated spores vary between 7.12 min at 120°C and 21.53 min at 110°C. The minimum pH obtained from the four isolated *B. cereus* spores varies between 4.70 and 5.10. Furthermore the minimum aw varies between 0.940 and 0.951. The studied revealed that processed cheese is contaminated by *B. cereus* spores in spite of the pasteurization or ultra-high temperature (UHT) treatment. The contamination origin can be raw material such as milk powder or starch or cheddar. The study focused on the importance of bringing out the presence of this *Bacillus cereus* in such products.

Key words: *B. cereus,* bacterial growth, heat resistance, processed cheese.

INTRODUCTION

Processed cheese is the most consumed dairy product in Algeria. The amount consumed was more than 101 273 tons in 2015 (2.51 kg/year/inhabitant) (CNIS, 2015). The Algerian cheese market is dominated by five brands which hold about half of the market share. It could be commercialized under several kinds in block, semi liquid and solid forms with several flavors. The rest of the production is mainly concentrated in the Western region. The best brands use cheddar cheese as raw material, but many small manufacturers use mixtures of different raw materials.

Processed cheese consists of more recent technology

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than conventional cheese. This technology stabilizes milk nutrients for a longer time and preserves the “cheese aspect” to get the final product (Boutonnier, 2000). Walter Gerber and Fritz Steller invented how to process cheese in 1911 in Switzerland. Its production consists of blending, heating and texturing dairy products (cheese, butter and milk powder) and non-dairy products (agents, emulsifiers and salt). Processed cheese melts at 70 to 95°C for 4 to 15 min (Richonnet, 2016), depending on stirring capacities, final product texture and qualities preservation (Fox et al., 1996).

In Algeria, the process consists of mixture of several raw materials such as: cheddar cheese, milk powder, fat, milk proteins, modified starch and chemical conservatives (salts of cast iron, citrate and poly phosphates). The mixture is heated at 72 to 86°C or 92 to 94°C for 10 min for pasteurized, dissolved cheese, while for sterilized cheese UHT, the heat treatment applied is about 140°C for 2 to 4 s. The heat treatment applied is generally sufficient to destroy vegetative bacteria, but might not be sufficient to eliminate spores forming bacteria (Warburton et al., 1986).

Some companies have effective means but many manufacturers do not have total control of hygiene. So there is an interest in assessing the health risk related to the consumption of processed cheese in Algeria. Thus, the cheese may be the cause of outbreak of food poisoning. In fact, dairy products are involved in 9.83 % cases of overall outbreak of food poisoning, with 19.56% of cases whose causal agent remains indeterminate according to Algerian Ministry of Trading (2015). The microbiological quality of processed cheese is not standardized in Algeria, but in a processed cheese quality control the standards used for this purpose are those applied to the soft cheese in the Algerian Official Journal No.35. Thus, the main researched micro-organisms are total Coliforms, fecal Coliforms, *Staphylococcus aureus*, *Clostridium* sulfite-reducers, *Salmonella* and *Listeria* (Ministerial order JO35, 1998). Otherwise, several microorganisms could be present in processed cheese as aerobic spores forming bacteria especially *Bacillus cereus*. In fact, *B. cereus* is an inevitable bacterium in dairy products (Lücking et al., 2013) and can survive processed cheese heating treatment; otherwise, it can be conveyed by different ingredients such as starch. In Algeria *B. cereus* is not researched as poisoning causal bacterium due to dairy products. So far, few or rare works have reported *B. cereus* and its toxins in processed cheese while some works have studied this bacterium on cheddar cheese, raw material used for manufacturing of processed cheese (Molva et al., 2009; Cherif-Antar et al., 2015).The *B. cereus* group presently consists of seven *Bacillus* species, that is, *Bacillus anthracis, B. cereus, Bacillus mycoides, Bacillus pseudomycoides, Bacillus thuringiensis, Bacillus weihenstephanensis,* and the most recently recognized member of the group, *Bacillus* cytotoxicus, which is thermotolerant. There are two types of *B. cereus* foodborne illnesses. The first type, which is caused by an emetic toxin, results in vomiting, whereas the second type, which is caused by enterotoxin(s), results in diarrhea. The emetic type is caused by a 45-kDa cyclic heat- and pH-stable peptide that is pre-formed in food “Cereulide”. The diarrheal type is caused by three component heat-labile enterotoxins, non-hemolytic enterotoxin (NHE), and hemolysin BL (HBL). Foods associated with the emetic type are commonly farinaceous while the diarrheal type is associated with meat, sauces, pudding, vegetables and milk products (Upasana and Labbé, 2016).

In this context, this study aims to research *B. cereus* in the commercialized processed cheese and its contamination level, its heat resistance, and the influence of some factors (aw, pH and lactic acid concentration) on *B. cereus* growth capacities.

MATERIALS AND METHODS

Samples of processed cheese

The processed cheese samples of four local trademarks (A, B, C and D) were bought directly from the local region of Mascara retail market (360 km west of Algiers) from February, 2011 to May, 2013. Samples were bought from refrigerated displays and had exceeded 50% of their shelf-life. The companies which make the marks A and D are situated in Oran (west of Algeria) whereas the companies making the marks B and C are located in Algiers. The companies manufacturing the marks C and D are considered as large-sized companies while those manufacturing the marks A and B are small-sized companies.

Determining processed cheese pH, aw and lactic acid

Processed cheese pH was measured with a pH-meter (Crisson microph 2001 model), introducing directly pH and temperature probes in a processed cheese sample at 20 to 25°C. Measures were recorded three times. The four processed cheese samples aw were measured with an aw-meter (FA-st/1, GBX France Scientific Instruments). D and L-lactic acids were analyzed adapting MEGAZYME D-LACTATE AND L-LACTATE kit recommendations and according to Noll (1984) and Gawehn (1988)’s methods.

Enumeration of *B. cereus* in processed cheese

Enumeration of *B. cereus* was carried according to the methods recommended through NF EN ISO 7932 norms (2005) in SAS (2010). It consists of the enumeration of presumptive *B. cereus* cells on whole selective Mossel agar (Tryptone (10.0 g) ; meat extract (1.0 g) ; D-mannitol (10.0 g) ; sodium chloride (10.0 g); phenol red (25.0 mg); sterile egg yolk emulsion (100.0 ml); bacteriological agar (13.5 g); and polymyxin B; pH 7.2±0.2) (Pasteur institute of Algeria). Twenty five grams of processed cheese were mixed with 225 ml of TSE (Biokar) and homogenized through a Stomacher (Lab-Blender 400). Decimal dilutions were prepared until 10-6. 0.1 ml of the each dilution was spread on whole

Mossel agar. The Petri dishes were inoculated for each dilution and then were incubated at 37°C for 24 to 48h. The presumptive colonies of *B. cereus* are big, pink (no fermentation of mannitol) and surrounded by a precipitation zone (lecithinase production). Then, the obtained presumptive *B. cereus* was streaked on blood agar and incubated at 37°C for 24h ± 2h. These colonies were supposed to belong to a *B. cereus* group.

Preparation of *B. cereus* spores suspension

Pre-cultures of *B. cereus* strains were realized in Brain Heart Infusion (BHI). 300 µl was spread on Fortified Nutrient Agar (FNA) supplemented with 50 mg/l of MnSO4,H2O and 60 mg/l of CaCl2. Cultures were incubated at 37°C for 5 to 7 days. Sporulation was checked daily by microscopic examination, and spores were harvested when at least 90% of the cells had produced spores. Then, agar medium surface was scraped with rake in a sterile water at 4°C. Spores were washed and centrifuged at 6500r/min for 5 min. Recovered supernatant spores are then centrifuged at 6500r/min for 30 min. The washed suspension was diluted in distilled water and centrifuged twice following the same procedure. The final washed spores were heated at 70°C for 15 min and then cooled in ice for 5 min. Finally, the spores stock was kept in 30% of glycerol. The final concentration could be at 1010 spores/ mL.

Molecular identification of isolates *B. cereus*

The isolated *B. cereus sensu lato* was identified by sequencing of the 16S rDNA gene. Genomic DNA was extracted by using Qiagen kit, PCR. The PCR was carried out by mixing 300ng of DNA and 0.2 mM of NTP (Eurogentec, Seraing, Belgium), 2.5 mM of MgCl2, 0.25 µM of every primer, 0.75 U of AmpliTaq polymerase (Perkin-Elmer, Courtaboeuf, France) and 9 µl of AmpliTaq 10X buffer with no MgCl2 (Eurogentec) for a final volume of 90µL. The PCR reaction was performed by using standard primers: Forward primer 27f (5'-GAGTTTGATCMTGGCTCAG-3') and reverse primer 192r (5'-GNTACCTTGTTACGACTT-3') (Weisburg et al., 1991). PCR cycle was realized in a thermo-cycler (BIO-RAD): a 5-minute start cycle at 94°C, followed by 30 15-second cycles at 94°C, a 30-second cycle at 55°C, a 30-second cycle at 72°C and a final 7-minute extension at 72°C. The obtained amplicons were sequenced in AGCT Biotech Company in Heidelberg, Germany. Finally, the obtained sequences were blasted with the NCBI database (http://www.ncbi.nlm.nih.gov/ BLAST /). The affiliation of *B. cereus* strains was determined according to Guinebretiere et al. (2008)'s procedure. PCR products were purified using "High Pure PCR Product" kit (Roche Diagnostics, Mannheim, Germany) and amplicon sequences were stringed together using Sym'Previus online software (https://www.tools.symprevius.org/bcereus/).

Determination of the minimum growth values for pH and aw

The minimal value of pH and aw allowing growth of the four strains of *B. cereus* were carried out in nutrient medium broth (Fluka, France). The studied pH range (4.51 to 9.35) was adjusted by adding HCl (0.1N) or NaOH (0.1N), and then sterilized by filtration. Otherwise, the aw was adjusted with NaCl according to Morales et al. (2006). The studied aw ranged between 0.940 to 0.982, and n was autoclaved. Finally, 5 ml of medium was inoculated with 10µl of spores suspension and then incubated at 37°C for 24 h. Growth ability is determined by visual observation.

***B. cereus* spore heat resistance**

The heat resistance studied was made by the method of capillaries after preparation of spore suspension. The spores stock was diluted at 1/100. 100 µl of spore dilution was then introduced in the sterile capillaries. The capillaries were sealed and then placed into a glycerol-thermostated water bath at different temperatures (105, 110, 115, 120 and 125°C). After heat treatment, capillaries were removed at regular time intervals and instantly cooled in ice water. Tube ends were scratched with a glass saw, blazed up and cracked. Tube content was expelled with 1 ml Tryptone-Salt dilution towards a 9 ml-tube with the same solution. Decimal dilutions were then realized. Finally, 1ml of every dilution was sown on nutrient agar at 37°C for 24 to 72 h.

Effect of pH on *B. cereus* heat resistance

Effect of pH on *B. cereus* heat resistance was studied in nutrient broth according to the same procedure as previously described for only LMBCF002 strain. The choice of this strain was based on the preliminary results of heat resistance: it seems to be the most representative and the pH change was made for only this strain. The pH of the medium was adjusted to different values (7, 6.5, 6, 5.5, 5 and 4.5) with HCl (1N). It was sterilized through a 0.22 µm filtration membrane. After inoculation of the pH adjusted nutrient broth with LMBCF002 strain, decimal dilutions were performed. Then, 1 ml of each dilution was sown on nutrient agar at 37° C for 24 to 72 h. The decimal reduction time (D) and sensitivity to treatment (z pH) of LMBCF002 *B. cereus* spores were estimated using GraphPad PRISM 6 (GraphPad Software, San Diego, CA, USA).

Determination of *B. cereus* heat resistance in processed cheese

The heat resistance of *B. cereus* in processed cheese was conducted as follows: 45 g of processed cheese was inoculated with spore suspension at 1% and then homogenized in a Stomacher (Lab-Blender 400). Each spore was inoculated in the cheese trademarks, which were isolated. Mixtures were filled into ampule of 1 mL type Wheaton (Sigma Aldrich, France) with sterilized syringe and needle (Spinal Needle quincke Type point 18GA3.00IN, 1.2x75mm). The ampule was sealed and then treated at different temperatures (110 and 120°C), at a rate of ten experimental points between 15 and 90 min. After the heat treatment, ampules were removed at time intervals and instantly cooled in iced water. The first series of ampules was removed, plated on nutrient agar and incubated for 24-48 hours at 37°C. In addition, two other Wheaton ampules were incubated at 37°C for 5 days. The surviving bacteria not affected by the incubation conditions have developed in the food matrix. The presence of surviving bacteria in the ampule is highlighted by seeding in agar nutrient medium. The time of decimal reduction (D) of *B. cereus* spores in processed cheese was estimated by GraphPad PRISM 6 (GraphPad Software, San Diego, CA, USA).

Determining heat resistance parameters

The time of decimal reduction (D) and sensitiveness to treatment (z) of *B. cereus* spores were estimated by GraphPad PRISM 6 (GraphPad Software, San Diego, CA, USA). D values for *B. cereus* were calculated using the average slope for a given treatment. The

Table 1. Characterization of four Algerian processed cheese.

Variable	A n=3	B n=3	C n=3	D n=3
pH	5.750±0.005	5.84±0.01	6.16±0.02	5.74±0.01
Water activity (a_w)	0.978±0.005	0.980±0.004	0.975±0.004	0.965±0.002
Lactic acid (g/100g)	0.151±0.002	0.294±0.005	0.095±0.006	0.211±0.004

Table 2. Enumeration of *B. cereus* in four Algerian processed cheese.

Variable		A n=15	B n=15	C n=15	D n=15
B. cereus (*CFU*/g)	Min	$9x10^2$	$1.5x10^4$	$1.9x10^4$	10^3
	Moy	$1.2x10^3$	$1.9x10^4$	$2.7x10^4$	$1.6x10^3$
	Max	1.4×10^3	2.5×10^4	3.4×10^4	1.9×10^3

value of the inverse slope obtained by plotting log10 D value versus temperature represents the Z value.

RESULTS AND DISCUSSION

Physicochemical and biochemical pH, aw and lactic acid dose of processed cheese

pH, aw and lactic acid concentration of processed cheese

Table 1 illustrates the results of physicochemical and biochemical analysis of four processed cheese samples. The results showed that pH varies from 5.74±0.01 to 6.16±0.02. The results are comparable to pH values (5.2 to 6.7) reported by Boutonnier (2000) and Roustel (2014). Even though pH values reported in this study were slightly superior to the product criterion of 5.6 recommended by the Canadian Food Inspection Agency (2014), they belong to 5.4 to 6 interval recommended by *Codex Alimentarius* (SAS, 2010). Concerning the aw results of the processed cheese, they varied from 0.965±0.002 to 0.980± 0.01. They are like those recorded (>0.970) by Rüegg et al. (1977). However, they are superior to values (0.840 to 0.940) indicated by the Canadian Food Inspection Agency (2014).

Moreover, for the food water activity, the lower microorganisms are heat resistant and therefore the heat treatment is ineffective. Generally, the minimum aw (causing bacterial sporulation is less than that for vegetative growth; it is estimated to *B. cereus* 0.950 (Lozach, 2001). This explains the presence and growth of *B. cereus* germ in the studied samples. Otherwise, the lactic acid concentration in processed cheese varied from 0.095 to 0.211 g/100g as summarized in Table 1. They were lower than the recommended values of cheese paste (<0.3g/100g) and traditional cheese (<1.2g/100g) reported by Arthur and Prashanti (2013).

The lowest lactic acid concentration was explained by the non- addition of lactic acid in processed cheese. The lactic acid could be produced by lactic acid bacteria from lactose. Lactic acid fermentation is generally a fast process. For certain types of cheese such as Cheddar, it must be completed before it is pressed (Henning et al., 2006). That clarifies an important presence of bacteria in studied products, because lactic acid in cheese might have antibacterial effects. This research field is quite new as mentioned by Puah et al. (2013).

Initial contamination of processed cheese

Results concerning searching and numbering of *B. cereus* on complete Mossel agar are shown in Table 2. The results showed that the four studied (A, B, C and D) trademarks processed cheese were contaminated by *B. cereus sensu lato* with maximal concentration equals to 1.4x103 to 3.4x104 CFU/g. These values are approximatively near to risky concentration assigned at 105spores/g of product according to Salustiano et al. (2009). This concentration could be increased depending on consumers' behaviors and high contamination can cause a harm to public health.

Identifying and determining molecular origin of *B. cereus*

The total four isolates selected show characteristic colonies on Mossel agar obtained from different processing cheese samples. After sequencing 16S rDNA gene, molecular identification confirmed that presumed *B. cereus* belonged to *B. cereus* group and the identified

Table 3. Minimal pH and aw (NaCl) for four B. cereus strains isolated from four Algerian processed cheese.

Strains	pH min	a_w min
LMB_CF001	4.72	0.940
LMB_CF002	4.93	0.940
LMB_CF003	5.10	0.951
LMB_CF004	5.10	0.951

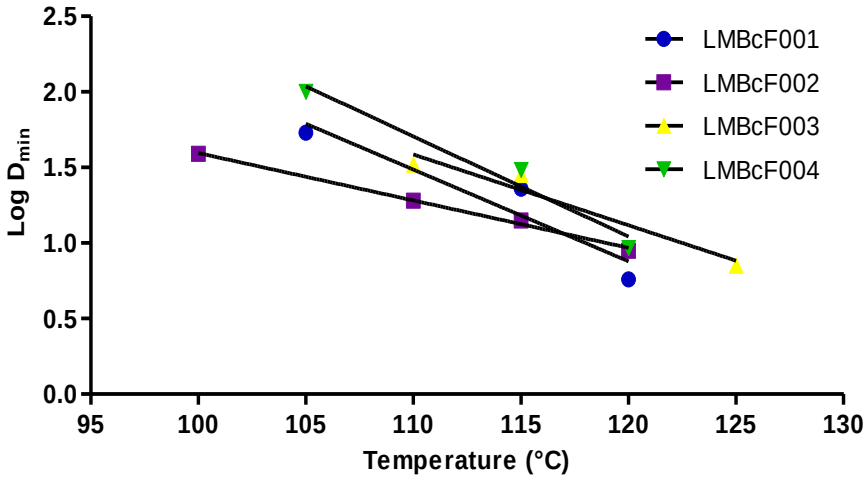

Figure 1. Survival curve for four B. cereus spores isolated from Algerian processed cheese in synthetic medium.

trains were codified as follows: LMBcF001, LMBcF002, LMBcF003 and LMBcF004.

On the other hand, after sequencing *pan*C gene according to Guinebretière et al. (2010), these four *B cereus* strains belong to group III with 97% homology. It consists in a mesophilic group matching with *Bacillus thuringiensis* III, *B. cereus* III or *Bacillus anthracis* species. This strain group is generally cytotoxic and the emetic strains are notably included in this group (Guinebretiere et al., 2008). The fact that isolates belong to group III does not necessary mean the strains harbor the emetic toxin gene. They produced the toxin in food product. The *B. cereus* strains of this group are not involved in the diarrheic syndrome but are involved in emetic syndrome (Guinebretiere et al., 2008; Kumari and Sarkar, 2014). *B. cereus* concentrations are toxicogenic above 105spores/g of the product (Salustiano et al., 2009).

Determining pH and aw minimum values

Few works have studied the impact of pH and aw on *B. cereus* growth. The minimal growth pH for studied strains varied from 4.72 to 5.10 (Table 3). They are higher than the values (4.63 and 4.65) reported for *B. cereus* group III by Carlin et al. (2013). Otherwise, minimal aw values obtained varied from 0.940 to 0.951. They are slightly superior to values (0.941 to 0.944) recorded by Carlin et al. (2013). This may due to the differences of used methods. *B. cereus* is a pathogen, Gram-positive, strictly aerobic or facultative anaerobic bacteria with a 25°C to 37°C optimal growth temperature (Abee et al., 2011) and a 4.9 to 9.3 pH growth acidity (Bermúdez-Aguirre et al., 2012).

Studying *B. cereus* spores heat resistance

Studying B. cereus spores and determining heat resistance parameters

Heat resistance results are shown in Figure 1. The results showed that when temperature of treatment increases, time of decimal reduction (D-value) decreases. At 115°C, the D115°C varied from 23.15 to 32.63min.

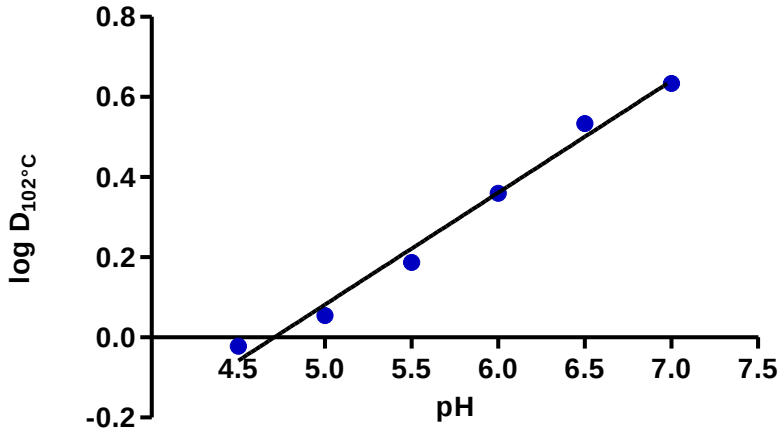

Figure 2. Influence of pH value on LMB_CF002 *B. cereus* heat resistance.

Otherwise, D110°C values ranged from 33.33min to 93.75 min higher than D110°C values documented by Gonzalez et al. (1999) which ranged from 0.080 to 0.36 min. The lowest D-values were observed at 120°C (4.76). D120°C values vary from 4.76 to 9.3 min and are higher than those mentioned by Russell (1999), indicating D120°C value 2,37min.

In this study, *B. cereus* LMBCF002 appeared as the most resistant strain, whereas *B. cereus* LMBcF003 was the most sensitive. D decimal value strain differs according to the phylogenetic group of bacterial species (ANSES, 2011).

The heat resistance depends on its physiological state, temperature and living environment, food composition (lipid…etc.) and characteristics (pH, aw, etc..). Else, the starch in some processed cheese types may explain increase of the heat resistance in heating medium (Oteiza et al., 2003). Nevertheless the study results do not match with those recorded by Mvou Lekogo (2010)'s works.

The sensitivity of heat treatment (ZT) values varies from 7.75 to 21.34°C. The z-values depend on bacteria strains. Diverse factors (surrounding environment during treatment, treatment parameters, etc.) can greatly influence microorganisms' heat resistance (Levy, 2010). ZT values for LMBCF002 match with ZT values reported in the literature for *B. cereus* spores. On the other hand, ZT values for LMBCF001, LMBCF003 and LMBCF004 strains are higher. The highest ZT value ever (21.34°C) was recorded with LMBCF003 *B. cereus*.

B. cereus Z value varies from 8 to 12.5°C, according to ANSES (2011); from 8 to 8.6°C according to Byrne et al., (2006) and from 6.7 to 10.1°C, according to Caudrillier (2008). Mayoraz (2006) reported a 15°C Z value, while characteristic group III ZT values are 8.4°C, according to Luu-Thi et al. (2014).

Studying pH effect on B. cereus heat resistance

It is documented that pH is used as a hurdle to limit microbial development. Food acidification is frequently used as a microbial development limiter. After studying pH influence (pH = 7, 6.5, 6, 5.5, 5 and 4.5) on LMBCF002 strain resistance to heat at 102°C, the results obtained (Figure 2) clearly assess that amount of *B. cereus* decreases as pH does: important when pH = 7 and reverse when pH = 4.5. By the same way, treatment length varies as pH does: decreasing from 15 minutes when pH = 7 to less than 5 minute when pH = 5 at 102°C. The same decrease as heat treatment increases with sample. The resistance decrease is related to proton concentration. Such decrease might not be abrupt but progressive (Weiss, 1921). At 102°C D value (D102°C) for the strain studied LMBCF002 decreased from 4.30 to 0.95min; for pH=7 and pH=4.5 respectively. The result suits those quoted by Lopez et al. (1997) and Couvert (1999). The obtained ZpH=3.58 value was close to ZpH reported by Gaillard et al. (1998). They record a 4.08 ZpH value for a pH range of 4.5 to 6.6 with a constant temperature.

Determination of *B. cereus* heat resistance in processed cheese

All studied *B. cereus* strains have the same surviving kinetic in processed cheese as well as in treatment medium with a temperature from 110 to 120°C (Figure 3). Bacteria heat resistance in food matrix differs in treatments processed in heating medium. The heat resistance of bacteria in food is different to that obtained in the laboratory environment since the decimal reduction time is higher in the food matrix for all samples studied

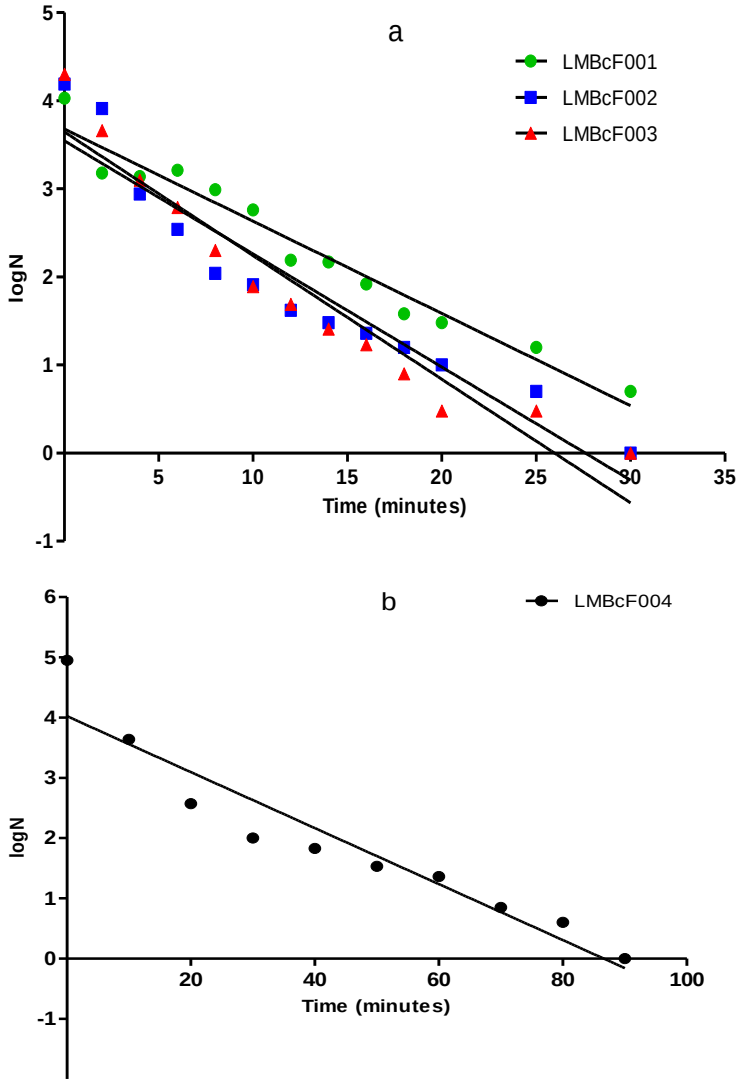

Figure 3. Survival curve for four B. cereus spores isolated from Algerian processed cheese in processed cheese (LMBCF001, LMBCF002 and LMBCF003 B. cereus at 120°C (a); LMBCF004 B. cereus at 110°C (b)).

compared to laboratory conditions. The D110°C and D120°C values appear superior to that obtained in nutrient broth : D120°C value was in the range of 7.12 to 9.55 minutes vs. 4.76 to 9.3 min in nutrient broth. The study notes that there is no total destruction. Bacteria persistence might be explained according to matrix type: in solid matrix, local microenvironments where aw is weaker. Bacteria located in these microenvironments are more heat-resistant compared to global population (Coroller et al., 2006). Otherwise, food products are more complex than nutrient broth and potentially include components that might protect spores (Leguerinel et al., 2005; Samapundo et al., 2014). It is therefore most likely that the macromolecular compounds in foods that is, fat, proteins, and starch may influence the effect of a heat treatment on the survival or outgrowth of spores in food products (Samapundo et al., 2014).

Conclusion

In Algeria, processed cheeses are the most popular cheese compared to other cheese types considered as luxury products. Nevertheless, processed cheese can be hygienically corrupted during preservation due to microbial contaminations. Among microbial contaminations, *B. cereus* is globally part of dairy products alteration flora. The *B. cereus* issue does not figure

among cheese microbiological specifications (Ministerial order JO35, 1998). Microbiological analyses assess presence of *B. cereus sensu lato* in all studied processed cheese samples. *B. cereus* isolated strains belong to group III according to Guinebretiere et al. (2010) classification.

Studies concerning resistance to heat assess that selected spores are resistant to heat treatment during processed cheese production. After validating results in food matrix, we know that these spores can grow in processed cheese. *B. cereus* spores have indeed demonstrated a considerable growth potential in processed cheese. Results might partly explain why unidentified agents responsible of food poisoning are so important in 2015 Algerian statistics. Percentage of unidentified agents represents 19.56% and could be caused by germs as *B. cereus*. Regulations should be amended to take in consideration a contaminant like *B. cereus*. *B. cereus* incidence on cheese can be due to its spore adhesion properties on milk industry surfaces (Marchand et al., 2012; Lücking et al., 2013).

Conflict of interests

The authors have not declared any conflict of interests.

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