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Full Length Research Paper

Production of novel antifungal compounds from actinomycetes isolated from waste dump soil in Western Uganda

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Drug resistant and opportunistic organisms are a problem to medical health due to the fact that most of the drugs that were used are now not effective. Currently, there is a need to search for new drugs that can enhance the control of these organisms. Actinomycetes and their secondary metabolites can be used as such drugs. This study was designed to isolate actinomycetes producing novel anti-fungal compounds from waste dump soil in Western Uganda. Fifty six (56) actinomycetes were isolated from 22 waste dump soil samples. All isolates were screened using modified spektra - plak method against *Candida albicans* ATCC1023, *Penicillium* sp., *Aspergillus* sp., *Fusarium* sp. and *Rhizopus* sp. Eight 8(14.29%) isolates showed antifungal activity to at least one test fungi during primary screening. Two isolates [KBRWDSa (FR) and KBMWDSb6] showed activity to all test fungi. Secondary screening was carried out by growing all 56 isolates in broth and their supernatant was tested for antifungal activity using agar well diffusion method. 11(19.64%) of these isolates showed activity against at least one test fungi with mean zone of inhibition 5.33 to 29.69 mm. Isolate KBMWDSb6 showed a broad spectrum activity against all test fungi. The remaining broths were extracted using ethanol. The ethanol extract at 2.5 mg/ml concentration was also tested for antifungal activity using agar well diffusion method. 13 (23.21%) isolates showed activity against at least one test fungi with mean zone of inhibition 6.33 to 30.67mm. The findings showed that some of these isolates had antifungal activity.

Key words: Waste dump soil, actinomycetes, novel anti-fungal compounds, Western Uganda.

INTRODUCTION

Infections caused by drug resistant and opportunistic fungi especially among immuno-compromised patients are now a global concern; this has caused a substantial morbidity and mortality (CDC, 2016). Most of these fungi developed resistance to commonly prescribed drugs (CDC, 2016; Howard et al., 2009). Chowdhary et al.

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(2013) reported that, *Candida auris* was the new emerging drug resistant fungus found in Japan (Satoh et al., 2009) and South Korea (Oh et al., 2011). This fungus causes fungemia and was reported to be resistant to fluconazole (Chowdhary et al., 2013).

However, other fungi like *Aspergillus*, *Fusarium*, and *Cryptococcus* species have been reported to be resistant to the azole group of antifungal compounds (Arendrup, 2014). Patients infected by these drug resistant fungi have poorer outcomes than those affected by drug susceptible fungi (Baddley et al., 2008; Lortholary et al., 2011). It was also reported that patients with fungemia stay longer in the hospital and consumed more healthcare facilities (Morgan et al., 2005; CDC, 2016). The long stay in hospitals affects the economy of the patient's family specifically and the concerned nation generally.

When the population of Uganda was 35 million, 1.1 million had HIV and about 9.2% (101,000) had a CD4 count <200 cells/μL out of which, 2783 developed Cryptococcal meningitis per year with approximated mortality rate of 2086 per year (Parkes-Ratanshi et al., 2013). Parkes-Ratanshi et al. (2013) also added that, fungal infections were estimated to be 1 million cases per year with exclusion of *Tinea capitis*. There are reports on increasing rate of motility due to fungal infections which was related to emergence of antifungal resistance (Smith et al., 2015). These put more demands for search of new antifungal compounds to face these global challenges.

Isolation and characterization of microorganisms from the most extreme habitat and unstudied areas or geographical location is one of the ways to discover novel antimicrobial agents as recommended by action-mycetologist (Monisha et al., 2011; Jagan et al., 2014). Actinomycetes are considered to be one of the golden microbes in the 20th century due to their ability to produce different kinds of bioactive compounds which includes antifungal agents (Lakshmipathy and Krishnan, 2010; Ensieh and Maryam, 2016; Rotich et al., 2017). Actinomycetes have produced 80% of the discovered antibiotics (Masna et al., 2016). Actinomycetes are gram positive bacteria, widely distributed in soil and most abundant microbes in the soil (Jemimah et al., 2012).

In Uganda, there is paucity of data on production of antifungal agents from soil actinomycetes (Nalubega et al., 2016). Therefore, this study was designed to isolate and screen actinomycetes from waste dump soil, from Western Uganda for novel antifungal compound(s).

MATERIALS AND METHODS

Study area and design

The samples were collected from two different temperate sites, cold areas (Bushenyi and Kabale districts) and warm areas (Kasese and Mbarara districts) Western Uganda (Figure 1). The study was experimental laboratory research that involved isolation, Identification and screening of actinomycetes for production of novel antifungal compounds.

Sample collection

A total of 22 waste dump soil samples were collected from market waste dump soil, residential waste dump soil and manure plantation farm soil. Samples were collected from two different temperate areas: cold (Bushenyi 6 samples and Kabale 6 samples) and warm (Kasese 4 samples and Mbarara 6 samples) regions of Uganda.

Two plots (120 cm × 120 cm) were mapped out from each sampling site and three samples (3 to 15 cm depth) were collected randomly from each of the plots using a sterile stainless spoon with hand core and sterile gloves to avoid contamination. The collected soils were mixed to have one representative sample per plot (Rinoy et al., 2012; Ekeke and Okonwu, 2013). The samples were placed in sterile polythene bags (Ismail et al., 2015) and transported to the Microbiology laboratory at Department of Microbiology and Immunology Kampala International University Western campus, Uganda for further study. The geographical location, soil temperature of each sampling site were recorded during sample collection while moisture content and pH of each sample were determined immediately using oven dried method and digital pH meter, respectively (Rinoy et al., 2012).

Isolation of actinomycetes

Isolation of actinomycetes was carried out according to the method described by Arifuzzaman et al. (2010) and George et al. (2012). Two grams of each soil sample was air dried for 10 days at room temperature, and approximately 1g of air dried soil sample was suspended in 9 ml of sterile distilled water supplemented with 0.9% of NaCl and incubated in a Gas bath thermostats oscillator (THZ-82B) for 1 h at 200 rpm and 55°C.

The suspension was serially diluted (10^{-1} to 10^{-7}). 100 μL from 10^{-2} dilution was spread on starch casein nitrate agar (composition in media g/L: starch 10, casein 0.3, KNO_3 5, $NaCl_2$ 2, K_2HPO_4 2, $MgSO_4.7H_2O$ 0.5, $CaCO_3$ 0.02, $FeSO_4.7H_2O$ 0.01 and agar 18; pH was adjusted to 7 ± 0.2) (Sengupta et al., 2015), glycerol casein agar (Kuster agar) (composition in media g/L: glycerol 10, casein 0.3, KNO_3 2, K_2HPO_4 2, $MgSO_4$ 0.05, $CaCO_3$ 0.02, $Fe_2(SO_4)_3.6H_2O$ 0.01, Agar 15 and pH was adjusted to 7 ± 0.2) (Lakshmanaperumalsamy et al., 1984) and yeast extract starch casein agar (YSCA) (composition in media g/L: yeast extract 3, peptone 3, casein 3, starch 8, K_2HPO_4 0.5, $MgSO_4.7H_2O$ 0.5, NaCl 2, agar 15 and pH 7.0 to 7.6) (Mincer et al., 2002).

The inoculated plates were incubated at 28°C for 7 to 14 days. Colonies with limiting growth, appeared dry powdery or velvety, tough leathery or chalky texture; dry or folded and branching filamentous with or without aerial mycelia and clear zone of inhibition were chosen and sub-cultured on clean starch casein nitrate agar plates to obtain pure cultures (Oskay et al., 2004). The pure cultures were maintained on starch casein nitrate agar at 4°C for short storage and 30% glycerol at -80°C for long storage (Oskay et al., 2004; Madigan et al., 1997).

Production of anti-fungal compounds

Test organisms

The test fungi were standard for *Candida albicans* ATCC1023, which was obtained from the Department of Medical microbiology Mekerere University, Kampala Uganda, while *Aspergillus* sp*, Fusarium* sp., *Rhizopus* sp. and *Penicillium* sp. were obtained from the Department of Microbiology and Immunology, Kampala International University Western Campus, Uganda.

Figure 1. Map of Uganda showing the study areas.

Primary screening

Antifungal activities of isolates were tested by modified spektra-plak method (Oskay et al., 2004; Madigan et al., 1997). Plates with potato dextrose agar supplemented with (g/l : K_2HPO_2 0.5, $CaCO_3$ 0.75, $MgSO_4.7H_2O$ 0.5, NaCl 2 and pH adjusted to 7 ± 4) were inoculated with 7 days old actinomycetes cultures by forming a circle of two to four different actinomycetes isolates on a plate and incubated for 7 days.

72 h cell and spores of tests fungi were inoculated and incubated for another 3 days. Antifungal activity was observed by formation of inhibition zone.

Secondary screening

Fermentation: All isolates were subjected to fermentation; this was to confirm the ability to produce bioactive compounds in solid and liquid media (Ensieh et al., 2015). Fermentation was carried out by the submerged culture in Erlenmeyer flask (500 ml).

The 7 days old culture of actinomycetes was inoculated in yeast extract starch broth (g/l: yeast extract 3, Peptone 3, Casein 3, Starch 8, Glycerol 3, $CaCO_3$ 0.75, K_2HPO_2 0.5, $MgSO_4.7H_2O$ 0.5, NaCl 12 and pH 7.4) and incubated in gas bath thermostats oscillator (THZ-82B) at 28°C and 200 ± 5 rpm for 7 days after which the broth was centrifuged at 3000 rpm for 20 min and filtered using filter paper (Whatman No 1) (Sohan et al., 2015).

Antifungal activity of fermented broth: Agar well diffusion method was employed to assess the antifungal activity of the fermented filtered broth. Cell concentration of *C. albicans* was adjusted at 0.5 McFarland turbidity standards and inoculated on potato dextrose agar plate while *Aspergillus* sp, *Penicillium* sp, *Rhizopus* sp and *Fusarium* sp were grown on Potato dextrose agar (PDA) for 72 h and spores were collected and inoculated on fresh PDA medium.

Wells were bored by sterile 1000 µl micropipette tip (Hotam et al., 2013). The wells were filled with 200 µl of supernatant of centrifuged broth and the plates were incubated at 28°C for 72 h. Amphotericin B at 50 µg/ml was used as positive control. All experiments were performed in triplicates.

Extraction of bioactive compounds: The fermented broths were centrifuged and filtered. The filtered broths were extracted using a solvent by adding equal volume (1:1) of ethanol (95%). The solution was shaken vigorously on a rotatory shaker for 24 h. The solvent phase was collected and evaporated in hot air oven at 40°C. The extracts were dried and stored at 4°C for further studies (Hotam et al., 2013; Raja and Prabakaran, 2011).

Antifungal activity of the solvent extract: The dried extracts were re-dissolved in 2.5% dimethyl sulphoxide (DMSO) at a concentration of 2.5 mg/ml and antifungal activity was determined by agar well diffusion method. Cell concentration of *C. albicans* was adjusted at 0.5 McFarland turbidity standards and inoculated on PDA plates

using sterilized cotton swabs. While *Aspergillus* sp, *Penicillium* sp, *Rhizopus* sp. and *Fusarium* sp. were grown on PDA for 72 h and spores were collected and inoculated on fresh PDA medium, wells were bored using sterile 1000 µl micropipette tip (Hotam et al., 2013).

The wells were filled with 200 µl of 2.5 mg /ml of the extract and the plates were incubated at 28°C for 72 h. Amphotericin B at 50 µg/ml was used as positive control. DMSO 2.5% was also used to serve as negative control. All experiments were performed in triplicates.

Identification of actinomycetes

The active actinomycetes isolates that showed activity during primary and secondary screening were identified using macroscopic, microscopic and biochemical methods.

Macroscopic study

The macroscopic features of the active isolates observed were colony colour, aerial mycelium, substrate mycelium, pigment production and texture.

Microscopic study

Surface appearances of the selected actinomycetes isolates were studied using dissecting microscope. While spores arrangements were studied using slide-culture method, blocks of Starch casein nitrate agar were cut and placed on sterile glass slides. The active actinomycetes isolate was inoculated on the block by streaking over the agar block surface, a cover slip was placed over the block, and the entire set up was incubated at 28°C for 7 days. The cover slip was removed and stained using Gram's staining techniques.

Cover slip was covered with crystal violet for 60 s and washed off with water, followed by Gram's iodine for 60 s, decolorized with alcohol for 05 s, and washed with water. Finally cover slip was stained with safranin counter stain for 1 min. After washing and drying, the test was microscopically observed under high power using phase-contrast microscope (X100) (Hotam et al., 2013; Kekuda et al., 2012).

Biochemical characterization

Gelatin hydrolysis: Gelatin test was carried out using methods described by Sundaramoorthi et al. (2011). Seven day old culture of active isolate was stabbed into nutrient gelatin tubes, using sterile inoculating needle. The tubes were incubated for 10 days at 30°C. Un-inoculated tube was used as a control.

After incubation, the tubes were placed in to refrigerator, at 4°C for 15 min. The refrigerated gelatin tubes that remained liquefied were considered positive test or solid to confirm negative test.

Starch hydrolysis: Starch hydrolysis test was carried out according to methods described by Remya and Vijayakumar (2008). Starch agar medium composed of (g/l): soluble starch, 20; yeast extract, 3; Peptone, 5; Agar 15 was prepared and autoclaved. Seven day old culture was inoculated in the medium and incubated at 30°C for 7 days. An un-inoculated plate was used as a control.

At the end of the incubation period, iodine solution was flooded on the plates to observe the clear zone of hydrolysis around the colony.

Esculin degradation: Esculin degradation test was conducted according to the method adopted by (Tiwarty, 2009). Seven day old culture was inoculated into Esculin agar slants (yeast extract, 0.3; ferric ammonium citrate, 0.05, agar 0.75, 0.1% of esculin and distilled water 50 ml) and incubated at 30°C for seven days. The test tube was observed for blackening the medium.

Methyl red – Voges – Proskauer test (MR-VP): Methyl red – Voges – Proskauer test (MR-VP) was carried out according to the method described by Cheesebrough (2006). It was used to determine the ability of the organism to ferment glucose with production of acid. Five millilitres (5 ml) of MR-VP broth was inoculated with the test organism and incubated for 48 to 72 h at 37°C.

After incubation, 2 to 3 drops of methyl red test was added to 1ml of the broth. A red colour signified a positive methyl red test while yellow colour signified negative test. To what remained, five drops of 4% potassium hydroxide (KOH) was added followed by fifteen drops of 5% α –naphthol in ethanol. The development of red colour within 1 h indicates VP positive test while no colour change indicated VP negative test.

Catalase test: Catalase test was carried out according to the method described by Cheesebrough (2006), to determine the ability of the isolate to produce the enzyme, catalase. A drop of 3% hydrogen peroxide was added to a loop full of the test organisms. Presence of bubbles indicates catalase activity.

Indole test: Indole test was carried out according to the method described by Cheesebrough (2006), to determine the ability of the isolate to degrade amino acid tryptophan and produce tryptophanase, enzyme was tested. 1% tryptophan broth in a test tube was inoculated with 7 days isolate and incubated at 37°C for 48 h.

After 48 h, 1 ml of chloroform was added to the broth. The test tube was shaken gently, and 2.1 ml of Kovac's reagent was added and again shaken gently, this was allowed to stand for 20 min. The formation of red coloration at the top layer, indicate positive while yellow coloration indicate negative.

Urease test: Urease test was carried out according to the method described by Cheesebrough (2006) to determine the ability to hydrolyse urea to produce ammonia and carbon dioxide. Test organism was inoculated into urease broth and incubated at 30°C for 72 h. Purplish pink coloration of the medium indicates positive reaction.

Nitrate test: Nitrate test was carried out according to the method described by Cheesebrough (2006), to determine the ability to hydrolyse nitrate to nitrite. Nitrate broth was inoculated with a loopful of active isolate and incubated at 28°C for 7 days. Un-inoculated test tube was used as a control. Two drops of sulphanilic acid and α – napthylamine solution was added to the broth. Presence of red colour indicates positive reaction.

Triple sugar iron test: Triple sugar iron test was carried out according to the method described by Vlab (2011); the test determines the ability of the organism to ferment the three sugar component of the medium: glucose, lactose and sucrose. The medium contains a pH indicator (phenol red) and a detection system (thiosulphate and ferrous sulphate) for hydrogen sulphide (H_2S). The medium was prepared as an agar slant.

The test organism was inoculated by stabbing the medium using sterilized straight wire loop and the surface of the slope was also streaked with the test organism. The test was incubated at 37°C for 3 days. After incubation, gas production was determined by observing the cracking of the medium, and production of H_2S was observed by the blackening of the butt (bottom) of the medium.

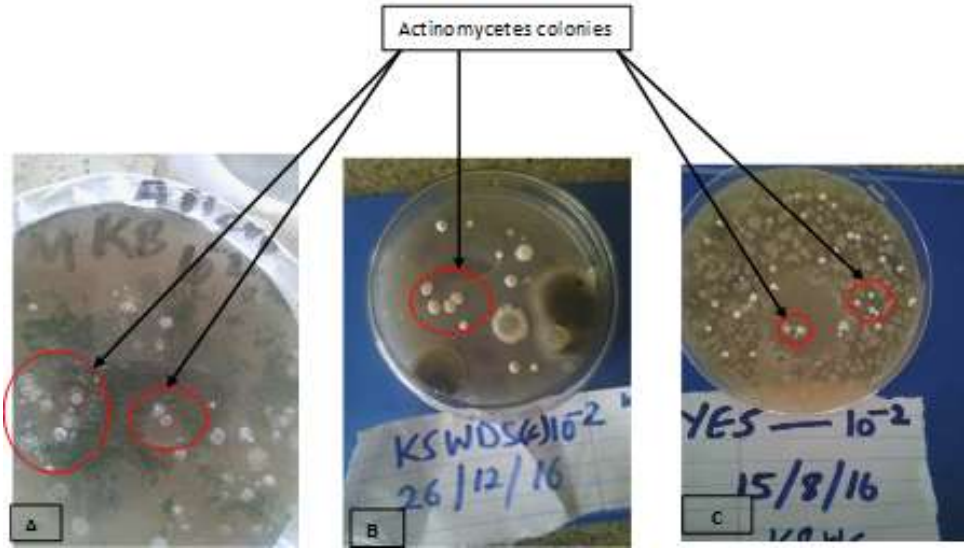

Figure 2. Suspected actinomycetes colonies in oval shape. A. *Actinomycetes spp* colonies on Glycerol casein agar, B. *Actinomycetes spp* colonies on Starch casein nitrate agar and C. *Actinomycetes spp* colonies on Yeast extract starch casein agar.

Glucose fermentation was determined by yellowing of the butt, the fermentation of lactose or sucrose or both was determined by the yellowing of both butt and slant.

Citrate utilization: This was carried out by inoculating the test organism in test tube containing Simon's citrate medium and incubated for 24 to 72 h. The development of deep blue colour after incubation indicated a positive result (Cheesebrough, 2006).

Ethical approval

Ethical approval of the study was obtained from Kampala International University (KIU), Institutional Research and Ethics Committee (IREC). All experiments were performed in accordance to the ethical standards of the microbiology laboratory operation.

Data analysis

Data was analysed using PAST software (Version 3.14). Pearson's correlation coefficient was used to compare between environmental factors (temperature, percentage moisture content and pH) with percentage colonies distribution, value at $p \leq 0.05$ was considered to be significant. One way analysis of variance (ANOVA) was used to compare between antifungal activity of fermented broth and ethanol extract, $p \leq 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Fifty six (56) actinomycetes isolates were isolated from 22 waste dump soil samples collected from four studied areas in Western Uganda. The samples were collected from market waste dump soil, residential waste dump soil and manure plantation farm. Three media (Starch casein nitrate agar, Glycerol casein agar and Yeast extract starch casein agar) were used for the isolation of actinomycetes (Figure 2). Glycerol casein agar was found to support high growth of actinomycetes followed by yeast extract starch casein agar (Result not shown). Although starch casein nitrate agar showed less growth, it which was chosen for the storage of pure isolates due to its ability to support the growth of both actinomycetes isolated from different media.

Table 1 below showed the results of pH, temperature, percentage moisture, colonies and percentage colonies distribution from the studied areas. The pH value ranged from 7.32 to 8.63 which are slightly alkaline, temperature was 18 to 57°C, percentage moisture content was 33 to 93% and colony and percentage colonies distribution ranged from 1 to 6 and 1.79 to 10.71%, respectively. Although the studied areas have high percentage moisture content and lower temperature, significant number of actinomycetes isolates (56) were isolated. There was positive weak correlation between percentage colonies distribution with moisture and negative weak correlation with temperature, but it was not statistically significant ($r = 0.146$, $p \leq 0.05$ and $r = -0.053$, $p \leq 0.05$, respectively). This was in accordance to the findings of Moselio and Joshua (2004) and George et al. (2012), who reported that environmental factors like temperature and moisture can affect the distribution of actinomycetes and tend to be abundant in wasteland than the moist soil. However, this was contrary to the findings of Rinoy et al. (2012), who reported negative correlation between actinomycetes loads and moisture content and positive correlation between actinomycetes loads and temperature.

The colonies and percentage colonies distribution were

Table 1 . The pH, temperature, moisture, number of isolates and percentage distribution from the studied areas.

Sampling site	Geographical location	pH	Temperature (°C)	Moisture (%)	No. of isolates	No. of isolates (%)
BRWDSa	00°32'31.17"S,30°08'36.16"E	7.81	20	36.98	3	5.36
BRWDSb	00°32'37.78"S,30°08'47"E	7.48	22	89.39	2	3.57
BRWDSc	00°32'34.75"S,30°08'31.86"E	8.24	25	65.56	1	1.79
BMWDSa	00°32'37.78"S,30°08'44.54'E	7.71	25	92.31	4	7.14
BMWDSb	00°32'33.54"S,30°07'57.55"E	8.53	29	47.06	2	3.57
BMWDSc	00°32'50.37"S,30°0943.66"E	7.36	32	74.83	1	1.79
KBRWDSa	01°14'53.04"S,29°58'06.23"E	8.33	20	52.85	3	5.36
KBRWDSb	01°1456.77"S,29°58'13.49"E	7.42	22	52.91	2	3.57
KBRWDSc	01°15'05.23"S,29°5907.86"E	7.65	24	93.82	2	3.57
KBMWDSa	01°15'18.34"S,30°01'12.63E	8.43	18	82.80	1	1.79
KBMWDSb	01°15'48.67"S,29°59'51.79"E	8.02	21	66.67	6	10.71
KBMWDSc	01°15'11.04"S,29°59'09.35"E	7.87	19	50.15	2	3.57
KSWDSa	00°10'35,44"N,30°4'43.76"E	7.81	49	60.77	5	8.92
KSWDSb	00°10'35,44"N,30°4'43.76"E	7.42	53	57.73	3	5.36
KSWDSc	00°10'14.26"N,30°04'56.29"E	8.01	56	48.81	1	1.79
KSWDSd	00°10'14.26"N,30°04'56.29"E	8.63	57	33.69	4	7.14
MBWDS a	00°36'13.88"S,30°39'41.29"E	7.78	29	38.12	2	3.57
MBWDS b	00°36'07.98"S,30°39'47.55"E	7.52	34	55.84	2	3.57
MBWDS c	00°36'21.73"S,30°38'36.65"E	7.57	36	48.37	1	1.79
MBWDS d	00°35'45.02"S,30°3945.64"E	8.36	39	37.36	5	8.92
MBWDS e	00°35'40.6"S,30°39'48.23"E	7.94	35	31.93	1	1.79
MBWDS f	00°35'28.13"S,30°40'03.03"E	8.01	33	56.21	3	5.36
Total					56	100

BRWDS: Bushenyi residential waste dump soil, BMWDS: Bushenyi market waste dump soil, KBRWDS: Kabale residential waste dump soil, KBMWDS: Kabale market waste dump soil, KSWD: Kasese waste dump soil, MBWDS: Mbarara waste dump soil.

found to have a positive weak correlation with pH (which is slightly alkaline) of the samples but, was not statistically significant ($r = 0.206$, $p \leq 0.05$). This was in line with findings of Basilio et al. (2003) and Rinoy et al. (2012), who reported that actinomycetes distribution is affected by pH and its desired alkaline pH condition than acidic environment. Basilio et al. (2003) also added that, actinomycetes loads drop at pH less than 5 which is acidic.

Eight actinomycetes isolates 8 (14.29%) showed antifungal activity to at least one test organism during primary screening. The isolates KBRWDSa (FR) and KBMWDSb6 showed antifungal activities to all test fungi (Table 2 and Figure 3). This result showed that 85.71% of the cultured actinomyctes isolates did not produce bioactive compounds in the solid medium. This could be due to the lacks of nutrients requirement or their cell structure as some actinomycetes have been reported not to produce bioactive compounds on the solid medium or the bioactive compound produced in the solid medium may be inactive due to presence of polar or nonpolar functional groups in the metabolites which may require particular amount of polar or nonpolar solvents to dissolve. This finding was similar to the previous studies carried out by different researchers where higher percentage number of the actinomycetes isolates subjected to primary screening failed to shows activity against test organism (Jemimah et al., 2012; Sohan et al., 2015; Alireza et al., 2010; Tara et al., 2009).

All actinomycetes isolates (56) were subjected to secondary screening involving two parts: Testing for antifungal activity of shake flask filtered broths and ethanol extracts. The results from the shake flask broth showed that some isolates that did not have activity during primary screening had activity after subjecting them into liquid broth culture and vice versa. Eleven actinomycetes isolate (19.64%) showed activity on at least one tested fungus (Table 3). The actinomycetes isolate KBMWDSb (M6) maintained its activity to all test fungi. The mean standard error antifungal activity of shake flask culture broth ranged from 5.33 ± 0.88 to 29.67 ± 0.88 mm. Although isolate KBMWDSb (M6) produced activity to all test organisms, isolates KBRWDSc (D1) produced the highest mean standard error zone of antifungal activity against *C. albicans* ATCC1023 (29.67 ± 0.88 mm).

The inability of isolate KBRWDSa (FR) to maintain its broad spectrum antifungal activity could be as result of

Table 2. Primary screening of antifungal activity of actinomycetes spp.

Isolates code	Test fungi				
	***Candida albicans* ATCC1023**	***Aspergillus* sp.**	***Fusarium* sp.**	***Penicillium* sp.**	***Rhizopus* sp.**
BRWDSc (SP)	+	+	-	+	-
KBMWDSb3 (N2)	-	-	+	-	+
KBMWDSb6 (M6)	+	+	+	+	+
KBRWDSc (D1)	-	+	-	-	-
KBRWDSa3 (RF)	+	+	+	+	+
KBRWDS (N1)	-	-	+	-	-
KSWDSc	-	-	+	-	-
MBWDS f2 (J)	-	-	-	+	-

BMWDS: Bushenyi market waste dump soil, BRWDS: Bushenyi residential waste dump soil, KBMWDS: Kabale market waste dump soil, KBRWDS Kabale residential waste dump soil, KSWDS: Kasese waste dump soil, MBWDS: Mbarara waste dump soil, +: positive and - : negative.

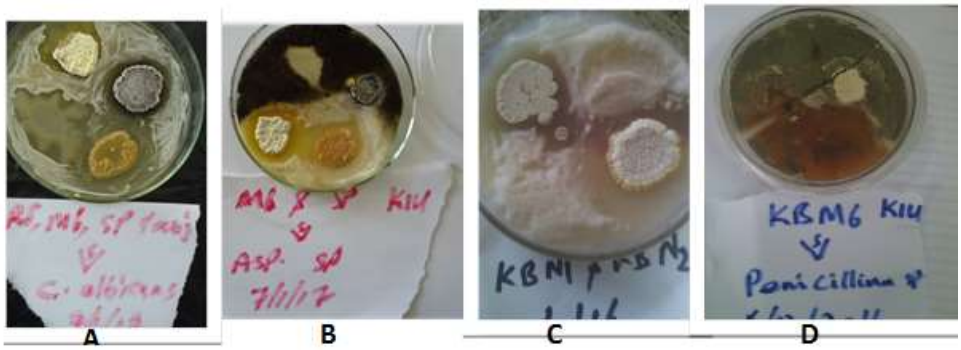

Figure 3. Primary screening: **A:** KBRWDSa3 (RF)**,** KBMWDSb6 (M6), BRWDSc (SP) and MBWDS f2 (J) against C. albicans ATCC1023, **B**: KBMWDSb6 (M6), BRWDSc (SP), MBWDS f2 (J) and KBRWDSa3 against *Aspergillus sp.* **C**: KBRWDS (N1) and KBMWDSb3 (N2) against Fusarium sp. and **D**: KBM6, KSWDSc and MBWDS f2 (J) against *Penicillium* sp.

cell structures or the bioactive compound produced in the liquid broth, contained non polar functional group which could not be dissolved in polar solvent (Tara et al., 2009). The result of solvent extract showed that some shake flask culture broth that failed to shows activity during screening, had activity when solvent was used for extraction of bioactive compounds. Thirteen (23.21%) actinomycetes isolates produced activity to at least one test organism (Table 3 and Figure 4).

The mean standard error zones of inhibition value of ethanol extract ranged from 6.33 ± 0.88 to 30.67 ± 0.88 mm. Although some fermented broth showed activity after solvent was used for extraction, there was a decreased in the zone of inhibition of antifungal activity of some fermented broth after ethanol was used for extraction. This could be as result of extraction processes, inability of solvent (ethanol) to extract the bioactive compounds or bioactive compounds were missing from ethanol segment due to presence of nonpolar compounds which could not dissolve in ethanol which is a polar solvent (Tara et al., 2009; Jirayut et al., 2012). Comparison between fermented broth and ethanol extract mean zone of inhibition using one way ANOVA showed no significant difference at ($p \leq 0.05$) between the two extracts.

Macroscopic, microscopic and biochemical features of the active actinomycetes isolates was presented in Table 4 and Figure 5. The macroscopic features of the isolates showed that majority were white chalky, grey and pink in colour, smooth to rough surface colonies with aerial and substrate mycelium and pigment production. Microscopically, the isolates were all Gram positive and had filamentous structures. The biochemical and phenotypical features of the isolates showed similarity to the genera Actinomycetes as described in Bergey's manual of determinative bacteriology 8th edition (Buchanan et al., 1974).

Conclusion

This finding shows that, waste dump soil of Western Uganda could be used as a rich source to explore novel actinomycete strains producing antifungal compounds.

Table 3. Mean standard error zone of inhibition of antifungal activity of fermented broth (FB) and ethanol extract (EE).

Isolate	Mean zone of inhibition (mm)									
	Candida albicans ATCC102		*Aspergillus* sp.		*Fusarium* sp.		*Penicilium* sp.		Rhizopus sp.	
	FB	EE 2.5 mg/ml	FB	EE 2.5 mg/ml	FB	EE 2.5 mg/ml	FB	EE 2.5 mg/ml	FB	EE 2.5 mg/ml
BMWDSc	0.00 ± 00	0.00 ± 00	0.00 ± 00	0.00 ± 00	0.00 ± 00	10.33 ± 0.88	0.00 ± 00	0.00 ± 00	0.00 ± 00	7.67±1.20
BRWDSc (SP)	16.67± 0.88	13.00 ± 2.08	19.33 ± 0.33	16.67±1.45	0.00 ± 00	0.00 ± 00	0.00 ± 00	0.00 ± 00	0.00 ± 00	0.00 ± 00
KBMWDSb3 (M18)	0.00 ± 00	0.00 ± 00	0.00 ± 00	9.67 ± 0.88	0.00 ± 00	0.00 ± 00	22.67 ± 1.45	24.00 ± 0.58	0.00 ± 00	0.00 ± 00
KBMWDSb6 (M6)	14.67 ± 0.33	16.67±1.20	10.33 ±1.45	11.33 ± 1.86	17.00 ± 1.15	18.00 ±1.15	15.33 ± 3.18	20.00 ± 2.65	19.67 ± 2.40	20.67 ± 2.91
KBRWDSc (D1)	29.67 ± 0.88	30.67 ± 0.88	0.00 ± 00	19.33 ± 1.20	0.00 ± 00	0.00 ± 00	11.67 ± 1.20	11.67 ± 0.88	0.00 ± 00	0.00 ± 00
KBRWDSa3 (RF)	26.67 ±1.45	25.33 ± 0.88	17.33 ±1.45	17.67 ± 1.20	0.00 ± 00	23.67 ± 2.33	12.67 ± 1.45	12.67 ± 1.45	0.00 ± 00	0.00 ± 00
KBRWDS (N1)	25.33 ± 1.45	22.33 ± 1.20	0.00 ± 00	0.00 ± 00	0.00 ± 00	0.00 ± 00	13.33 ± 1.76	13.00 ± 1.73	0.00 ± 00	0.00 ± 00
KSWDSc (G)	0.00 ± 00	0.00 ± 00	0.00 ± 00	0.00 ± 00	0.00 ± 00	0.00 ± 00	0.00 ± 00	18.67 ± 3.18	21.67 ±1.20	23.00 ± 2.08
KSWDSd2	0.00 ± 00	0.00 ± 00	0.00 ± 00	0.00 ± 00	0.00 ± 00	11.33 ±1.33	0.00 ± 00	0.00 ± 00	14.00 ± 2.08	14.67 ± 1.20
MBWDSc	0.00 ± 00	15.33 ±1.86	0.00 ± 00	18.00 ± 2.31	0.00 ± 00	22.67 ± 1.45	0.00 ± 00	0.00 ± 00	0.00 ± 00	0.00 ± 00
MBWDSd4	0.00 ± 00	0.00 ± 00	0.00 ± 00	0.00 ± 00	0.00 ± 00	0.00 ± 00	18.33 ± 0.88	19.00 ±1.15	15.00 ± 2.65	14.33 ± 2.33
MBWDSe	0.00 ± 00	0.00 ± 00	0.00 ± 00	0.00 ± 00	5.33 ± 0.88	6.33 ± 0.88	0.00 ± 00	0.00 ± 00	0.00 ± 00	0.00 ± 00
MBWDS f2 (J)	0.00 ± 00	0.00 ± 00	0.00 ± 00	0.00 ± 00	19.00 ± 0.58	20.67 ± 1.76	16.33 ± 2.19	18.00 ± 1.15	15.33 ± 2.73	16.33 ± 1.76

FB: fermented broth, EE: Ethanol extract. The result was not statistically significance ($P \leq 0.05$).

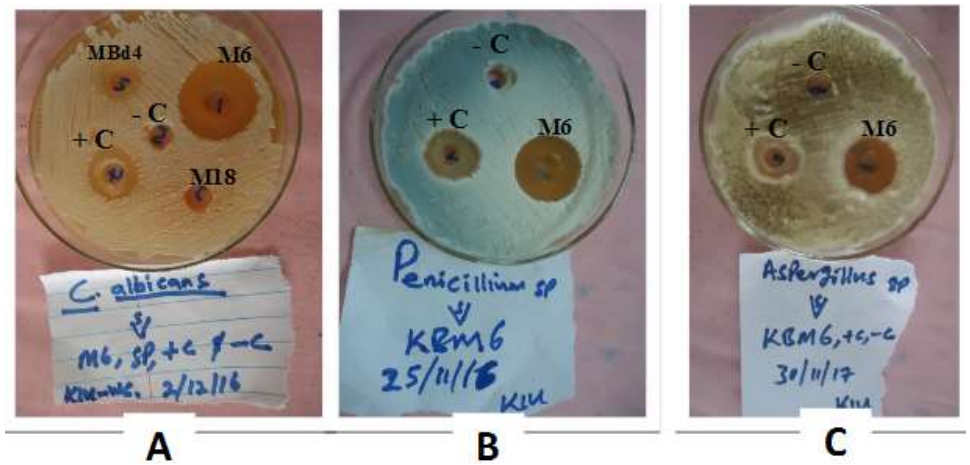

Figure 4. Antifungal activity of ethanol extract: A. *C. albicans* ATCC1023, B. *Penicilium* sp., C. *Aspergillus* sp.

Table 4. Macroscopic, microscopic and biochemical features of the active actinomycetes isolates.

Active isolate	Colony colour	Aerial mycelium	Substrate mycelium	Pigment production	Colony Surface	Gram's reaction	Catalase test	Citrate test	Indole test
BMWDSc	White chalky	Khaki	Brown	Nil	Rough	+	+	-	-
BRWDSc (SP)	Light yellow	Light yellow chalky	Khaki2	Light yellow	Smooth	+	-	-	-
KBMWDSb3 (M18)	Light pink	White	Light pink	Nil	Rough	+	-	+	-
KBMWDSb6 (M6)	Pink	Pink	Red	Red	Rough	+	-	-	-
KBRWDSc (D1)	White chalky	White chalky	Tan2	Nil	Rough	+	+	+	-
KBRWDSa3 (RF)	Grey 98	Grey 94 chalky	Light yellow	Yellow2	Smooth	+	-	-	+
KBRWDS (N1)	White chalky	White chalky	Khaki2	Nil	Smooth	+	+	+	-
KSWDSc (G)	Light yellow	Light yellow chalky	Black	Black	Smooth	+	+	-	-
KSWDSd2	White chalky	White	Light yellow	Light yellow	Rough	+	-	-	+
MBWDSc	White chalky	Khaki	Tan	Nil	Smooth	+	-	-	+
MBWDSd4	Light yellow	Light yellow chalky	Khaki	Light yellow	Smooth	+	+	-	-
MBWDSe	White chalky	Whit	Tan	Nil	Smooth	+	+	+	-
MBWDS f2 (J)	Grey	Grey	Tan	Nil	Rough	+	-	-	-

+: Positive, - : negative, H_2S: hydrogen sulphide.

Table 4. Contd.

Active isolate	Urease test	Gelatin hydrolysis	Methyl red test	Voges – Proskauer test	Nitrate test	Esculin degradation	Starch hydrolysis	Triple sugar iron test				
								Glucose	sucrose	Lactose	Gas production	H_2S
BMWDSc	-	+	-	+	-	-	-	+	-	-	-	+
BRWDSc (SP)	-	+	-	-	-	+	+	+	+	+	+	+
KBMWDSb3 (M18)	+	-	+	-	+	-		+	-	-	-	-
KBMWDSb6 (M6)	+	-	-	-	-	+	-	+	-	-	-	-
KBRWDSc (D1)	-	-	-	-	+	-	+	-	-	-	-	-
KBRWDSa3 (RF)	-	-	+	-	-	+	+	+	+	+	+	+
KBRWDS (N1)	-	+	-	-	-	+	+	+	+	+	+	-
KSWDSc (G)	+	+	-	-	+	-	-	-	-	-	-	-
KSWDSd2	-	-	-	+	-	+	+	+	-	-	-	+
MBWDSc	-	+	-	-	-	+	+	+	+	+	-	+
MBWDSd4	+	+	-	-	+	-	-	-	-	-	-	-
MBWDSe	+	-	+	-	-	-	-	-	-	-	-	-
MBWDS f2 (J)	-	+	-	-	+	-	+	+	-	-	-	-

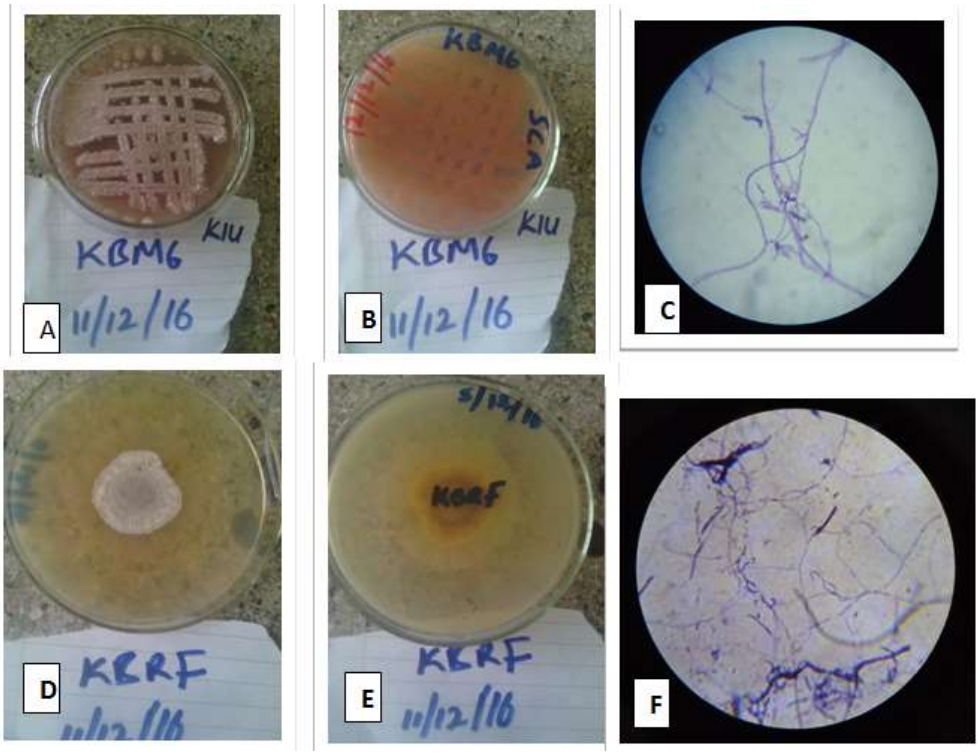

Figure 5. Morphological features of the some active *actinomycetes spp.* A. colony morphology of KBMWDSb6 (M6) B. Pigment produced by KBMWDSb6 (M6) C. Spore arrangement (rectiflexibiles) *of* KBMWDSb6 (M6), D. Colony morphology of KBRWDSa3 (RF), E. Pigment produced by KBR WDSa3 (RF) and F. Spores arrangement (rectiflexibiles) of KBRWDSa3 (RF).

Among the isolates obtained, isolate KBMWDSb6 (M6) produced broad spectrum activity against all test fungi. This organism will be subjected to further studies in order to discover its novelty as an antifungal agent.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Microbiological monitoring of water and *Crassostrea rhizophorae* in a mangrove ecosystem in Brazil

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Mangroves are considered as natural nurseries and classified as permanent protected areas. The study area is the municipal ecological site of Ilha do Lameirão, covering 891.83 ha of mangrove forest. This area persistently suffers from anthropic pressure. For this reason, there is the need to study and monitor the microbiological characteristics and recognition of the main factors involved in the degradation of mangroves process. This study was done in the year 2014. Samples were taken every month during dry and rainy seasons. Two areas (Maria Ortiz and Canal dos Escravos) were chosen based on the degree of their vulnerability to anthropic pressure. The results show there is a microbiological contamination both in water and *Crassostrea rhizophorae* tissues, mainly in samples taken from Maria Ortiz. According to the results, the Canal dos Escravos estuary, known as Class 1 is an environment that is able to maintain aquatic communities and fishing activities. In oyster tissues, microorganism strains that indicate the pathogens which cause diseases in humans were identified.

Key words: Environmental quality, microbiology, anthropic, pollution, oyster.

INTRODUCTION

Mangroves ecosystems maintain biodiversity offering the right conditions for feeding, reproduction and protection of several species and have an important role in nutrients and organic matter recycling (Schaeffer-Novelli, 1995). Over ecological aspects, mangroves provide direct and indirect benefits for population, especially for communities that use resources of those environments to survive (Wilson, 2002; Caffrey et al., 2016).

The transitional aquatic environment of Ecological Site Ilha do Lameirão suffers direct and indirect effects of

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water contamination; mainly because of illegal drains and domestic wastes (Moura et al., 2009). This contamination can cause the growth of fecal bacteria in the estuary waters that can exceed acceptable limits for drinking, recreation and irrigation (Brazil, 2005).

Together with mangroves root system, bivalve's mollusks of genus *Crassostrea rhizophorae,* generally known as mangrove oyster, build great communities both in the estuary edges and stuck to the substrate in the inner mangrove forest (Sroczyńska et al., 2012). Some of those mollusks are able to filter roughly 100 L of water per day (Suplicy, 2000); because they absorb toxins, pollutants and microorganisms (Corporeau et al., 2012), the concentration of biotic and abiotic elements in their tissues is a reliable indicator of environmental conditions.

Some cases of intoxication and microbiological contamination are associated with bivalves; recently, a particular attention has been given to oysters because of the habit to eat them in nature (Potasman et al., 2002; Iwamoto et al., 2010).

Concentration of pathogens in the estuary of monitoring can help you pick out certain parameters to protect public health and the environment. This study was conducted to assess the impacts of the release of domestic sewage and industrial waste under water and microbiological conditions of oysters from the mangrove forest on the island of Lameirão (EEMIL). The results of this work will provide basic information and a source of scientific knowledge on the levels of water contamination, and identify the main pathogens that cause food poisoning in oysters that are usually consumed in nature and can pose a risk to public health. The study will also help to generate a data base for fishermen, indicating the most suitable places for the collection of these organisms.

MATERIALS AND METHODS

Area of study

The Municipal Ecological Site Ilha do Lameirão (EEMIL), is placed in Vitória – Espirito Santo State - latitude 20° 14' S to 20° 17' S and longitude 40° 16' W to 40° 20' W.

Sampling points were chosen according to the class of vulnerability. The Maria Ortiz estuary (Points 1 and 2) shows a high or very high degree of anthropic pressure, mainly due to the closeness to neighborhood Maria Ortiz from that, probably, illegal drains discharge of domestic wastes (Tulli, 2007). The Canal dos Escravos estuary (Points 3 and 4) shows a low or very low degree of anthropic pressure, far from any possible drain of waste waters, and roughly 2.5 km from Points 1 and 2.

Water and oysters sampling

In the wet season samples were collected from January to March while in the dry season they were collected from June to August.

During each sampling and in each sampling Point 3 (three), water samples were manually collected, using 50 ml falcon tubes, at an approximate depth of 20 cm, in order to collect more than 20 ml of water, leaving an empty volume for a further shaking, before processing samples. Tubes filled up with samples were packed up in a thermic box for further analysis.

Oysters were manually removed from roots of trees found in the mangrove forest. 6 (Six) oysters with an average size of 8 cm were collected in each point (n=90). Oysters were placed in sterilized plastic bags with hermetic lock, and then stored in a thermic box for further processing and microbiological laboratory analysis.

Microbiological analysis

Microbiological analysis of water and oyster tissues was carried out within four hours from the field sampling. The chosen method was the most probable number per milliliter (MPN/mL) based on consecutive dilutions technique (APHA, 1970). The first step of this technique is a presumptive test in which there is the identification of lactose fermenting bacteria (through the Lauryl Sulfate Tryptose Broth). The second step is a confirmative test, recognizing the presence of total coliforms (through VB broth) and / or the presence of thermotolerant coliforms, through EC broth (APHA, 1970).

For the presumptive test, aliquots of 25 g of each sample were prepared and then diluted in 250 mL of 0.1% peptone water. Serial dilution was followed in tubes containing 9 mL of 0.1% peptone water up until 10^{-5}. Then, 1 ml of each dilution was transferred into tubes, in triplicate, containing Lauryl Sulfate Tryptose broth (LTS) and these were incubated for 24 to 48 h at 37°C. After incubation, tubes showing turbidity and gas production were selected for the confirmatory test. For this step, approximately 10 µL of each tube was transferred to a tube containing Brilliant Green Bile broth (VB) and a tube containing *Escherichia coli* (EC) broth. The tubes contained VB broth were incubated for 24 to 48 h at 37°C and the tubes with EC Broth were incubated for 24 to 48 h at 45°C. The tubes with turbidity and gas production were registered to calculate the most probable number, according to the table established by APHA (Morelli and Vieira, 2003; ISO, 2015).

Oyster microbiological analysis needs before the inoculation, the preparation of a solution containing bivalves tissue: Only after that, it is possible to carry out microbiological analysis (Whitman, 2004). Preparation steps were: Valves cleaning; shell detachment; weighing 25 g of bivalves tissue; tissues liquefaction; intervalvar liquids put into sterilized blender, then inoculated in a growth medium according to most probable number (MPN) method. To detect microorganisms indicating the presence or the absence of pathogens dangerous for food sanity, like *Salmonella*, *Shigella* and other bacteria, agar *Salmonella Shigella* (agar SS) and agar MacConkey (Himedia laboratories) growth media were employed, characterized by a medium selectivity and able to isolate those genera. Although culture media makers explain how to detect pathogens presence through colony color and smell, biochemical analysis of the material are required to confirm the results. The samples were subjected to bacterial analysis in the laboratory of Microbiology and Biotechnology of the University Vila Velha (UVV).

Data were analyzed by mean and standard deviation. All sampling data points were compared each other considering different sampling stations and they were analyzed by non-parametric tests Kruskal-Wallis and student's t-test, considering significant difference in $p < 0.05$ (Zar, 1999). Values were compared with water quality standard set by the national environmental Council (CONAMA) and for the quality of oysters RDC 12/2001 of the national health surveillance agency (ANVISA), which defines criteria and microbiological standards for foodstuffs.

RESULTS

Water microbiological parameters evaluation

Total coliforms values change significantly between each sampling point and within each different season (dry and wet) (p=0.01). In the Points 1 and 2 (localized in Maria

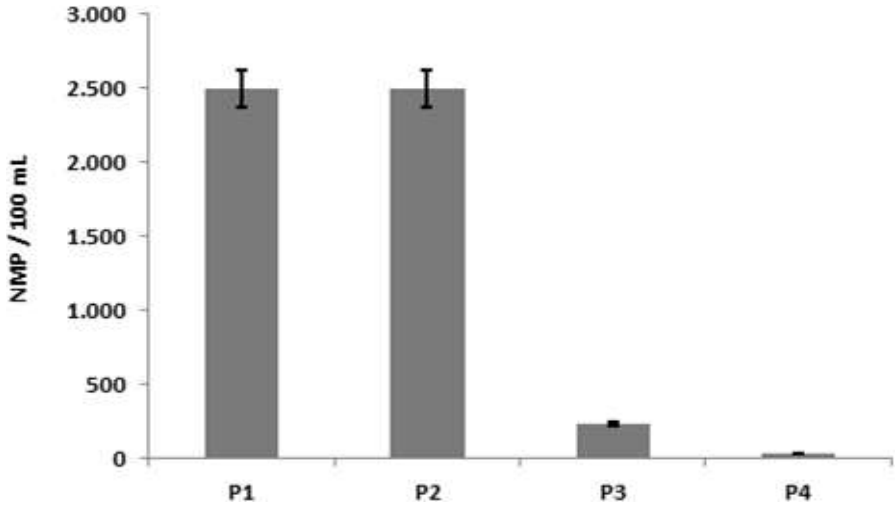

Figure 1. More probable number (MPN) of total coliforms per 100 mL in water (Y axis), in 4 sampling points (X axis), localized in Municipal Ecological Station, Ilha do Lameirão, in rainy season. Points 1 to 2 correspond to Maria Ortiz area, and 3 to 4 to Canal dos Escravos. Averages followed by the same capital letter in the different points do not have significant differences according to Tukey non paramentric test with a degree of confidence of 5%.

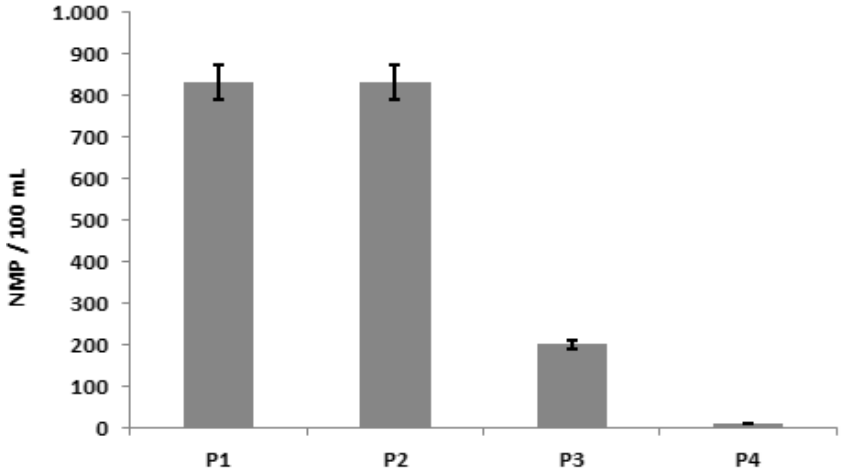

Figure 2. More probable number (MPN) of total coliforms per 100 mL in water (Y axis), in 4 sampling points (X axis), localized in Municipal Ecological Station, Ilha do Lameirão, in dry season. Points 1 to 2 correspond to Maria Ortiz area, and 3 to 4 to Canal dos Escravos. Averages followed by the same capital letter in the different points do not have significant differences according to Tukey non paramentric test with a degree of confidence of 5%.

Ortiz area) values were greater than 2000 MNP/100 mL during the wet season and 800 MNP/100 mL during the dry season, while in the Points 3 and 4 localized in Canal dos Escravos area (characterized by a lower anthropic pressure) total coliforms values change from 210 to 28 MNP/100 mL in both seasons, respectively for Point 3 and 4 (Figures 1 and 2).

The results of thermotolerant coliforms quantification show significant changes between sampling points ($p=0.01$). During the rainy season, those values exceeded 2000 MNP/100 mL for the point 1 and remained up to 1100 MNP/100 mL in point 2 (localized in the area classified as affected by high and very high human pressure). During the dry season, these values decreased to a value over the 1100 MNP/100 mL for the point 1 and over 460 MNP/100 mL in Point 2. About Points 3 and 4 values did not change across the two seasons ($p \leq 0.95$), remaining over 210 MNP/100 mL for the Point 3 and over 28 MNP/100 mL in the Point 4 (Figures 3 and 4) but with significant differences between

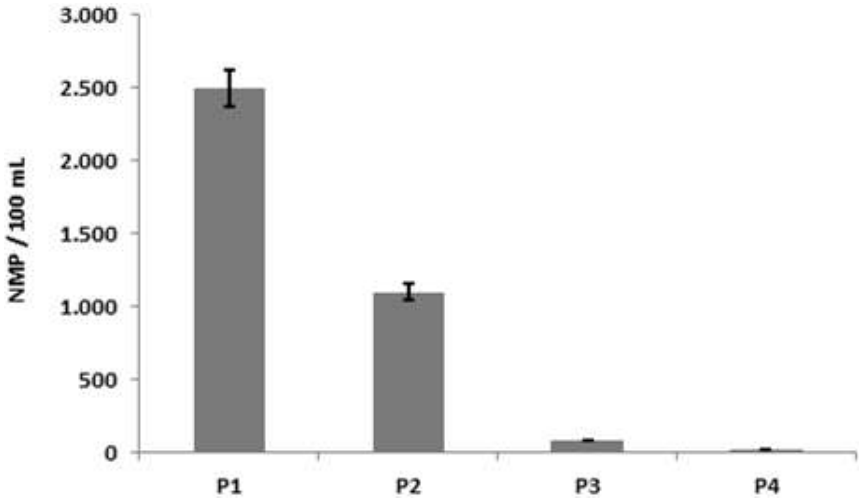

Figure 3. The most probable number (MPN) of thermotolerant coliforms per 100 mL in water (Y axis), in 04 sampling points (X axis), localized in Municipal Ecological Station, Ilha do Lameirão, in rainy season. Points 1 to 2 correspond to Maria Ortiz area, and 3 to 4 to Canal dos Escravos. Averages followed by the same capital letter in the different points do not have significant differences according to Tukey non paramentric test with a degree of confidence of 5%.

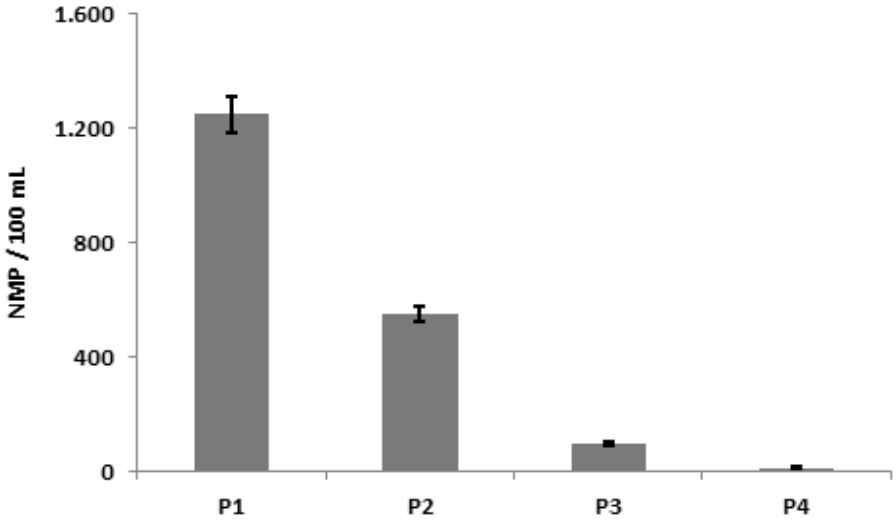

Figure 4. The most probable number (MPN) of thermotolerant coliforms per 100 mL in water (Y axis), in 04 sampling points (X axis), localized in Municipal Ecological Station, Ilha do Lameirão, in dry season. Points 1 to 2 correspond to Maria Ortiz area, and 3 to 4 to Canal dos Escravos. Averages followed by the same capital letter in the different points do not have significant differences according to Tukey non paramentric test with a degree of confidence of 5%.

both points (p=0.01).

Evaluation of mangrove oyster microbiological parameters

Total coliforms values change between sampling points within the two evaluated seasons (dry and rainy) (p≤0.002). The MO point, localized in Maria Ortiz area, showed values greater than 600 MNP/100 mL during the rainy season and 1200 MNP/100 mL in the dry season (Figures 5 and 6), while in the CE point, localized in the Canal dos Escravos area (suffering a lower anthropic pressure), total coliforms values changed from 550 to 1200 MNP/100 mL in the both seasons (Figures 5 and 6).

As shown in Table 1, pathogenic strains like: *Salmonella* sp. (an amount of three strains found during

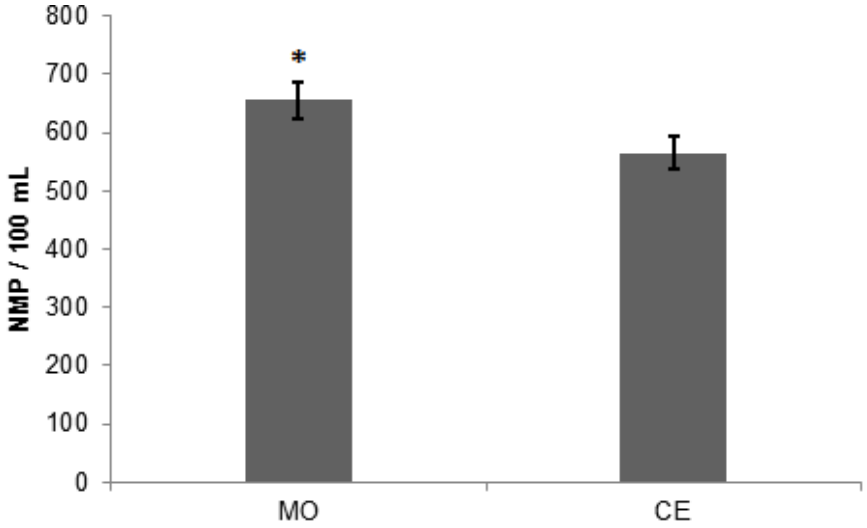

Figure 5. The most probable number (MPN) of total coliforms per 25 g of oyster tissue (Y axis), in two sampling areas (X axis), localized in Municipal Ecological Station, Ilha do Lameirão, during the rainy season. MO corresponds to oysters collected in Maria Ortiz and CE corresponds to oysters collected in Canal dos Escravos. Averages followed by the same capital letter in the different points do not have significant differences accordingly to Tukey non paramentric test with a degree of confidence of 5%.

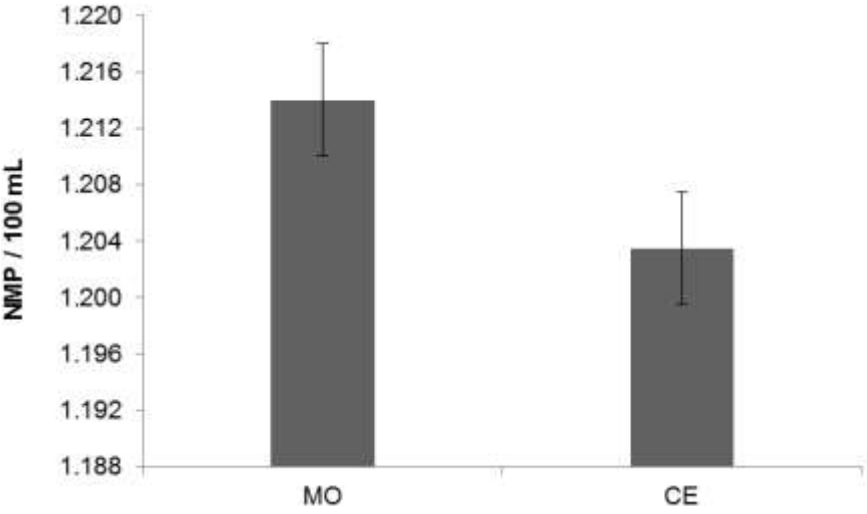

Figure 6. The most probable number (MPN) of total coliforms per 25 g of oyster tissue (Y axis), in two sampling areas (X axis), localized in Municipal Ecological Station, Ilha do Lameirão, in dry season. MO corresponds to oysters collected in Maria Ortiz and CE corresponds to oysters collected in Canal dos Escravos. Averages followed by the same capital letter in the different points do not have significant differences accordingly to Tukey non paramentric test with a degree of confidence of 5%.

February, April and June in MO area), *Pseudomonas* sp. (an overall of six strains found across all months in the MO area) and *E. coli* (an overall of eight strains: six found in the MO area and two in the CE area were detected). The types of bacterial were determined by serial dilution and plating of samples on differential culture media. The isolates were identified and characterized accordingly.

DISCUSSION

The CONAMA resolution nº 357/2005, characterizes the environmental classification according to the microbiological quality of the environment. The indices of total coliforms and thermotolerant found in the Canal dos Escravos are classified as Class 1 area (in which the

Table 1. Quantitative analysis for confirming presence or absence of strains with microorganisms indicating pathogens presence in oyster tissue.

Microrganism	MO	CE
Salmonella sp.	+	-
E. coli	+	+
Pseudomonas	+	-

-, Negative; +, Positive.

different uses of water are allowed for thermotolerant coliforms values not exceeding 1000 MPN/100 ml water). This class allows all activities which involve a direct use of water for protection of aquatic communities, aquaculture, fishing and supply for human consumption after standard treatment.

The values found in the estuary region of Maria Ortiz, allow you to classify it as Class 2, including the limit of 2500 MNP/100 ml. In this area in which activities are allowed sporadic or accidental direct contact with the water and the possibility of drinking water is very low as in navigation. In this estuary, the bivalves must not be collected for human consumption, mainly because it is an area that exceeds the limits allowed for the preservation of water aquatic communities and their maintenance in condition to be sold.

The high values of coliforms in Maria Ortiz can be related with the influence of anthropogenic pressure on the ecosystem. Human activities involving improper discharge of domestic waste are prevalent in coastal areas, negatively affecting the quality of water (Edun and Efiuvwevwere, 2012). High values of thermotolerant coliforms are often found in low quality organic matter enriched environments (Kolm and Miquelante, 2011). Evangelista-Barreto et al. (2014) assessed the microbiological characteristics of water and oysters from estuary of Maragogipe, Bahia, where they performed the analyses of total coliforms count, thermotolerants and isolation of *E. coli*. By comparing their results with the 357/2005 of CONAMA resolution allows the authors concluded that the environment in which the oysters were being drawn was with a high degree of contamination.

Severe or punctual pollution is a kind of environmental degradation and negatively affects the environmental and human health, reducing the human lifespan and exposing people to potentially dangerous pathogens. Health and quality of life are strictly related to the environment, since it is impossible to show conditions of good health, living in a degraded and polluted environmental plan (Komeily and Srinivasan, 2015).

Oysters as a bioindicator is efficient, being sensitive to the parameters evaluated through microbiological analysis of water from the estuary. The National Agency, in resolution Nº 12 of 2001 does not indicate parameters about the group of total coliforms and nor has assessment criteria for shellfish consumed in nature, considering only bivalve molluscs cooked or industrially chilled or frozen, that establishes a limit for thermotolerant coliform of 5×10/g. In accordance with the results presented in this work, Vieira et al. (2008) was able to assess the microbiological quality of the water habitat of oyster, the Pacoti River estuary, and succeeded in isolating 29 strains of *E. coli* samples collected. The presence of *E. coli* in fresh foods indicates fecal contamination and demonstrates environmental contamination by sewage drainage system.

In this study three strains of bacteria of the genus Salmonella were isolated, all from the region of Maria Ortiz, a fact very worrying, since the legislation requires total absence of this bacterium to any random sample of 25 g. Lee and Younger (2003) analyzed the United Kingdom coast oysters and realized that the presence of *Salmonella* was greatly influenced by the collection location. The influence varied according to the disposal of sewage and the type of agriculture performed in the region. These factors influenced directly the amount of bacteria present in the marine environment and therefore in oysters.

Bacteria of the genus *Salmonella* are Gram-negative bacteria and can be used as indicators of faecal contamination in samples of *Crassostrea rhizophorae* (Silva et al., 2004), and the consumption of these fresh oysters can be a serious health hazard, being responsible for cases of salmonellosis and colibacillosis (caused by *E.coli*) in the human population (Sanchez-Vargas et al., 2012).

Salmonella is a bacterium widely distributed in nature, being the main reservoir for these bacteria in the intestinal tract of humans and warm-blooded animals and cold. Molluscs and crustaceans are among the animals which pollute more easily with this type of bacteria (Rampersad et al., 1999). These molluscs are not capable of being used for human consumption from the point of view of health when compared to microbiological standards established by RDC n° 12 (Brazil, 2001). In America and Southeast Asia, salmonella is a common cause of gastrointestinal disease and accounts for about 1.4 million cases of infections annually (Ponce et al., 2008). These pathogens, as well as heavy metals and other toxic compounds can be bioaccumulated in the tissues of these organisms and be biomagnified along the trophic chain (Mok et al., 2015). Machado et al. (2001) suggest that the determination ofthermotolerant coliforms in soft tissues and intravalvular liquid, to assess the quality of the molluscs, produced for commercial purposes.

Conclusion

The high average of total coliforms and thermotolerant organism in the region of Maria Ortiz demonstrated that the level of proximity to urban centers and the degree of

degradation is directly related to the microbiological quality of the environment, jeopardizing the life of the ecosystem. The results obtained from the analysis of the water support with the results found in the analysis of the tissue of oysters, which were found in pathogens microorganisms collected at the nearest region bivalve. The definition of management measures of harvesting areas of oysters and other bivalves can be optimized through data like this that allow the evaluation of environmental quality by using microrganisms indicators of brackish waters.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Contamination of meat products by coagulase positive staphylococci in the Algiers, Algeria

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The meat is regarded as one of the main sources of food-borne diseases; its evaluation can constitute a valuable source of information that can be used in the design of the collective prophylaxis programs in public health. In order to assess the level of contamination of certain meat products by coagulase-positive staphylococci and the influence of the storage temperature of these products, we conducted a study in three types of trade in the region of Algiers (Algeria). In total, 25 samples divided between meat products from red meat (minced meat, Merguez) and white meat (turkey, chicken) were taken at three different sales outlets (covered market, supermarkets and independent butchers), for bacteriological analysis. The mean of coagulase positive staphylococci were 1.60 ± 0.33 $\log_{10}$ cfu/g for minced meat, 2.02 ± 0.46 $\log_{10}$ cfu/g for Merguez, 2.02 ± 0.47 $\log_{10}$ cfu/g for turkey and 1.63 ± 0.21 $\log_{10}$ cfu/g for chicken. In addition, the descriptive analysis of the storage temperature data for these meat products revealed that, these temperatures have low correlations with variations in bacterial levels for minced meat, Merguez and turkey (minced). These results reflected insufficient hygienic conditions in the preparation, preservation and sale of these meat products. Thus, the consumption of these products can present a real health risk to public health.

Key words: Algeria, coagulase positive staphylococci, contamination, meat products, storage temperature.

INTRODUCTION

Meat is considered as a food of choice because of its nutritional value. Its richness in protein and the nature of these make it an indispensable food for a balanced diet (Bender, 1992). However, because of its nutritional qualities, meat is a very favorable ground for most microbial contamination (Bender, 1992).

Meat and meat products are ranked among the foods, most involved in collective food-borne outbreaks (TIAC) in Algeria (Mouffok, 2011). These diseases are responsible for serious health problems worldwide and the World Health Organization (WHO) estimates that, diarrhea kills 1.5 million people, and 70% of these cases

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are attributed to food consumption. In addition, food-borne outbreaks have a great social and economic impact: work stoppages, stoppages of agribusiness companies and restaurants involved, which can go as far as bankruptcy, court costs, bacteriological analysis and medical costs (Corpet, 2014).

In Algeria, the treatment of a banal poisoning costs the state around 3000 DA. This figure will increased in case of hospitalization and reaches a minimum of between 20,000 and 30,000 DA per day (Maïdi, 2012).

This study investigates bacterial contamination by assessing and counting coagulase positive staphylococci and taking the storage temperature of certain meat products, widely consumed in Algeria in three different sales outlets (independent butchers, covered markets and supermarkets), in order to assess their risks to public health.

MATERIALS AND METHODS

Sampling

A total of 25 samples of meat products from bovine red meat (minced meat, Merguez) and white meat (turkey minced, chicken) were studied. The samples were taken randomly between April and June 2015 from two different communes: Bab Ezzouar and Mohammadia. Three types of trade were targeted: independent butchers, covered markets and supermarkets.

The storage temperature of these meat products was recorded using a digital food thermometer (thermometer for food use) for each sample taken. The samples (200 g) were packaged in sterile bags, clearly identified and kept in a cooler under cold conditions. These bags were immediately sent to the veterinary and quality control laboratory (AVCQ-LAB) of Algiers. Their contents were analyzed as soon as they arrived at the laboratory.

Search and enumeration of coagulase positive staphylococci

In the laboratory, 10 g of each sample was placed in sterile stomacher bags. Then, 90 ml of the PSE diluent (Peptone, salt, water) (Institut Pasteur, Algeria) was introduced into the bags. The whole was crushed for 2 min in the stomacher to thereby make the stock solution 10^{-1}. The supernatant obtained after the grinding was recovered in a sterile flask. The latter was left to stand for 45 min, to allow the revivification of the stressed bacteria.

Coagulase positive staphylococci (CPS) were grown on Baird Parker agar (Institut Pasteur, Algeria) supplemented with egg yolk and potassium tellurite. The bacterial number is evaluated after 48 h of incubation at 37°C and their identity is confirmed by the search for catalase on H_2O_2 and coagulase on the plasma of rabbit (Arrêté ministériel, 2014).

The results were compared with the criteria required by interministerial decree N°35-1998 of 24 January 1998 on the quality of foodstuffs. The maximum accepted concentrations for the bacteria counted are: 2 $\log_{10}$ cfu/g for minced meat and Merguez, 2.70 $\log_{10}$ cfu/g for turkey and chicken (Arrêté interministériel, 1998).

Statistical analysis

The average bacterial burdens were calculated by type of trade and type of levy. The type of trade (independent butchers, covered markets and supermarkets) and storage temperature were used as a source of variation.

A factorial analysis of variance (ANOVA) was used to compare the results of coagulase positive staphylococci means between the three types of trade. It is also used to compare the means of enumeration of these bacteria according to the type of sampling.

The correlation coefficient (r) was calculated between the coagulase positive staphylococci means and the storage temperature of the four meat products at the time of sampling. The Student test was used to compare the mean number of bacterial colonies with the acceptability threshold for coagulase positive staphylococci. It was thus used for comparison between the two types of meat.

Before carrying out the statistical analysis, the first step was to test normality. To do this, the most powerful test is that of Shapiro-Wilk.

All calculations were carried out using the free statistical software of the latest version (R 3.3.3 of March 2017), after transformation decimal logarithmic of the results expressed as cfu/g to normalize the distribution.

RESULTS

Overall bacteriological quality of the meat products

Table 1 reports the concentration of coagulase positive staphylococci according to the meat product considered, expressed as $\log_{10}$ cfu/g. In the light of the results obtained, Merguez (2.02 ± 0.46 $\log_{10}$ cfu/g) and turkey (2.02 ± 0.47 $\log_{10}$ cfu/g) are more likely to be loaded with coagulase positive staphylococci compared to the minced meat (1.60 ± 0.33 $\log_{10}$ cfu/g) and chicken (1.63 ± 0.21 $\log_{10}$ cfu/g).

Depending on meat quality, Merguez (2.02 ± 0.46 $\log_{10}$ cfu/g) is more contaminated with coagulase positive staphylococci compared to minced meat (1.60 ± 0.33 $\log_{10}$ cfu/g) in the case of red meats. At the same time, turkey (2.02 ± 0.47 $\log_{10}$ cfu/g) is more contaminated with coagulase positive staphylococci than chicken (1.63 ± 0.21 $\log_{10}$ cfu/g) in the case of white meat.

The interpretation of the results obtained according to the thresholds of acceptability, set by interministerial decree N°35, 1998, respectively gave non-conformity rate of 20.0% for minced meat and 41.7% for Merguez. For other products (turkey and chicken), the enumeration results are below the threshold set by the Algerian regulations (2.70 $\log_{10}$ cfu/g), therefore they are 100% compliant (Arrêté interministériel, 1998).

The comparison between the averages of the coagulase positive staphylococci with the acceptability threshold for each type of sampling showed a non-significant difference ($p > 0.05$) for minced meat and Merguez, and a significant difference ($p < 0.05$) for turkey and chicken. The statistical analysis (ANOVA) revealed a non-significant difference ($p > 0.05$) between the bacterial composition of coagulase positive staphylococci of the four foodstuffs (minced meat, Merguez, turkey and chicken).

Comparison of coagulase positive staphylococci enumeration results for the two types of meats studied

Table 1. Variation in the amount of coagulase positive staphylococci of the meat products analyzed (Mean ± SD in log_{10} cfu/g).

Variable	Red meat		Mean	White meat		Mean
	Minced meat	Merguez		Turkey (minced)	Chicken	
CPS	1.60 ± 0.33	2.02 ± 0.46	1.89 ± 0.46	2.02 ± 0.47	1.63 ± 0.21	1.92 ± 0.44
PS<CR (%)	80.0%	58.33%	64.7%	100%	100%	100%
PS>CR (%)	20.0%	41.7%	35.3%	0%	0%	0%

CPS: Coagulase positive staphylococci; PS<CR: Percentage of samples which present a bacterial burden lower than the criterion fixed by Algerian standards; PS>CR: Percentage of samples which present a bacterial load higher than the criterion fixed by the standard; (%): Prevalence.

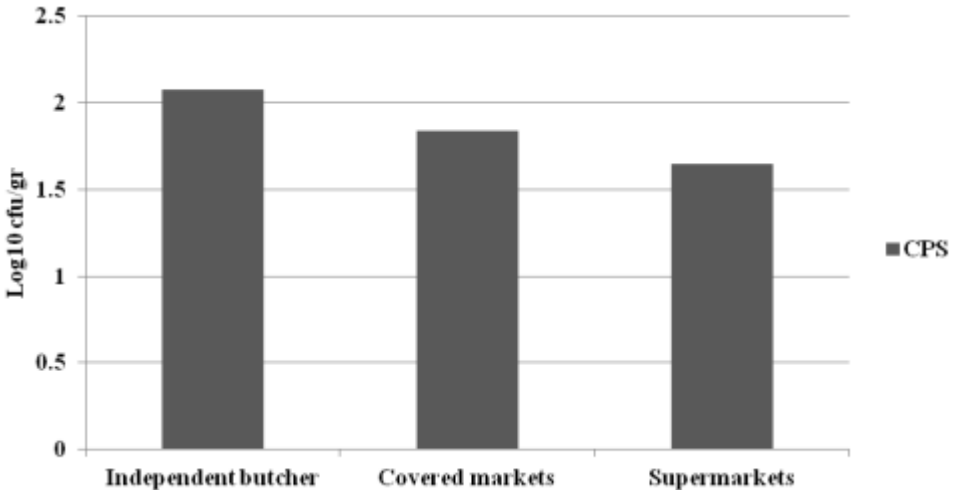

Figure 1. Distribution of coagulase positive staphylococci according to business type.

showed a non-significant difference ($p > 0.05$) between the bacterial load of red and white meats.

Distribution of the results according to the type of business

According to the results, meat products from independent butcheries (2.08 ± 0.40 log_{10} cfu/g) are more heavily loaded by coagulase positive staphylococci than those on the covered markets (1.84 ± 0.45 log_{10} cfu/g) and that of supermarkets (1.65 ± 0.44 log_{10} cfu/g) (Figure 1).

Statistical analysis showed a non-significant difference ($p > 0.05$) between the three types of trade.

Relationship between coagulase positive staphylococci contamination and the storage temperature of meat products at the point of sale

According to meat products, the storage temperature recorded in the various sites interacted with the contamination processes. From a minimum of, 7.40°C for minced meat, 2.90°C for Merguez, 7.20°C for turkey and 8.30°C for chicken while the maximum 22.5°C for minced meat, 20.5°C for Merguez, 13.2°C for turkey and 9.10°C for chicken. Mean storage temperatures were 13.7 ± 5.90°C for minced meat, 11.2 ± 5.87°C for Merguez, 11.3 ± 2.36°C for turkey and 8.70 ± 0.57°C for chicken (Figure 2).

The storage temperatures were very close to the Algerian standard (+4°C) at the level of supermarkets.On the other hand, the majority of the samples made in covered markets and independent butchers far exceeded the temperature recommended by the Algerian standards.

The statistical analysis shows that coagulase positive staphylococci have a development with a slightly weak correlation for minced meat ($r = 0.48$, $R^2 = 0.23$), large correlations for Merguez ($r = 0.04$, $R^2 = 0.002$) and the other for turkey ($r = -0.02$, $R^2 = 0.0002$) (Table 2).

DISCUSSION

The meat products selected from the present work are products which have been manipulated during their preparation, thus allowing evaluation of the contamination

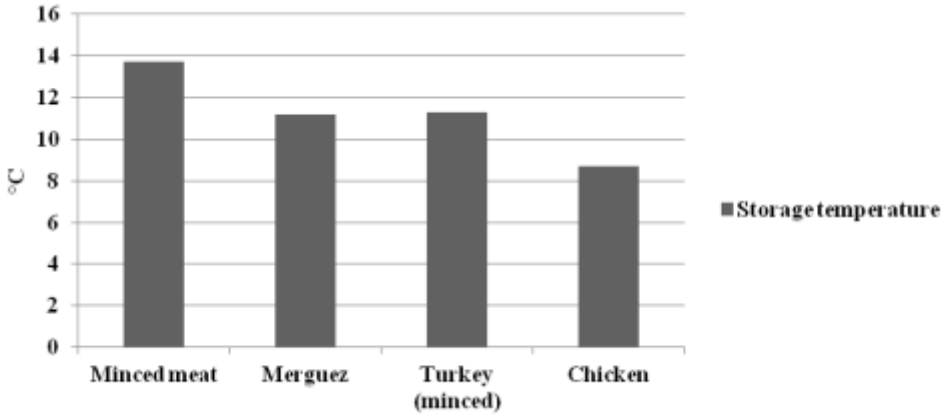

Figure 2. Distribution of the storage temperature according to the type of sampling.

Table 2. Correlation between the coagulase positive staphylococci enumeration results and the storage temperature.

Type of sampling	Relationship between the parameters	r	R^2
Minced meat	CPS-TS	0.48	0.23
Merguez	CPS-TS	0.04	0.002
Turkey (minced)	CPS-TS	-0.02	0.0002
Red meat	CPS-TS	0.03	0.0008
White meat	CPS-TS	0.21	0.05
General	CPS-TS	0.05	0.002

CPS: Coagulase positive staphylococci; TS: Temperature of storing; r: Coefficient of correlation; R^2: Coefficient of determination.

by human manipulation which adds to the initial contamination. In addition, three different types of sales outlets were targeted to assess the degree of contamination according to the hygiene status of each type of trade.

Coagulase positive staphylococci are considered to be pathogenic bacteria and their presence in food is due to poor handling conditions during preparation as well as poor hygienic quality of the material used in the food chain (Salihu et al., 2010).

The results obtained have an average of 1.60 ± 0.33 $\log_{10}$ cfu/g for minced meat, 2.02 ± 0.46 $\log_{10}$ cfu/g for Merguez, 2.02 ± 0.47 $\log_{10}$ cfu/g for turkey and 1.63 ± 0.21 $\log_{10}$ cfu/g for chicken. The frequency of results exceeding the legal criterion is 20.0% for minced meat and 41.7% for Merguez. For meat products derived from white meat (turkey, chicken), the enumeration results are below the threshold, set by the Algerian regulations (2.70 $\log_{10}$ cfu/g), that is they are 100% compliant. These results (1.60 ± 0.33 $\log_{10}$ cfu/g) are clearly inferior than those of Bouzid et al. (2015), who reported an average level of contamination of 4.61 ± 1.41 $\log_{10}$ cfu/g for fresh minced meat.

In a similar study, Chaalal (2013) confirmed the isolation of 55.5, 60 and 20% strains of *Staphylococcus aureus* from minced meat and Merguez samples, respectively, in Tiaret region (Algeria), while in the study conducted by Cohen et al. (2006) on Moroccan, meat products taken from different places of sale, *S. aureus* was detected in 16% of the samples distributed in red meat and offal, poultry meat, bovine minced meat, fresh sausages and fishery products. Whereas Sebban (1995) detected higher rates ranging from 33 to 52% (between 10^2 and 10^6 cfu/g) out of a total of 133 samples including raw, bovine, equine, cooked minced meat and fresh sausages of the Merguez type, obtained from different places of preparation or sale of the city of Rabat (butchers, supermarkets, restaurants). These results are similar to those detected by Normanno et al. (2005) in Italy, particularly for minced meat at a rate of 31.2%, which shows the importance of contamination of meat products by *S. aureus* both in developing countries (Algeria, Morocco) than in industrialized countries such as Italy.

Depending on the type of trade, the majority of samples taken from supermarkets recorded a low level of

contamination (1.65 ± 0.44 log_{10} cfu/g) compared with independent butcheries (2.08 ± 0.40 log_{10} cfu/g) and the covered markets (1.84 ± 0.45 log_{10} cfu/g), which can be explained by the observations taken at the time of sampling. It was noted that the hygiene conditions in these places of sale were better than those noted in independent butcher shops and those located in the covered markets.

In these modern supermarkets, staff wears suitable outfits, including gloves, cutting rooms and worktops, which were clean and equipped with a knife sterilizer and non-manual hand wash. The products were served in food trays and wrapped with a cellophane film. In addition, the checkout place is separated from the place of preparation, which may reduce the contamination.

The storage temperature of foodstuffs plays a major role in bacterial growth. Our results show that the majority of samples taken from covered markets and independent butchers exceeded by far temperature of 10°C. This temperature is considered as a threshold from which the *S.aureus* bacterium can start producing the enterotoxin responsible for the disease (Hennekinne, 2009). On the other hand, storage temperatures were very close to the norm at the level of supermarkets.

The correlation between the coagulase positive staphylococci contamination level of the four meat products and the storage temperature of these products showed a slightly weak correlation for minced meat (r = 0.48) and two other largely weak correlations, one positive for Merguez (r = 0.04) and the other negative for turkey (r = -0.02).

According to De Buyser (1996), coagulase positive staphylococci multiply at temperatures between 6 and 46°C with an optimal temperature of 37°C and toxinogenesis occurs under conditions somewhat more restrictive than those required for growth.

Conclusion

The results of this study confirm the importance of contamination of meat products by coagulase positive staphylococci in different places of sale (independent butchers, covered markets and supermarkets). Contamination by these bacteria is considered as an important indicator of hygiene, since the contamination can be of human origin during the manufacture of food or its domestic preparation.

In addition, this work sheds light on the conditions of conservation of these products by evaluating the temperature of storage at the point of sale. This factor is primordial in staphylococcal foodborne disease. However, a change can be noticed in practice food of Algerians and a development of the sector of the fast food.

Therefore, it is important to respect the cold chain and hygienic conditions when preparing meat products. Sensitization and popularization of consumers and other stakeholders in the food chain on the dangers of eating and preparing meat products is therefore necessary.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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