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Full Length Research Paper

Heat shock impact on the growth of *Bacillus* spp. (SUBB01) and its surveillance in minimal medium under shaking condition

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Heat shock proteins (HSPs) are a family of proteins that are produced by all living organisms in response to exposure to stressful condition. The present research aims to study the physiology of *Bacillus* spp. (SUBB01) under aeration in diverse culture media and temperature at 47, 48, 49, 50, 52, 53 and 54°C. Bacterial growth was measured through enumeration of the viable and culturable growing cells that are capable of producing the colony-forming units (CFUs) on Luria–Bertani (LB) and nutrient agar (NA) plates for 24 to 48 h. The work also focused on the repercussion of *Bacillus* spp. against excessive temperatures (37, 45 and 50°C) in minimal media under shaking condition. Isolated microbes were demonstrated under a light microscope to observe their cellular morphology, shape and organization. There was high-temperature shock up to 50°C in both LB, NA, and agar media, with the presence of a demanding defense mechanism against heat shock in these bacterial cells. However, further molecular studies on the genetic makeup of such stress responses as well as the growth retrieval mechanisms of *Bacillus* spp. through the exogenous organic factors would be very important.

Key words: Heat stress, *Bacillus* spp. (SUBB01), viable cells, minimal media, bacterial growth.

INTRODUCTION

Spore-forming pathogenic bacteria known as *Bacillus* spp. are repeatedly found in the environment. *Bacillus* spp. are hostile; they like *Escherichia coli have* with a number of growth defending stress factors like heat shock stress plus nutrient depletion, temperature fluctuation, variation in pH and redox potential (oxidation and reduction), limited water activity (a_w), elevated level of reactive oxygen species (ROS), osmotic disequilibrium

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along with unusual solute concentrations (Givskov et al., 1994; Kabir et al., 2004; Nystrom, 2005; Den Besten et al., 2009; Den Besten et al., 2013). Many of the heat shock proteins (HSPs) have been described as having heat shock, nutrient depletion, osmotic shock, toxic chemicals, and so on. Such HSPs have been reported to be induced in response to several stress factors. During heat shock stress the regulation of HSPs is specifically controlled by a single transcription factor; in eukaryotes, this regulation is accomplished by heat shock factor (HSF) (Klein et al., 2003). To withstand high-temperature stress, it is known as CspB and CspE (cold shock protein B and cold shock protein E) in *Bacillus* cells, and elevated levels of GroEL and DnaK proteins are reported in *E. coli* and *Salmonella* (Mayr et al., 1996).

Similar studies have shown that variation of temperature has an effect on the bacterial growth rates (Murata et al., 2009a, b; Noor et al., 2013). The impulsion of ROS provides hydrogen peroxide (H_2O_2), not just at the beginning of the early stage, but also in the growing culture (Kabir et al., 2004; Murata et al., 2009a; Munna et al., 2013; Nur et al., 2014) and the effect of several aeration speed on the cellular capability to produce the colony-forming units (CFUs) on agar plates (Munna et al., 2014). Above all interpretation, the physiological response of *E. coli* (SUBE01), *Pseudomonas* spp. (SUBP01), *Salmonella* spp. (SUBS01) and *Bacillus* spp. (SUBB01) was determined against oxidative stress through their sustainability in retaining the viable and culturable cells (Nitta et al., 2000; Noor et al., 2009a; Murata et al., 2011, 2012; Noor et al., 2013; Munna et al., 2013, 2014; Nur et al., 2014). Also the accumulation on the defense strategy of these bacteria peculiarly belonging to *Bacillus* spp. (SUBB01) under the static condition was detected through their phenotypic behavior (Nur et al., 2014). The recent investigation was conducted to further anatomize the heat-shock response in *Bacillus* spp. (SUBB01) under shaking condition (100 rpm) in minimal media.

MATERIALS AND METHODS

Bacterial strain, medium and culture condition

In this prominent experiment, the laboratory stock cultures of *Bacillus* spp. (SUBB01) were used for possible findings. Minimal agar (MA) and Minimal broth (MB) as well as nutrient agar, Luria-Bertani agar, nutrient broth and Luria-Bertani broth were used for the exploration of culture ability (Noor et al., 2013). After incubation for 24 h at 37°C in MA plates, one loopful of each bacterial culture was added into 5 ml of MB medium imitated by 100 rpm for 4-6 h (pre-culture) at 37 °C. Each bacterial isolate was introduced into 30 mL of MB and incubated at 37, 45 and 50°C, under shaking condition (100 rpm), after adjusting the optical density of the pre-culture at 600 nm (OD_{600}) to 0.1, 30 µl. At every 12 h interval, bacterial cell growth was observed by measuring at OD_{600} every 12 h, and the formation of CFUs was estimated by counting the bacterial viable and non-viable colonies up to 60 h at every 24-h intervals (Noor et al., 2009b, 2013; Munna et al., 2013, 2014). All the experiments were executed more than three times. Statistical analysis regarding bacterial growth was performed by determining P-value through t-test. The standard deviations were also measured.

Spot test

Each bacterial culture suspension was serially diluted in 9 mL nutrient broth to obtain up to 10^{-4} fold dilution described earlier, (Noor et al., 2013; Munna et al., 2013, 2014). An aliquot of 5 µl was dropped onto the nutrient agar and Luria-Bertani agar from each dilution. Then it was dried off for 15 min, and finally the agar plates were incubated at 37°C for 24 h. Spotting tests were done at every 12 h intervals of bacterial growth (Noor et al., 2013; Munna et al., 2013, 2014).

Demonstration of culturable *Bacillus* spp. (SUBB01) under heat stress

Laboratory stock culture of *Bacillus* spp. (SUBB01) and *E. coli* (SUBE01) were used in this experiment. Demonstrating the bacterial growth considering as cell turbidity (optical density at 600 nm) and CFUs were conducted as described in previous similar studies (Nur et al., 2014). To assay of culture ability of *Bacillus* spp. (SUBB01) and *E. coli* (SUBE01) by growing under the heat stress condition; nutrient agar (NA), Luria–Bertani (LB) agar, nutrient broth (NB) and Luria–Bertani broth were used as per standard policy. One loopful of each bacterial culture was added into 5 ml of MB medium mixed by 100 rpm for 4-6 h (pre-culture) at 37°C. Each bacterial isolates were introduced into 30 ml of MB and incubated at 37, 45 and 50°C at shaking condition (100 rpm), after adjusting the optical density of the pre-culture at 600 nm (OD_{600}) to 0.1, 30 µl. At every 12 h intervals, bacterial cell growth was observed by measuring OD_{600} at every 12 h, and the formation of CFUs was estimated by counting with the bacterial viable and non-viable colonies up to 60 h at every 24 h. All the experiments were conducted by three times. Statistical analysis regarding bacterial growth was performed by determining P-value through t-test. Standard deviations for all data have been indicated by error bars.
Assessing bacterial cell viability was further confirmed by the spot tests intervals (Noor et al., 2013; Munna et al., 2013, 2014; Nur et al., 2014). As delineated previously, each culture suspensions were serially diluted in 9 mL nutrient broth to obtain up to 10^{-4} fold dilution. From each dilution, an aliquot of 5 µL was dropped on to NA and LB agar, dried off for 15 min, and finally the plates were incubated at 37°C for 24 h. Spotting on the agar was accomplished at 24 h of growth.

Microscopic analysis of morphological changes in bacterial strains

Simple staining (Crystal Violet, Hucker's Solution) was used to observe the viability and cellular morphology of bacterial cells as previously described. Spore staining (malachite green oxalate, safranin O) was conducted to differentiate the bacterial spores from vegetative cells following standard procedures. An aliquot of 10 µl from the bacterial culture suspension was removed at 24 h of growth, and the cellular morphology, shape and organization were observed under the light microscope (Optima Biological Microscope G206, manufactured in Taiwan) at 1000× magnification (Munna et al., 2013).

RESULTS AND DISCUSSION

Previous study demonstrates the effective defense

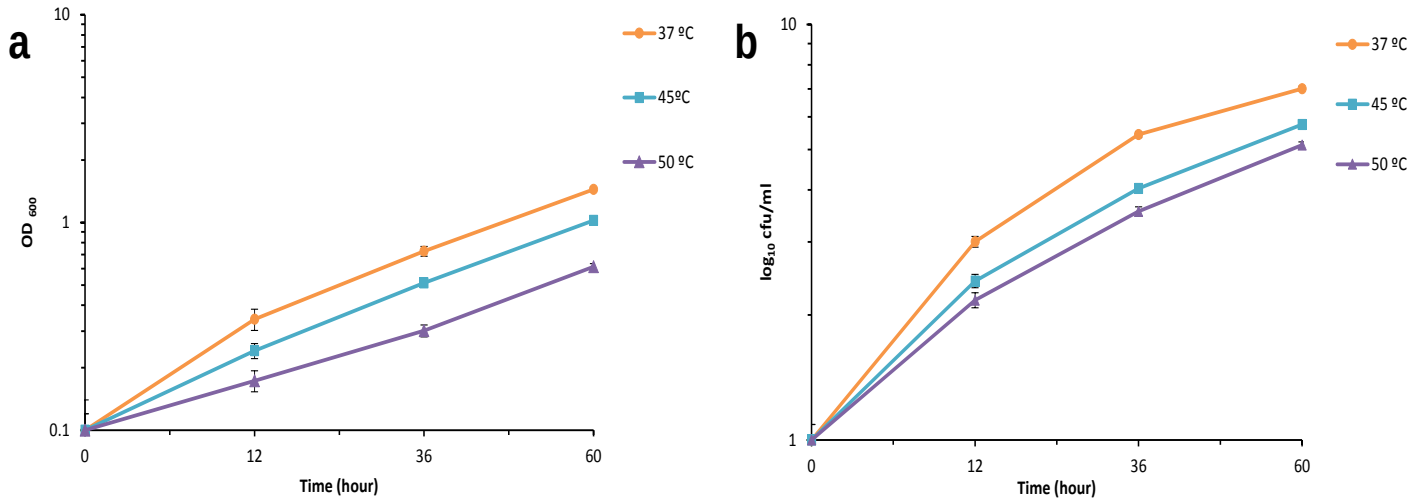

Figure 1. Categorization of growth of *Bacillus* spp. (SUBB01) at 37, 45 and 50°C. (a) Cell turbidity was monitored by measuring cells optical density and (b) Colony-forming units CFU/ml were determined at 100 rpm (rotation per minute) in minimal media.

strategies of *Bacillus* spp. (SUBB01) in response to the oxidative stress unnaturally produced by the supplementation of 6 mM H_2O_2 in the growing culture at an aeration speed of 100 rpm. Interestingly, the culturable cells of *Bacillus* spp. (SUBB01) decreased in their amount when these bacterial cells were compared with a nascent concentration (21 mM) of H_2O_2 (Nur et al., 2014). On the other hand, apart from this stressed condition, current investigation also engaged with a state of heat stress in the *Bacillus* cells since the increase in temperature is known to induce the accumulation of ROS inside the cells. This is turned into losing of cellular viability and culturability of bacterial cell largely (Yamada et al., 2009). In addition, when the *Bacillus* spp. (SUBB01) cells were grown at 37, 45 and 50°C, around 4-log reduction in cell turbidity (Figure 1a) as well as in the generation of the CFUs they were monitored (Figure 1b) up to 24 h of incubation periods in both nutrient and Luria–Bertani (LB) agar and broth. Surprisingly, bacterial growing cells produced CFUs up to 10^2 CFU/mL at 53°C. Interestingly, when the bacterial cells were compared with high temperature at 54°C, a complete excretion in both cell turbidity and CFUs was determined. This indicates the critical temperature for *Bacillus* spp. (SUBB01) is 53°C (Figure 1). Coherent with the results acquired in the growth-related experiments, no morphological changes were observed under the light microscope when cells were susceptible to growth temperatures of 48, 50, 52 and 53°C in both LB and nutrient broth for up to 24 h of incubation (Figure 2). Sporulation was also observed at 54°C. Previous studies showed that the stress of the signaling complex of *Bacillus* spp. is activated in response to several environmental stresses including the housekeeping heat shock stress (Munna et al., 2015).

Demonstration of growth viability and culturability of *Bacillus* spp. (SUBB01) under heat stress

Our previous study demonstrated that *E. coli* strain showed typical response against heat stress and cold stress conditions in minimal media (Noor et al., 2013; Shahriar et al., 2019). The consequence of our recent investigation indeed portrayed the phenotypic behavior of *Bacillus* spp. (SUBB01) in response to heat stress at 37, 45 and 50°C under shaking condition (100 rpm) in minimal media. No significant alterations were observed in cell turbidity (Figure 1a) as well as in CFU, Figure 1b) in minimal media. A steady growth at 37, 45 and 50°C was shown by *Bacillus* spp. (SUBB01) under shaking condition (100 rpm) up to 60 h of incubation periods (Figure 1). All the data found in this study were estimated as significant ($P < 0.1$). Aliquots were removed from respective bacterial cultures. There is no significant morphological or arrangement change observed with heat shock at 48, 50, 52, 53 and 54°C at 100 rpm (a–j). After every 12 to 60 h the *Bacillus* spp. (SUBB01) had steady growth at 37, 45 and 50°C.

The findings of the present investigation are quite consistent with earlier study on the critical growth temperature of *Bacillus* strain depending on the discernible impression of the descriptive nature of research condition. The results distinctly exposed the strong defense scheme of *Bacillus* spp. (SUBB01) in response to the heat shock at 37, 45, 47, 48, 49, 50, 52, 53 and 54°C in minimal media respectively. A quick drop in both cell turbidity and CFUs along with spores was observed after 12 to 24 h of incubation periods when cells were grown at 54°C in both Luria-Bertani and nutrient broth and agar.

In addition, the cells growth and viability spot tests were

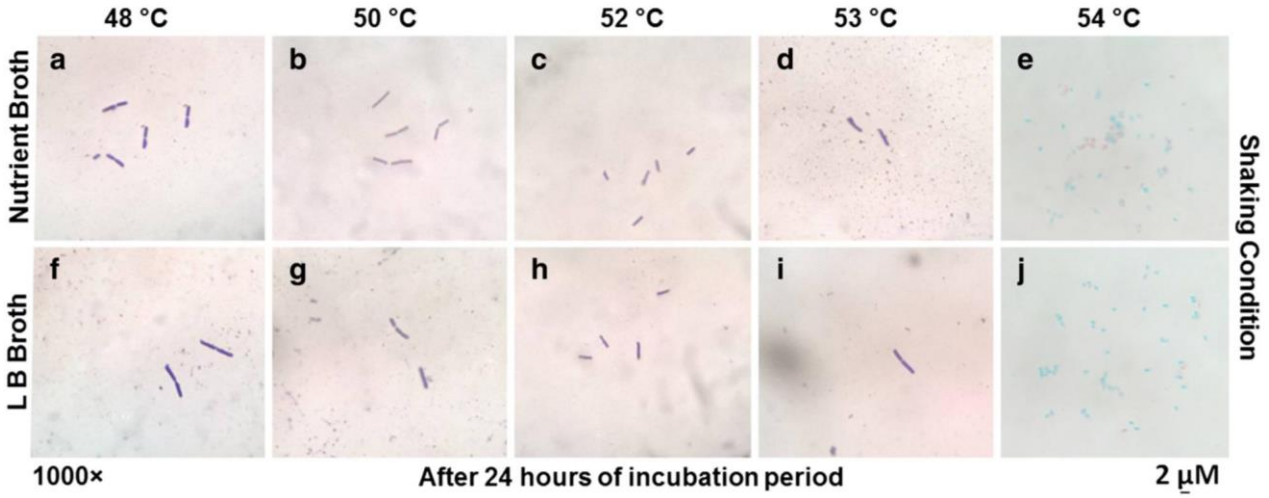

Figure 2. Microscopic analysis (morphology, size and shape) of *Bacillus* spp. (SUBB01) grown cells.

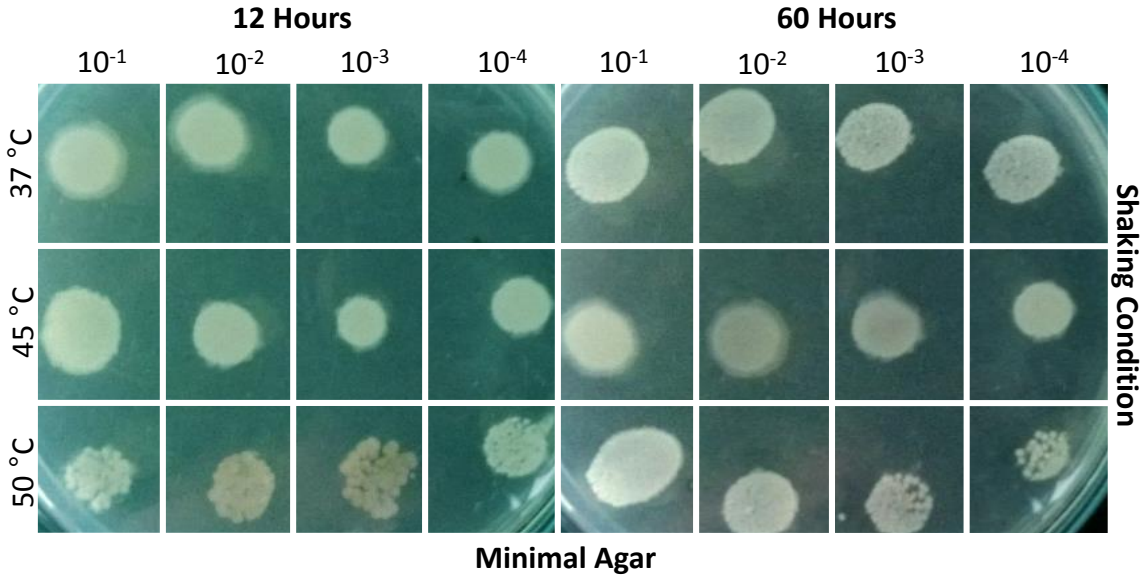

Figure 3. The confirmative manifestation of culturability and survival potential of *Bacillus* spp. (SUBB01) at 37, 45 and 50°C at 100 rpm in minimal agar media.

performed. Systematically, up to 10^{-4} dilution of MB culture, *Bacillus* spp. (SUBB01) on MA at 37, 45 and 50°C showed a steady growth (Figure 3). *Bacillus* spp. have six classes of heat shock proteins (HSPs) including HrcA and GroE chaperons of the class I category, RsbV, RsbR, RsbW and RsbX of the Class II category (regulated by the alternative sigma factor σ^B and σ^F), Class III HSPs, HtpG of Class IV, HtrA and HtrB of Class V, and finally the VI HSps to survive heat shock (Price et al., 2001; Phillips and Strauch, 2002; Huillet et al., 2012).

However, the present experiment depicts changing the temperatures of growth surveillance that impact *Bacillus* spp. (SUBB01) strain remained uninfluenced. This corroborates with previous reports (Price et al., 2001; Phillips and Strauch, 2002; Periago et al., 2002; Huillet et al., 2012). It is well-known that upon shifting from optimum to high temperatures, *Bacillus* spp. and other bacteria have been found to synthesize increased amounts of the HSPs.

The study also clearly illustrated the phenotypical changes in the bacterial cell caused by the sudden heat stress at the optimum velocity of aeration, which is contemporary unlike any other bacteria such as *E. coli*. These findings are relatively new in the field of heat

shock response on the growth of *Bacillus* cells with their surveillance under shaking condition. Such preliminary findings could be worth increasing the existing knowledge on bacterial cell biology and signal transduction. Spore forming bacteria, *Bacillus* spp. (SUBB01) usually have heat resistance which can be considered to have two components: temperature alteration distinguishing the species and the stabilization conferred by the heat shock state.

The heat shock response in bacteria is a protective mechanism to cope with heat-induced damage to proteins by synthesizing a specific set of proteins known as HSPs (Lindquist, 1986). The alternative sigma factor σ32 mediates the heat shock response. Under stress conditions, an elevated environmental temperature causes a transient increase in *σ*32 transcription and transient stabilization of *σ*32 protein levels, which is usually unstable. The *σ*32 directs transcription of RNA polymerase (RNAP) from the heat shock promoters and, thus, results in the induction of HSPs. Most HSPs behave as molecular chaperones that function to bind to and stabilize non-native polypeptides that are generated during protein synthesis or by heat denaturation of existing proteins, modulate protein folding pathways to prevent miss-folding or aggregation of proteins, and promote protein refolding and proper assembly (Georgopoulos and Welch, 1993). HSPs play a crucial role in this stress response.

Conclusion

Heat stress resistance of bacterial spores such as in *Bacillus* spp. (SUBB01) were compared with the temperature adaptation of each strain as weighed by the optimum and maximum growth temperatures (37 to 54°C) and the heat stress resistance of its vegetative cells. The extensive variation of temperature like sudden heat shock that affected *Bacillus* spp. (SUBB01) was estimated to be 53°C. The findings of the present investigation are quite consistent with earlier study on the critical growth temperature of *Bacillus* strain. The results distinctly exposed the strong defense scheme of *Bacillus* spp. (SUBB01) in response to the heat shock at different temperatures in minimal media. A quick drop in both cell turbidity and CFUs along with spores was observed after 12 to 24 h of incubation periods when cells were grown at 54°C in both Luria-Bertani and nutrient broth and agar. However, further molecular studies on the genetic makeup of such stress responses as well as the growth retrieval mechanisms of *Bacillus* spp. (SUBB01) through the exogenous organic factors would be important.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Passive surveillance of clinical poultry diseases in an Upazila Government Veterinary Hospital of Bangladesh

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The present study is aimed at investigating clinical poultry diseases in an Upazila Government Veterinary Hospital of Bangladesh through passive surveillance and to know the frequency distribution of antimicrobial drugs prescription pattern. The study was conducted in Ramu Upazila Government Veterinary Hospital under Cox's Bazar district of Bangladesh from February to March, 2016. A total of 180 cases including 73 broiler, 84 layer, 18 duck and 5 pigeon were included in this study. Diseases were diagnosed based on clinical history, clinical signs, post mortem lesions and rapid kit test. The most prevalent diseases were Newcastle disease (ND) 17.8% (95% CI 12-24%), colibacillosis 11.5% (95% CI 6-17%), infectious bursal disease (IBD) 10.8% (95% CI 6-16%) and aspergillosis 9.6% (95% CI 5-14%) in broiler and layer chickens; duck plague 69.6% (95% CI 49-90%) in duck and pigeon pox 13% (95% CI 0.2-24%) in pigeon. Regarding production type, the prevalence of colibacillosis, IBD and aspergillosis was significantly higher in broiler chickens (17.8, 19.2 and 16.4%) when compared with layer chickens (6.0, 3.6 and 3.6%) ($p<0.05$). On the contrary, ND and avian influenza (AI) were significantly greater in layer (27.4 and 10.7%) than broiler chickens (6.8 and 1.4%). Within age group, aspergillosis and omphalitis were higher at 1 to 10 days age groups while IBD and ND were greater in older age groups. In layer chickens, the prevalence of ND, AI, salmonellosis and fowl cholera were recorded higher in number between the ages of 9 and 83 weeks. Among the antibiotic drugs, ciprofloxacin (46.7%), colistin (42.2%), trimethoprim and sulphur drug (37.8%) were mostly prescribed to treat the diseases. Poor biosecurity practices and lack of appropriate laboratory diagnostic facilities are associated with these disease distributions in the study area. Strict biosecurity and continuous surveillance program can minimize the disease prevalence.

Key words: Poultry diseases, veterinary hospital, antimicrobial drugs, Bangladesh, surveillance.

INTRODUCTION

Nowadays, poultry sub-sector is playing a significant role in the national economic growth and employment generation in Bangladesh (Hamid et al., 2017). According to United States Department of Agriculture (USDA), around one million entrepreneur and eight million people are directly or indirectly involved in this sector. In the last

few decades, the poultry industry has been the fastest fostering livestock sub-sector in Bangladesh with a 20% annual growth rate (Islam et al., 2014). Poultry rearing in Bangladesh started in the 80s with backyard poultry farming (Begum, 2005). However, progress mainly happened in the 90s when a lot of private farms invested in this industry and started producing day-old chicks (DLS, 2016). This sector plays a great role in the nutrition sector of Bangladesh and is reported to contribute 37% of the total meat supply and also meet 22 to 27% of total human protein demand (Hamid et al., 2017). In Bangladesh, there are 8 grandparent stock breeder farm, 82 parent stock breeder farm and hatchery, 53112 commercial broiler farm, 18222 commercial layer farm and 6554 commercial duck farm (DLS, 2013; Hamid et al., 2017). According to DLS (2018), in Bangladesh, out of 337.99 million poultry population, 282.21 million are chickens and 55.85 million are duck population.

Despite the rapid growth of the poultry industry in Bangladesh, several factors reduce the growth rate and cause mortality in chickens (Badruzzaman et al., 2015). Among such factors, poultry disease is one of the major constraints that hinder the productivity and decline the economic improvement of poultry farmer (Islam et al., 2016). It is noted that about 30% of total chickens in Bangladesh die due to several disease outbreak (Badruzzaman et al., 2015). Poultry diseases thrive due to several factors such as climate, geographical position, farm hygiene, biosecurity, immunity status, chick quality, hatcheries, and management practices (Abbas et al., 2015; Badruzzaman et al., 2015; Chakma, 2015; Hassan et al., 2016). Along with species of chickens, production type, age and sex play significant role in disease prevalence (Yunus et al., 2009; Rashid et al., 2013; Talukder et al., 2017; Rahman et al., 2019).

Diseases such as Newcastle disease (ND), infectious bursal disease (IBD), colibacillosis, aspergillosis, avian influenza (AI), coccidiosis, infectious bronchitis, fowl cholera, salmonellosis, mycoplasmosis, chronic respiratory diseases, necrotic enteritis in broiler and layer (Choudhary et al., 2012; Islam et al., 2014, 2016; Badruzzaman et al., 2015; Hassan et al., 2016; Matin et al., 2017; Rahman et al., 2017, 2019), duck viral hepatitis, duck plague in duck (Hossain et al., 2005; Hoque et al., 2006, 2010; Ahamed et al., 2015; Khan et al., 2018) and pigeon fox (Paul et al., 2015; Munmun et al., 2016; Elina et al., 2017) have been reported in Bangladesh. These poultry diseases have also been recorded in other countries like India, Nigeria, China and Pakistan (Balami et al., 2014; Abbas et al., 2015; Borah et al., 2017; Ghosh et al., 2017; Xi et al., 2017).

In Bangladesh, antibiotics in poultry farms are frequently prescribed for prophylactic and therapeutic purpose (Islam et al., 2014). Due to frequent uses, pathogens have become resistant to most antibiotics. These resistant bacteria can easily be transmitted to humans through food chain and pose a serious human health risk (Parvez et al., 2016; Islam et al., 2018). The present study aimed to investigate clinical poultry diseases through passive surveillance in an Upazila Government Veterinary Hospital of Bangladesh, and to know the frequency distribution of antimicrobial drugs prescription pattern.

MATERIALS AND METHODS

Study area

The study was carried out in Ramu Upzilla Government Veterinary Hospital (RUGVH), Cox's Bazar, Bangladesh from February to March 2016. This area is well known for the poultry sector and the numbers of broiler and layer poultry farms are quite high. Apart from that, people in this area also rear other poultry species such as pigeon, duck, myna and deshi chicken. So, farmers from different broiler and layer farms and also household's people around Ramu Upazila brought their dead or sick birds for the diagnosis and treatment in RUGVH. Ramu Upazila consists of 11 different union from where poultry cases are originated.

Sample size

A total of 180 poultry cases were reported in RUGVH, among which 73 were from broiler, 84 from layer, 18 from duck and 5 from the pigeon.

Examination procedure

In RUGVH, there was a designated area where clinical examination and postmortem of sick and dead birds were carried out. Postmortem examination was performed based on the standard procedure and protocol described in the Atlas of Avian Necropsy (Majó and Dolz, 2011). During postmortem, personal protection was ensured to prevent contamination. The birds were examined systematically and gross pathological lesions were observed and recorded carefully. As there was no laboratory support available, the final diagnosis of all bacterial, viral and fungal diseases was done based on clinical history, clinical signs, and postmortem lesions as mentioned in the Manual of Poultry Diseases (Brugere-Picoux et al., 2015). Rapid test was performed for the diagnosis of *Salmonella* (Serotext®SP, S & A Reagents Lab Ltd., Part Thailand) and Avian Influenza detection (AIV Ag Test Kit, BioNote Inc., Korea) as these two kits were available in hospital. Few samples were preserved and taken to the microbiology laboratory of Chattogram Veterinary and Animal Sciences University (CVASU) for isolation of pathogens and antibiotic sensitivity testing. After performing postmortem, the necropsied birds were thrown into the dumping pit immediately.

Data collection

During the study period, a structured questionnaire was developed

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Table 1. Frequency and distribution of different poultry diseases obtained from RUGVH.

Disease	Frequency	Percent	95% CI
Newcastle disease	28	17.8	12-24
Colibacillosis	18	11.5	6-17
Infectious bursal disease	17	10.8	6-16
Aspergillosis	15	9.6	5-14
Avian influenza	10	6.4	3-10
Salmonellosis	10	6.4	3-10
Coccidiosis	8	5.1	2-9
Mycoplasmosis	7	4.5	1-8
Chronic respiratory disease	7	4.5	2-9
Fowl cholera	6	3.8	1-7
Necrotic enteritis	5	3.2	0-5
Omphalitis	5	3.2	0-5
Infectious coryza	4	2.5	0-5
Fowl pox	3	1.9	0-4
Infectious bronchitis	2	1.3	0-3
Visceral gout	3	1.9	0-3
Ascites	2	1.3	0-3
Toxicity	2	1.3	0-3
Femoral head necrosis	2	1.3	0-3
Cannibalism	3	1.9	0-4
Other poultry disease (n=23)			
Duck Plague	16	69.6	49-90
Duck Viral Hepatitis	2	8.7	0.4-21
Pigeon Pox	3	13	0.2-24
Pigeon Newcastle disease	2	8.7	0.4-21

for the purpose of the study. Information collected include type of birds, number of birds, farm size, age, sex, history of vaccination, rearing system, clinical history, morbidity and mortality, postmortem lesions, a drug used previously, tentative diagnosis and drug prescribed. Before and during each postmortem, farmers were asked for the aforementioned questions and recorded carefully.

Statistical analysis

Data was incorporated into Microsoft excel 2010 (MS-10) and imported into Statistical Package for the Social Sciences (SPSS) software (IBM SPSS-25.0, USA) for further analysis. Descriptive analysis was carried out to calculate the prevalence according to different variable such as diseases, species, type, age and drug users. The results were presented as percentages with 95% confident interval. Pearson's Chi-square and Fisher's exact test were performed to evaluate the significant relationship between clinical poultry diseases with different variables. The probability (P) value of less than 0.05 was considered a significant relationship.

RESULTS

Overall prevalence of clinical poultry diseases

The overall prevalence of different poultry diseases are shown in Table 1. Among the diagnosed diseases in broiler and layer, Newcastle disease (ND) was higher (17.8%) followed by colibacillosis 11.5%, infectious bursal disease (IBD) 10.8%, aspergillosis 9.6%, avian influenza (AI) 6.4%, salmonellosis 6.4% and coccidiosis 5.15%, respectively. Other clinical diseases such as fowl cholera 3.8%, necrotic enteritis 3.2%, omphalitis 3.2%, infectious coryza (IC) 2.5%, fowl pox 1.9%, and infectious bronchitis (IB) 1.3% were found in small number of birds. Duck plague was more frequent in duck 69.6% than duck viral hepatitis 8.7%. Between two observed pigeon diseases, pigeon pox has a higher prevalent of 13% when compared with ND 8.7%.

Prevalence of clinical poultry diseases according to production type

The prevalence of colibacillosis, infectious bursal disease and aspergillosis were significantly ($p<0.05$) higher in broiler chickens (17.8, 19.2, and 16.4%) when compared with layer chickens (6.0, 3.6, and 3.6%) (Table 2). The prevalence of Newcastle disease and avian influenza in layer chickens was significantly ($p<0.05$) greater in

Table 2. Prevalence of clinical poultry disease based on production type.

Disease	Broiler (n=73)	Layer (n=84)	P value
Newcastle disease	5 (6.8)	23 (27.4)	0.001
Colibacillosis	13 (17.8)	5 (6.0)	0.020
Infectious bursal disease	14 (19.2)	3 (3.6)	0.002
Aspergillosis	12 (16.4)	3 (3.6)	0.006
Avian influenza	1 (1.4)	9 (10.7)	0.017
Salmonellosis	-	10 (11.9)	-
Coccidiosis	3 (4.1)	5 (6.0)	0.600
Mycoplasmosis	4 (5.5)	3 (3.6)	0.563
Chronic respiratory disease	7 (9.6)	1 (1.2)	0.017
Fowl cholera	-	6 (7.1)	-
Necrotic enteritis	1 (1.4)	4 (4.8)	0.227
Omphalitis	4 (5.5)	-	-
Infectious coryza	1 (1.4)	3 (3.6)	0.383
Fowl pox	-	3 (3.6)	-
Infectious bronchitis	-	2 (2.4)	-
Visceral gout	3 (4.1)	-	-
Ascites	2 (2.7)	-	-
Toxicity	1 (1.4)	1 (1.2)	0.920
Femoral head necrosis	2 (2.7)	-	-
Cannibalism	-	3 (3.6)	-

percentages (27.4 and 10.7%, respectively) than broiler chickens (6.8 and 1.4%, respectively). No significant difference ($p>0.05$) was observed for coccidiosis, mycoplasmosis, necrotic enteritis and infectious coryza in both broiler (4.1, 5.5, 1.4, and 1.4%) and layer chickens (6.0, 3.6, 4.8, and 3.6%). Omphalitis (5.5%), visceral gout (4.1%), ascites (2.7%), and femoral head necrosis (2.7%) were observed in broiler chickens. On the other hand, salmonellosis (11.9%), fowl cholera (7.1%), fowl pox (3.6%), infectious bronchitis (2.4%), and cannibalism (3.6%) were recorded in layer chickens.

Prevalence of clinical poultry diseases according to age groups

Among the observed diseases in the broiler, aspergillosis and omphalitis were higher at 1 to 10 days age groups (42.1 and 21.1%). Newcastle disease, infectious bursal disease, and chronic respiratory disease were higher between the ages of 11 and 20 days (8, 36, and 16%). Prevalence of colibacillosis and mycoplasmosis was greater at later age groups (20.7 and 10.3%) than earlier age (10.5 and 5.3%). Diseases such as avian influenza, necrotic enteritis, infectious coryza, visceral gout, ascites, and femoral head necrosis were prevalent between the ages of 2 and 35 days (Table 3).

In layer chickens, avian influenza, salmonellosis and fowl cholera varied significantly based on age groups and their prevalence was higher (20, 22.5, and 19.2%, respectively) between the ages of 9 and 83 weeks. Among the three age groups, ND was more prevalent between the ages of 20 and 83 weeks of age groups (32.5%) than the other two age groups (16.7 and 26.9%). Most of the diseases in layer chickens such as necrotic enteritis, infectious coryza, fowl pox, infectious bronchitis, toxicity, and cannibalism were recorded between the ages of 57 and 581 days, respectively (Table 4).

Frequency of antibiotic used in poultry diseases

Some of the antibiotics that were frequently prescribed in the treatment of the various poultry diseases are ciprofloxacin (46.7%), colistin (42.2%), trimethoprim and sulphur drug (37.8%), metronidazole (36.1%), erythromycin (28.9%), spiramycin (26.7%), enrofloxacin (25.6%), sulfadiazine (17.8%), linofloxacin (13.9%), tylosin tartrate (12.2%) and neomycin (11.1%), respectively (Table 5).

DISCUSSION

The current findings of poultry disease support previous studies conducted in Bangladesh (Uddin et al., 2010; Badruzzaman et al., 2015; Hassan et al., 2016; Rahman et al., 2017, 2019) and other countries like Nigeria and Pakistan (Balami et al., 2014; Abbas et al., 2015).

The prevalence of colibacillosis, IBD and aspergillosis

Table 3. Prevalence of clinical diseases in broiler based on age category.

Disease	1-10 (n=19)	11-20 (n=25)	21-35 (n=29)	P value
Newcastle disease	1 (5.3)	2 (8.0)	2 (6.9)	0.939
Colibacillosis	2 (10.5)	5 (20)	6 (20.7)	0.627
Infectious bursal disease	-	9 (36)	5 (17.2)	0.010
Aspergillosis	8 (42.1)	4 (16)	-	0.001
Avian influenza	-	-	1 (3.4)	-
Salmonellosis	-	-	-	-
Coccidiosis	-	1 (4)	2 (6.9)	0.500
Mycoplasmosis	1 (5.3)	-	3 (10.3)	0.250
Chronic respiratory disease	3 (15.8)	4 (16)	-	0.078
Fowl cholera	-	-	-	-
Necrotic enteritis	-	-	1 (3.4)	-
Omphalitis	4 (21.1)	-	-	-
Infectious coryza	-	-	1 (3.4)	-
Fowl pox	-	-	-	-
Infectious bronchitis	-	-	-	-
Visceral gout	-	-	3 (10.3)	-
Ascites	-	-	2 (6.9)	
Toxicity	-	-	1 (3.4)	-
Femoral head necrosis	-	-	2 (6.9)	-
Cannibalism	-	-	-	-

Table 4. Prevalence of clinical diseases in layer based on age category.

Disease	1-56 (n=18)	57-140 (n=26)	141-581 (n=40)	P value
Newcastle disease	3 (16.7)	7 (26.9)	13 (32.5)	0.456
Colibacillosis	2 (11.1)	2 (7.7)	1 (2.5)	0.397
Infectious bursal disease	3 (16.7)	-	-	-
Aspergillosis	3 (16.7)	-	-	-
Avian influenza	-	1 (3.8)	8 (20)	0.029
Salmonellosis	-	1 (3.8)	9 (22.5)	0.016
Coccidiosis	5 (27.8)	-	-	-
Mycoplasmosis	-	-	3 (7.5)	-
Chronic respiratory disease	1 (5.6)	-	-	-
Fowl cholera	1 (5.6)	5 (19.2)	-	0.012
Necrotic enteritis	-	3 (11.5)	1 (2.5)	0.136
Omphalitis	-	-	-	-
Infectious coryza	-	2 (7.7)	1 (2.5)	-
Fowl pox	-	1 (3.8)	2 (5.0)	0.636
Infectious bronchitis	-	2 (7.7)	-	-
Visceral gout	-	-	-	-
Ascites	-	-	-	-
Toxicity	-	1 (3.8)	-	0.323
Femoral head necrosis	-	-	-	-
Cannibalism	-	1 (3.8)	2 (5.0)	0.635

was found significantly higher in broiler chickens than the layer in the present study. Similar findings were observed in earlier studies such as Rahman et al. (2017) who observed increased prevalence of colibacillosis and IBD in the broiler (33.4 and 15.3%, respectively) than the layer chickens (15.9 and 8.4%, respectively) in Gazipur

Table 5. Frequency of antibiotic used in poultry cases.

Name of the antibiotic	Frequency	Percentage
Ciprofloxacin	84	46.7
Colistin	76	42.2
Trimethoprim and sulphur drug	68	37.8
Metronidazole	65	36.1
Erythromycin	52	28.9
Spiramycin	48	26.7
Enrofloxacin	46	25.6
Sulpudiazine	32	17.8
Linofloxacin	25	13.9
Tylosin tertrate	22	12.2
Neomycin	20	11.1
Doxycycline	17	9.4
Oxytetracyline	11	6.1
Streptomycin	8	4.4
Sulphequinoxaline	5	2.8
Chlortetracycline	3	1.7
Amoxicillin	2	1.1

district of Bangladesh. This finding is also in agreement with several other published studies conducted in Bangladesh and India (Uddin et al., 2010; Choudhary et al., 2012; Hassan et al., 2016; Borah et al., 2017). The difference in disease prevalence between broiler and layer may be due to poor biosecurity management, vaccination failure, improper use of the vaccine, low vaccination success rate, education level of farmers, subclinical disease prevalence and geographical location (Hamid et al., 2017; Rimi et al., 2017; Rahman et al., 2019).

In the present investigation, Newcastle disease was found in both broiler and layer chickens but the prevalence was significantly higher in layer chickens. The present findings correlate with several other previous findings (Geresu et al., 2016; Islam et al., 2016; Rahman et al., 2017; Das et al., 2018). There are several factors that may influence the greater prevalence of ND in layer chickens such as long term production period, types of breed and strain, failure to maintain bio-security for longer period of time, vaccine use, vaccine failure due to stressful condition and sometimes subclinical state of disease (Sarker et al., 2012; Munmun et al., 2016).

Colibacillosis was significantly higher in broiler chickens compared to layer chickens. This finding is in agreement with the previous study such as Rahman et al. (2017) who recorded 33.4% colibacillosis in broiler chickens than 15.9% in layer chickens of Gazipur district of Bangladesh. Colibacillosis was also recorded in different poultry farms in Bangladesh and other countries (Balami et al., 2014; Islam et al., 2014; Hassan et al., 2016; Matin et al., 2017). To improve the situation, biosecurity and hygiene measures should be followed strictly.

The prevalence of IBD was significantly higher in broiler chickens which agreed with several previous findings (Islam et al., 2016; Das et al., 2018). The prevalence was higher between the age of 11 and 35 days which is congruent with the earlier published research articles (Chakma, 2015; Islam et al., 2016; Hassan et al., 2016; Rahman et al., 2019). Initial 2 weeks of ages, chicks get maternally derived antibody from mother, after that proper vaccination should be ensured to protect from IBD (Rashid et al., 2013).

Aspergillosis was found mostly in the early stage of birdlife (1-10) days. Similar findings were also noted in other studies (Badruzzaman et al., 2015; Rahman et al., 2019). The prevalence of aspergillosis higher in earlier age may be due to inhalation of spore from litter, contaminated hatching incubator, higher moisture containing litter and sawdust litter (Sultana et al., 2015). Avian influenza is endemic in Bangladesh causes huge economic losses in the poultry sub-sector through reduced egg production and high mortality (Xia et al., 2017). The strain H5N1 causes high mortality in birds and poses a serious health risk to the human population (Sarker et al., 2016).

Salmonellosis, zoonotic disease, was frequently observed in layer chickens similar to several previous studies (Akter et al., 2007; Barua et al., 2012). Within different age groups, the prevalence of salmonellosis was higher in later age (above 21 weeks) which is comparable to other researches (Hossain et al., 2010; Sabuj et al., 2019). Both coccidiosis and necrotic enteritis are correlated with each other cause severe loss to poultry farm owners (Badruzzaman et al., 2015). Fowl cholera, fowl pox, infectious bronchitis, and cannibalism found

only in layer chickens and hand omphalitis, visceral gout, ascites, and femoral head necrosis in broiler chickens corresponding to the previous findings (Hassan et al., 2016; Rahman et al., 2017).

Duck plague considers an economic disease in Bangladesh causes higher mortality in the duck population (Hoque et al., 2010). Several studies have so far been conducted on duck plague elsewhere in Bangladesh (Hoque et al., 2011; Ahamed et al., 2015; Khan et al., 2018). The vaccine is available for duck plague but people did not use it regularly which leads to disease occurrence. Pox and ND were found as most of the pigeon which agree with other findings (Lawal et al., 2016; Elina et al., 2017; Rahman et al., 2019). Improved husbandry practices and regular vaccination can reduce the disease prevalence in the pigeon.

Various types of antibiotics were prescribed to treat healthy and sick birds in the study area. Antibiotics used in birds based on clinical signs and post mortem history and experience of the veterinary surgeon which may lead to antimicrobial resistance (Rahman et al., 2019). Combine drugs are mostly used to treat the clinical poultry disease as resistance had been developed to most of the antibiotic. Thus, with increased antibiotic resistance, resistant pathogens can transmit to humans through contact as well as food chain (Hasan et al., 2010; Khan et al., 2014; Rahman et al., 2019). The veterinarian should have to prescribe antibiotic drug careful and try to avoid it if not necessary. Through this study, poultry practitioners, veterinarian, researcher, and the government officials can get a real picture of the disease prevalence in this area which helps in future research and decision making. The shorter period of study, lower sample size and consider one veterinary hospital, these are the limitation in the present study.

Conclusion

Poultry diseases are more prevalent in the study area and antimicrobial drugs are used on an experience basis without testing sensitivity. For precise diagnosis of diseases, laboratory support must be ensured and a sensitivity test should be performed to detect the resistant pattern of an antibiotic.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Assessment of bacterial contamination and milk handling practices along the raw milk market chain in the north-western region of Rwanda

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This study was conducted to assess raw milk bacterial loads and micro-organisms associated with milk handling practices and raw milk chain in the North-western region of Rwanda. A multistage sampling method was used to collect sixty-seven raw milk samples that were analyzed for milk quality at four stages of the raw milk chain: dairy farmers, milk hawkers, milk collection centres (MCC) and milk kiosks. Total bacterial counts (TBC) at different stages of the chain were determined and microorganisms were isolated. A questionnaire was distributed to gather information on factors and milk handling practices that influence milk quality at farm level. The study revealed a TBC mean values of 1.2×10^6 CFU/ml (dairy farmers), 2.6×10^7 CFU/ml (milk hawkers), 1.5×10^6 CFU/ml (MCC) and 6.9×10^6 CFU/ml (kiosks/restaurants). The prevalent micro-organisms were: *Escherichia coli* (*E. coli*), 26.9%; *Salmonella* spp., 16.4%; *Streptococcus* spp., 16.4%; coagulase- negative staphylococci (CNS), 14.9%. Bacterial load was highly associated with containers used for milk transport, cleaning time of milk containers and source of water used to clean containers. It is, therefore, highly recommended that all concerned parties in the raw milk value chain improve their milk handling and storage practices.

Key words: Bacterial contamination, milk handling, milk quality, north-western Rwanda, raw milk.

INTRODUCTION

Human health is greatly threatened by the pathogenic microorganisms commonly isolated from milk and milk products. Raw milk quality is determined by various factors including composition (butterfat, protein, lactose, milk solids, etc.), udder health (mastitis infection, somatic cell count (SCC)) and hygiene (total bacterial count, thermoduric bacteria, psychotropic bacteria) (O'Brien et al., 2009). Microbial contamination in milk may result in milk spoilage and milk-borne diseases spreading to humans (Ngasala et al., 2015). Microbial contamination

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of milk may be primary, secondary, or tertiary (Parekh and Subhash, 2008). Primary infection is usually from infected lactating cows. Secondary infection can be from milkers during milking, milk handlers during transportation and storage, milk handling procedures, water used in cleaning, unclean utensils and/or milking equipment. Tertiary infection is mainly due to re-contamination of milk after processing due to unhygienic conditions, improper handling and poor storage of milk before serving.

Raw milk is a means of transmission of milk-borne pathogens to humans (Addo et al., 2011). However, Harding (1995) demonstrated that in milk stored at a standard temperature of 4°C for up to 15 h bacteria do not multiply appreciably. He also demonstrated that it is possible to store milk for 7 days at 1.5-2°C without spoilage provided the initial quality is good (Harding, 1995). Total plate count of microorganisms in milk provides useful general information on the microbiological quality and indirectly the conditions under which it was produced (Jay, 2000).

According to Boor et al. (1998) TBC for raw milk must be less than or equal to 10^5/ml; for retailed milk, it must be less than or equal to 2×10^4/ml or gram; while for frozen desserts it must be 5×10^4/gram or less. The USA has a standard of 10^5 bacterial cells per milliliter; however, other countries take 2×10^5 colony forming units/milliliter as the acceptable bacterial limit (Lore et al., 2005). The microbial limit of total plate counts is used to grade milk as follows: Grade I or A (< 2×10^5 bacterial cells/ml), II or B (>2×10^5– < 10^6 bacterial cells/ml) and III or C (>10^6– < 2×10^6bacterial cells/ml) in Rwanda (COMESA, 2006). In Kigali, Rwanda, Doyle et al. (2015) reported unacceptably high TBC in milk from three major segments of the dairy value chain, milk transporters, MCCs and milk kiosks.

There are pathogens which cause milk contamination. These include *Escherichia coli, Staphylococcus aureus, Salmonella* spp*., Listeria monocytogenes, Brucella abortus, Mycobacterium* spp*., Campylobacter* spp*., Leptospira* spp*., Clostridium* spp*., Pseudomonas aeruginosa* and *Proteus* spp. (Farah et al., 2007; FAO, 2010). The microorganisms that are principally involved in milk spoilage are psychrotrophic organisms, most of which are destroyed by pasteurization temperatures (Goff et al., 1989). However, some, such as *Pseudomonas fluorescens* and *Pseudomonas fragi*, can produce proteolytic and lipolytic extracellular enzymes which are heat stable and capable of causing spoilage (Goff et al., 1989). In addition, some species and strains of *Clostridium, Microbacterium, Micrococcus, Bacillus, Corynebacterium, Arthrobacter, Lactobacillus,* and *Streptococcus* can withstand pasteurization temperatures and grow at refrigeration temperatures leading to spoilage of treated milk (Banwart, 1989).

Ideally, milk meant for human consumption must be free from any pathogenic organisms (Bertu et al., 2010). Considering that milk is normally sterile when it comes from a normal cow's udder, contamination occurrs during milking, transportation, cooling, storage and processing (Farah et al., 2007). There is need for good management of raw milk and milk products in order to minimize the contamination. This will subsequently enable the dairy sector serve as a contributing tool for alleviation of poverty and creation of wealth in developing countries (FAO, 2010).

Food safety is an area of great concern in relation to public health management and particularly from an economic perspective (Kaiza, 2011). Microbial contamination of milk is a risk to the public health through transmission of food borne diseases (Pires et al., 2009). Raw milk is and continues to be a major distress in the epidemiological data of campylobacteriosis, salmonellosis, tuberculosis, brucellosis, hemorrhagic colitis, Brainerd diarrhoea, Q fever, listeriosis, among others (Alvarez, 2009). It is known that, microbial contamination of milk could be reduced by adhering to effective hygienic practices at the farm level. Many developing countries are ignorant about the existence of milk-borne infections and consuming raw milk predisposes small-scale livestock farmers, consumers and the general public at risk of contracting these infections (Mosalagae et al., 2011). Therefore, this study was conducted to establish microbial contamination and assess factors and management practices associated with bacterial contamination along raw milk value chain in three districts (Musanze, Nyabihu and Rubavu) of Rwanda.

MATERIALS AND METHODS

Study area

This study was carried out in the North-western region of Rwanda, specifically in Musanze, Nyabihu and Rubavu districts. Musanze District is located in Northern Province (1°30′6.94″S; 29°37′59.75″E at 1850 m above sea level) whereas Rubavu (1°40'52.54"S; 29°19'45.55"E and 1,830 m above sea level) and Nyabihu (1°39'9.90"S; 29°30'24.62"E and 2,437 m above sea level) districts are located in Western Province.

The average temperatures for Musanze, Rubavu and Nyabihu districts are 19.4, 18.1 and 15°C, respectively. The average annual rainfall is 1 100, 1 377 and 1 400 mm for Musanze, Rubavu and Nyabihu districts, respectively. Soil types in the region consist of volcanic, lateritic and humus-bearing and clayey soils (MINALOC, 2011). There are two wet seasons in the North-western region of Rwanda, the first being from February to May and the second from September to November. This region has 14 MCCs and has over 70% of the cheese processors in Rwanda. More than 91% of the human population in the North-western region of Rwanda is engaged in agriculture.

Sample size and sampling procedure

A multistage sampling method was used involving all entities of the milk value chain as applied in Rwanda. The sampling procedure was based on the schematic presentation of raw milk commodity in the value chain (Figure 1). A total of sixty-seven samples were

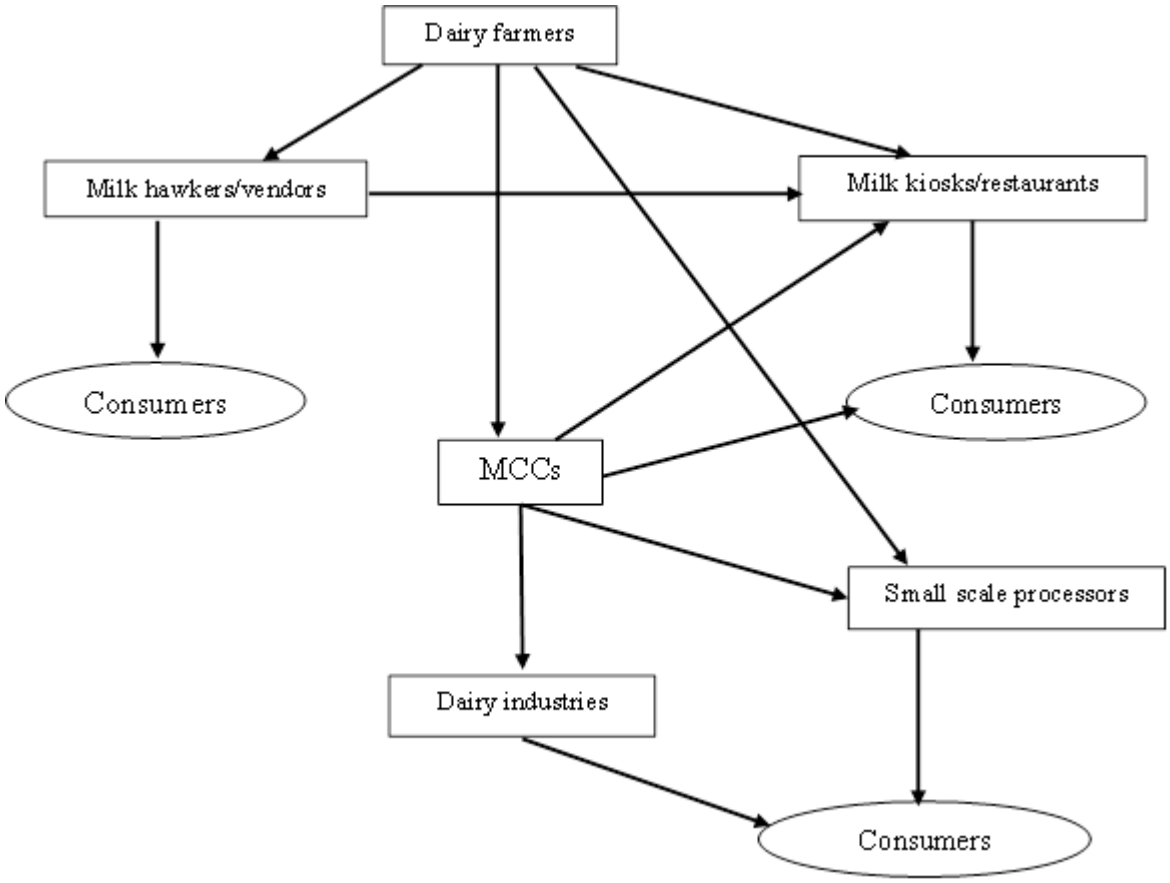

Figure 1. Raw milk value chain in the study area.

collected from September 2016 to March 2017 at four different levels of the value chain. These were 36 dairy farmers (26 in Nyabihu, 10 in Musanze and ten in Rubavu district), 15 milk hawkers, 12 milk kiosks (four in Nyabihu, five in Musanze town and three in Rubavu town) and four MCCs (CEMO and CEZONYI in Nyabihu, CODERU in Rubavu and IWACU ZIRAKAMWA in Musanze).

A milk collection centre was defined as a place with milk quality testing equipment (alcoholmeter; lacto densimeter; thermometer; antibiotic residue test kit and mastitis test kit) and cooling facilities, where milk from different dairy farmers and milk transporters within the same location was gathered and cooled before sale. Milk kiosk/restaurant was defined as a safe/certified commercial place where milk was sold either as fresh-raw, skimmed or mixed with tea (African tea) (MINAGRI, 2016).

Milk sample collection and transportation

At the dairy farmer's level, milk was sampled from the bulked milk containers prior to carrying it to the MCC. Additionally, designed questionnaires were sdministered to individual farmers to get more information regarding factors and milk handling practices that influenced milk quality at their farms. At the MCC level, milk was sampled from the bulk tank. At the hawker's level, milk was sampled from milk containers at the selling point (public road, market, etc.) whereas at the kiosks/restaurants milk samples were collected from previously boiled and cooled milk, ready to be served. At each stage, 15 ml of milk was aseptically collected into sterile labelled tubes, kept on ice in cooler box (4°C) and then taken to the University of Rwanda, Busogo campus Microbiology Laboratory within 1-2 h, for further analysis. The analysis was performed immediately after reaching the laboratory.

Total bacterial count and bacteriological analysis

Total bacterial count was performed as described previously (Campbell et al., 2015). The standard plate count (SPC) agar (Oxoid-CM0325, UK) was cooled to 50°C before 15-20 ml was poured onto sterile Petri dishes and left to solidify. Then ten-fold dilutions of the milk samples were prepared, up to a dilution of 10^{-7}, using sterile peptone water (Oxoid-CM0509, UK) and sterile test tubes; and mixing was done using an electronic vortex. A standard volume (1 ml) of milk sample was spread in duplicate onto the solid agar, prepared earlier; this was then incubated at 37°C for 48 h. Colony count was made using an electronic colony counter (Galaxy 230 Colony Counter, ROCKER SCIENTIFIC CO., LTD, New Taipei,Taiwan), focusing mainly on plates containing 30-300 colony forming units (Campbell et al., 2015). The bacterial concentration (colony forming units) in the respective original milk sample was then calculated using the formula given by Campbell et al. (2015).

$$\text{cfu/ml} = \frac{\text{Average count (number of colonies)}}{\text{(Dilution plated) x (Volume plated)}}$$

For bacteriological analysis, milk samples were inoculated

Table 1. Summary of bacterial count (in CFU/mL) and mean correlation between milk hawkers and other levels of the raw milk chain.

Source of samples	No. of samples	Mean counts	D.F	SEM	*P-value*
Dairy farmers	36	1.2×10^6	35	2×10^5	<0.001
MCCs	4	1.5×10^6	3	2.6×10^5	0.008
Kiosks	12	6.9×10^6	11	1.8×10^6	0.003
Milk hawkers	15	2.6×10^7	14	8.5×10^6	-

D.F: Degrees of freedom
SEM: Standard Error of the Mean.

separately onto MacConkey agar and blood agar plates by streaking method. Inoculated plates were then incubated aerobically at 37°C for 24-48 h. After 24 h, primary bacteriological identification was made based on colony morphology, colour and haemolytic characteristics; after which pure cultures were prepared through subculturing and incubation. The purified isolates were then subjected to Gram staining and further biochemical testing as previously describe by Quinn et al. (2011). Staphylococci were identified based on catalase and tube coagulase tests. Streptococci were identified based on catalase production and the Christie, Atkins, and Munch-Peterson (CAMP) test as previously described by Quinn et al. (2011). Gram negative isolates were identified based on growth characteristics on MacConkey agar and reactions to oxidase test, catalase test, Triple sugar Iron (TSI) agar and the "IMViC" tests (Indole, Methyl-Red, Vogas Proskaur and Citrate utilization) (Quinn et al., 2011).

Statistical analysis

All data collected throughout the survey were encoded into Microsoft Excel, 2013 and analyzed using Statistical Package for Social Sciences (SPSS). Bacterial counts at different levels of the value chain were determined by comparing respective means using ANOVA. Total bacterial counts were subjected to Least Significance Difference (LSD), Levene's test (Page et al., 2003), Welch test (Welch, 1951), and Games-Howell post-hoc test (Games and Howell, 1976) to determine homogeneity of variance and overall statistical significance. Association between farming/milk handling practices with milk grades was determined using Chi-square, while strength of association was determined using Cramer's "V" test (Cramér, 1946). Distribution of microorganisms invading milk at different levels of the raw milk chain was performed using descriptive statistics.

RESULTS

Total bacterial counts

Total bacterial count (TBC) was interpreted according to COMESA milk grading standards. The mean TBC at dairy farmers' level was 1.2×10^6 CFU/ml (SE± 2×10^5); at milk hawkers' level, it was 2.6×10^7 CFU/ml (SE± 8.5×10^6); while at MCC's and kiosks/restaurants' levels, it was 1.5×10^6 CFU/ml (SE± 2.6×10^5) and 6.9×10^6 CFU/ml (SE± 1.8×10^6), respectively (Table 1). ANOVA tests showed that there was a significant difference (p <0.001) in the mean values among the four levels of the value chain considered. There was also significant difference in TBC mean values between milk hawkers, dairy farmers (p <0.001), MCC (p =0.008) and kiosks (p =0.003) (Table 1). Additional statistical analysis showed a significant difference between dairy farmers and kiosks (p =0.044). However, there was no significant difference observed between dairy farmers and MCC (p =0.975) and between MCC and kiosks (p =0.551).

Based on COMESA milk grades, it was found that all tested hawker's milk (15/15; 100%) and 10/12 (83.3%) of the milk samples collected from kiosks were above the lowest COMESA grade (>2,000,000 CFU/ml) (Table 2). This study also showed that 3/4 (75%) of the milk samples collected from MCC were within COMESA Grade III/C (1 000 000-2 000 000 CFU/ml) whereas (15/36; 41.7%) of the milk samples from dairy farmers were within COMESA Grade I/A (<200 000 CFU/ml).

Isolated bacteria

Of the microorganisms which contaminated milk at different levels of the raw milk chain coliforms were the most predominant, at 34.4%. With respect to individual bacteria, *E. coli* was the most predominant isolate, followed by *Salmonella* spp., *Streptococcus* spp, coagulase-negative staphylococci, *Enterobacter* spp., *Bacillus* spp., *S. aureus* and *Yersinia enterocolitica* (Table 3).

Bacterial isolates were distributed across the market chain in such a way that high prevalence rates were recorded at milk hawkers for gram negative bacteria (Table 3). This predominance can be partially explained by the fact that milk hawkers store their milk at ambient temperatures for a long time before they reach their customers.

Factors associated with bacterial contamination along market chain

When statistically evaluated, it was revealed that bacterial contamination of milk was significantly and strongly associated with containers used for milk transport (p <0.0001), milk containers cleaning time (p <0.0001) and source of water used to clean containers

Table 2. Quality grade of milk samples tested on the basis of bacterial load (TBC).

Source of samples	No. of samples	Milk quality grade			
		Grade Ior A	Grade II or B	Grade IIIor C	Above COMESA standards
Dairy farmers	36	15 (41.7%)	6 (16.9%)	8 (22.2%)	7 (19.4%)
MCCs	4	0	0	3 (75%)	1 (25%)
Kiosks	12	2 (16.7%)	0	0	10 (83.3%)
Milk hawkers	15	0	0	0	15 (100%)

Grade I or A: <2 x10^5 cfu/ml
Grade II or B: 2 x10^5-1 x10^6 cfu/ml
Grade III or C: 1-2 x10^6 cfu/ml
TBC – Total bacterial count
MCC – Milk collection centre.

Table 3. Distribution of isolated bacteria according to the sampled sites.

Bacterial isolates	n	Farms	MCCs	Kiosks	Milk hawkers	Prevalence (%)
Escherichia coli	18	4	1	4	9	26.9
Streptococcus spp.	11	5	2	0	4	16.4
Salmonella spp.	11	2	1	2	6	16.4
CNS	10	4	3	0	3	14.9
Bacillus spp.	5	3	2	0	0	7.5
Enterobacter spp.	5	0	0	1	4	7.5
Staphylococcus aureus	5	3	0	0	2	7.5
Yersinia enterocolitica	2	0	0	0	2	3.0

CNS – coagulase-negative staphylococci.

Table 4. Association between Milk Grade and bacterial contamination risk factors at farm level.

Selected factors	Chi-square value	D.F	Significance level (P-Value)	Cramer's V
Production system	17.308[a]	1	0.001	0.693
Milking space	17.308[a]	1	0.001	0.693
Time to supply to MCC	3.441[a]	1	0.328	0.309
Containers for Transport	25.435[a]	1	0.000	0.841
Milk containers cleaning time	23.813[a]	1	0.000	0.813
Source of water for cleaning containers	20.034[a]	1	0.000	0.746
Type of water used in cleaning	28.832[a]	2	0.000	0.633

D.F: Degrees of freedom.

(p <0.0001) (Table 4). Milk which was transported from farms to MCC in aluminum containers had lower TBC mean values than milk transported in plastic Jeri cans/containers; farmers who cleaned their utensils (milking and milk transport equipment) five to eight hours before milking delivered milk that had lower bacterial counts than those who cleaned one to two hours before milking, and farmers who used tap water to clean utensils had reduced bacterial contamination of their milk than those who used stream water; farmers who used warm water with disinfectant (soap) to clean utensils had milk with lower bacterial contamination (TBC mean values) than those who used cold water with disinfectant (soap). There was a correlation and moderate strength of association between bacterial contamination of milk and production system (p <0.001), milking space (p <0.001) and type of water used to clean utensils (p <0.001). Results indicated that there were lower TBC values in milk from cows kept under intensive system than from those kept in extensive system, milk from cows milked from Kraal showed lower TBC values than from those milked in open space. Although milk supplied to MCC

Table 5. Factors influencing milk quality on dairy farms: number and percentage of milk samples showing contamination at stated grades.

Selected factors		Milk grades			
		Grade I	Grade II	Grade III	Above COMESA Standards
Production system	Intensive (n=10)	10 (100%)	0	0	0
	Extensive (n=26)	6 (23.1%)	6 (23.1%)	8 (30.8%)	6 (23.1%)
Milking space	Open (n=26)	6 (23.1%)	6 (23.1%)	8 (30.8%)	6 (23.1%)
	Kraal (10=2)	10 (100%)	0	0	0
Time to supply to MCC	Immediately after milking (n=34)	16 (47.1%)	6 (17.6%)	7 (20.6%)	5 (14.7%)
	One (1) hour after milking (n=2)	0	0	1 (50%)	1 (50%)
Containers for milk transport	Aluminum (n=13)	13 (100%)	0	0	0
	Plastic (n=23)	3 (13%)	6 (26.1%)	8 (34.8%)	6 (26.1%)
Milk containers cleaning time	Five to eight hours before milking (n=24)	16 (66.7%)	5 (20.8%)	3 (12.5%)	0
	One to two hours before milking (n=12)	0	1 (8.3%)	5 (41.7%)	6 (50%)
Source of water for cleaning containers	Tap water (n=19)	15 (78.9%)	2 (10.5%)	1 (5.3%)	1 (5.3%)
	Stream water (n=17)	1 (5.9%)	4 (23.5%)	7 (41.2%)	5 (29.4%)
Type of water used in cleaning	Warm water only (n=17)	1 (5.9%)	5 (29.4%)	6 (35.3%)	5 (29.4%)
	Warm water with disinfectant (soap) (n=17)	15 (88.2%)	2 (11.8%)	0	0
	Cold water with disinfectant (Soap) (n=3)	0	0	2 (66.7%)	1 (33.3%)

Grade I or A: $<2 \times 10^5$ cfu/ml
Grade II or B: 2×10^5-1×10^6 cfu/ml
Grade III or C: 1-2×10^6 cfu/ml
MCC: Milk collection centre.

immediately after milking had lower bacterial counts than that which was kept for more than one hour after milking; there was no statistical association between raw milk bacterial contamination and time taken to supply milk to the MCC ($p > 0.328$) (Table 5).

DISCUSSION

The mean TBC values for the hawkers' milk samples (15/15; 100%) and kiosks/restaurant's samples (10/12; 83.3%) were above COMESA's acceptable levels. This contamination at hawkers' level is influenced by different factors, including: storage and transport in unclean milk containers, prolonged time for milk storage and uncontrolled temperature along transportation. In the study area, some farmers milked their cows in the morning hours and stored milk (for about 5 h) at ambient temperature. They then waited for milk hawkers who came to collect it for distribution to different customers like individual consumers, milk kiosks/restaurants. The hawkers also had tendency of selling milk in the afternoon hours on public roads or milk "markets". Indeed, 80% of Rwanda's milk market is designated as "informal" due to the fact that the milk coming from a majority of small-holder farmers does not enter the regulatory food chain (MINAGRI, 2013).

According to bacterial multiplication and growth curve, at ambient temperature, for example *E. coli* divides into two after every twenty minutes

(Harding, 1995); this seems to be the most likely scenario at hawker's level, where milk undergoes prolonged storage time at ambient temperature and *E. coli* isolates were found to be many. Furthermore, bacterial contamination was exacerbated when the milk was stored in unhygienic plastic containers, and when it was subjected to poor handling practises during distribution to different customers. This observation is in agreement with the results of Grimaud et al. (2007), when they analysed raw milk marketed through the informal subsector in Uganda. They reported that unhygienic conditions from the production source to the consumer combined with an improper milk storage were associated with increased bacterial contamination.

The findings of the current study corroborate with those obtained by Doyle et al. (2015), who recorded TBC mean values at kiosks of 9.8×10^6 CFU/ml; they were slightly higher than 6.9×10^6 CFU/ml obtained in the current study but also above COMESA's acceptable level). Normally, in the study area, milk sold in kiosks/restaurants is obtained either directly from dairy farmers, or from MCC and/or milk hawkers. It is normally boiled before consumption; served either hot or cold, however it needs to be noted that, even though boiled before consumption, the milk still carry pathogenic bacteria which it can transmit to respective consumer(s); that is, contamination can occur after boiling. This possibility of contamination after boiling of the milk is also highlighted by other scientists (Kilango et al., 2012); this is influenced by contamination level of storage utensils, storing temperature and hygienic status of milk handlers. Kiosks/restaurants in the study area also served raw milk which was found to be of very poor quality. This could have been due to milk contamination at source, poor hygienic milk handling conditions after boiling, inadequate refrigeration and recontamination during milk storage and serving (consumption). Though the current study did not evaluate the impact /safety hazards of poor milk quality on human health on consumption, in the United States, Oliver et al. (2009) did so and showed that several documented milk-borne disease outbreaks which occurred within the years 2000-2008 were traced back to consumption of raw unpasteurized milk.

The TBC mean values of MCC's and dairy farmer's milk (1.5×10^6 CFU/ml and 1.2×10^6 CFU/ml, respectively) laid in grade III/C according to COMESA's milk grades. Similar results were found by Doyle et al. (2015); they recorded MCC milk samples' TBC mean values of 1.5×10^6 CFU/ml. In this study, it was found that, once milk reached the MCC, it was directly tested via platform tests for organoleptic properties, added water and then cooled in cooling tanks at 4°C in compliance to a ministerial order (MINAGRI, 2016). The slight increase of the TBC mean values from dairy farmers to MCC, observed in this study, could have been caused by use of contaminated transportation containers and/or time the utensils were cleaned after supplying milk to a MCC. These findings are supported by Grimaud et al. (2007) in Uganda (with similar milk handling and transport conditions); they demonstrated increase of bacterial load in milk during transportation.

The highest TBC mean values obtained at dairy farmer's level was found to be associated with the investigated factors and milk handling practices used by farmers in the study area. These include source of water used to clean containers, milking space, production systems, milking hygiene and cleanliness of milk containers used during milking. This is in agreement with findings of (Banwart (1989); he concluded that poorly cleaned and sanitized milking utensils are the source of many microorganisms which transform high quality milk to an unacceptable product. The current findings also corroborate those of Grimaud et al. (2007), who during an evaluation of milk quality in Uganda, noted that milk contamination took place as early as at the farm level, the beginning of the value chain. They concluded that raw milk contamination along the value chain is associated with storage and milk handling conditions, especially during transportation from the primary production area to the urban market place.

In this study, coliforms (*E. coli* and *Enterobacter* spp.) were the most isolated bacteria from different levels of the raw milk chain, representing 34.4%. The results are in agreement with those of Garedew et al. (2012) who found *E. coli* (29.6%) to be the most isolated Gram-negative staining bacterial pathogen. Adams and Moss (2008) concluded that *E. coli* is the most prominent fecal coliform and that its presence indicates fecal contamination of raw milk and its products; which is in agreement with the findings of the current study. Mellenberger and Roth (2009) also stated that coliform bacteria are normal inhabitants of soil and the intestines of cows. They accumulate and multiply in manure, polluted water, dirt and contaminated bedding. Iraguha et al. (2015), from their study in eastern Rwanda, found that the predominance of coliform bacteria were largely of environmental origin, at farm level.

The predominance of coliforms found in the current study seems to be associated with milking practices such as unclean water use during milking and cleaning of milk utensils, poor milker's hygiene, milking space and non-use of teat dips. The coliform contamination at other levels of the value chain (milk hawkers and kiosks) increased largely due to poor hygiene of milk handlers and poorly cleaned utensils used for milk transport. *Salmonella* organisms were found at 16.4% in the current study. These findings corroborate those found by Lubote et al. (2014) in Arusha, Tanzania; they reported prevalence of *Salmonella* organisms at 37.3% of all the bacterial isolates along the raw milk chain. This high prevalence is explained by factors such as poor animal husbandry and hygienic practices, inappropriate transportation and storage facilities, lack of cooling systems and use of unclean water. The presence of *E.*

coli and *Salmonella* organisms is also an indication of fecal contamination by milk handlers as previously reported (Kamana et al., 2014).

The current results have also revealed high prevalence of coagulase-negative staphylococci (CNS), at 14.9%. Coagulase-negative staphylococci are commonly considered to be teat skin opportunists that normally reside on the teat skin (Radostits et al., 2007). So, they may contaminate milk during udder washing and milking, which also explains their association with poor milking hygienic practices and non-use of teat desinfectant(s) before milking. Other bacteria isolated in this study were: *Streptococcus* spp. at 16.4%, *Bacillus* spp. at 7.5 % and *Staphylococcus aureus* at 7.5%. These bacteria could originate from mastitic milk (O'Brien et al., 2009).

Although the current study did not go further to establish diseases associated with consumption of raw milk; De Buyser et al. (2001) did so when they explored the implication of milk and milk products in food-borne diseases in France and in different industrialized countries. They found that 37.5% of the food vehicles were from raw milk where *Salmonella* spp. were responsible for 29 outbreaks, *L. monocytogenes* for 10 outbreaks, pathogenic *E. coli* 11 outbreaks, and *Staphylococcus aureus* 10 outbreaks. Furthermore, Rohrbach et al. (1992) reported that 68 of 195 (34.9%) dairy producers in East Tennessee and Southwest Virginia consumed raw bulk-tank milk produced on their farm. Twenty-five percent (17 of 68) of the bulk-tank milk samples were shown to contain *Listeria monocytogenes*, *Campylobacter jejuni*, *Y. enterocolitica* and/or *Salmonella* (Rohrbach et al., 1992).

The high mean values of TBC that placed milk in the third grade at farm level (according to COMESA's grading) was mainly due to poorly cleaned milk containers used by farmers during milking, the time milk containers were cleaned, poor hygiene practices of milkers, use of stream water to clean containers and udder, milking from open space, and use of cold water without disinfectant to clean utensils. The study also revealed that milk contamination took place as early as at the farm level-the beginning of the value chain; increasing along the rest of raw milk chain.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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The bacterial diversity in infected tissue pus of an East Asian finless porpoise

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Two East Asian finless porpoises (*Neophocaena asiaeorientalis sunameri*) in Ningbo, East China Sea, China, were observed to be bacterially infected between the fat layer and muscle layer. The microbial communities in pus samples were identified by metagenome sequencing 16S in the PacBio platform and explored the relationship between emaciation and bacterial infection. The present paper was the first report on bacterial diversity in infected tissue pus of finless porpoise. In total, 101 bacterial species were identified, and the top nine species were *Rhodococcus qingshengii* (26.25%), *Rhodococcus jialingiae* (22.43%), *Ralstonia pickettii* (16.03%), *Moraxella osloensis* (9.97%), *Psychrobacter cibarius* (2.97%), *Flavobacterium johnsoniae* (2.11%), *Flavobacterium chungbukense* (1.80%), *Stenotrophomonas maltophilia* (1.73%) and *Serratia marcescens* (1.62%). These main bacteria could cause various diseases or metabolic disorder, such as endocarditis, meningitis, bacteraemia, septicaemia, and so on; they also involve microbiome metabolism of amino acids (12.47%), carbohydrates (11.58%), xenobiotic biodegradation (7.81%), lipids (6.01%) and energy (4.98%). Regardless of the type of disease metabolic disorder will firstly cause body weight loss. In conclusion, these bacteria could cause diseases or metabolic disorders, resulting in emaciation of East Asian finless porpoises. Admittedly, pathogenic assay is furthermore needed to determine the mechanisms regarding the pathological phenomena.

Key words: Bacterial diversity, East Asian finless porpoises, Metagenome sequencing 16S.

INTRODUCTION

Infectious diseases caused by bacteria were thought to be the primary cause of mortalities in aquatic mammals (Dunn et al., 2001). These bacteria contributed to different degrees of pathogenicity. For example, a *Helicobacter* sp. isolated from stomachs may play a role in the etiopathogenesis of gastritis and gastric ulcers in dolphins (Harper et al., 2000). Pathogenic vibrios have been found, which might lead to, secondary infections in debilitated (e.g., stranded) marine mammals was a distinct possibility (Buck and Stephen, 2010). *Brucella* sp. infections and associated lesions were described in a harbour porpoise (*Phocoena phocoena*) (Jauniaux et al.,

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2010). 20 bacterial pathogens were identified in captive and free-ranging killer whales or sympatric odontocetes species, and they were considered to affect fecundity or reproductive success (Gaydos et al., 2004).

Studies on infectious disease in marine mammals have seldom been conducted in China. Although nearly 45 marine mammal species harbor in Chinese waters (Zhou, 2004), the specimens of most animals were difficult to obtain except for finless porpoise. The East Asian finless porpoise (*Neophocaena sunameri*) was one of three finless porpoises species (Zhou et al., 2018), and most common marine mammal was found stranded or by caught. 170 porpoises were accidentally caught in Bohai and the Northern Yellow Sea from 2004 to 2008 (Liu, 2009). At present, bacterial infections in wild finless porpoises has rarely been reported due to scarce infected specimens and limitation of technology.

To identify these bacteria/microorganism species, 16S ribosomal RNA (rRNA) gene sequencing was commonly used as these genes comprise of highly conserved domains, interspersed with variable regions (You, 2005; Pootakham et al., 2017). To begin with, 16S rRNA gene profiling was performed using clone-based Sanger sequencing, which provides accurate, full-length or almost full-length sequences. Due to the high cost and low-output nature of the approach, the number of 16S rRNA sequencing used in Sanger-based bacterial profiling studies were often lower than 200 sequences per sample, which was insufficient to cover the complete diversity of the bacterial community (Sharp et al., 2012). Currently, various next generation sequencing platforms such as Roche 454 (Bayer et al., 2013), Illumina (Schmidt et al., 2013) and Ion Torrent PGM (Salipante et al., 2014) have gradually replaced the use of the Sanger sequencing method. Of these new sequencing platforms, the Pacific Biosciences (PacBio) single-molecule, real-time (SMRT) sequencing technology was recommended to be capable of analyzing the bacterial profiles based on the full length 16S rRNA gene (Amir et al., 2013; Pootakham et al., 2017). This technology favours metagenomic analysis and affords the researcher a relatively unbiased view of the microbial communities at different taxonomic levels of a sample (Charuvaka and Rangwala, 2011).

In Ningbo, East China Sea, China, two East Asian finless porpoises were observed to be bacterial infections between the fat and the muscle layers. The present paper focused on the bacterial diversity in the tissue pus sample, and the relationship between bacterial composition and infections symptoms. The recommended SMRT sequencing of the full-length 16S rRNA gene were applied.

MATERIALS AND METHODS

Sample collection

Within February and May 2009, 12 dead East Asian finless porpoises from fisheries by catch accidentally, in Ningbo, East China Sea were collected. The collection and research on specimens were authorized by Ningbo Ocean and Fisheries Bureau. Then pathological anatomy was conducted later on. During the necropsy, the green pus was found between the abdominal fat and muscle layers of two dead finless porpoises (Figure 1). Except of green pus, the fat and muscle tissues were normal, no lesions were found. The pus samples of one finless porpoise were collected in a sterile valve bags and stored at -20°C until its DNA was extracted.

Both infected porpoises were male with a body length of 114 and 118 cm. To explore the differences in nutritional status between infected porpoises and non-infected porpoises, six morphological characteristics, that is, dorsal blubber thickness, lateral blubber thickness, ventral blubber thickness, cervical blubber thickness, axilla girth and maximum girth were chosen. Meanwhile, to minimize the influence of aging and gender, data on five dead male finless porpoises with similar body lengths (113 to 119 cm) were collected in the same period, used for comparison (Table 1).

16S rRNA gene amplification and sequencing

Genomic DNA was extracted using the MoBio PowerSoil® DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The DNA quality was assessed on 0.8% agarose gel to ensure no degradation or rRNA contamination. The DNA samples were subsequently quantified using a spectrophotometer (Eppendorf, model RS-232C, Hamburg, Germany).

The diversity of bacterial communities was analyzed using single molecule real-time PacBio sequencing technology (Pacific Biosciences, Menlo Park, CA, USA). The full-length 16S ribosomal RNA gene was amplified from genomic DNA using the bacterial-specific primer. The primer sequences were 5'-AGAGTTTGATCMTGGCTCAG-3' and 5'- ACCTTGTTACGACTT-3'.

To obtain barcoded 16S rRNA amplicons, the amplifications in two steps was performed. A first round of PCR was performed in a 25 µL PCR solution containing 5 µL NEB Q5 High Fidelity DNA polymerase (New England Biolabs, Ipswich, MA), 5 µL GC Enhancer (New England Biolabs, Ipswich, MA), 2 µL DNTP mixture (2.5 mmol/L^{-1}), 1 µL DNA template (20 ng/μL^{-1}), 1 µL of each primer (10 µmol/L^{-1}), and 10 µL sterilized distilled water. The thermal cycling conditions were as follows: 98°C for 2 min; 25 cycles of 98°C for 30 s; 50°C for 30 s; and a final extension time of 90 s at 72°C. The primer of the second round of PCR contained a set of 16-nucleotide barcodes for the barcoded SMRT sequencing of the full-length 16S rRNA gene. The primary PCR products were diluted to 2 ng/μL^{-1}, and the diluted products were used as templates for the secondary amplification. A second round of PCR was performed in a 25 µL PCR solution containing 5 µL NEB Q5 High Fidelity DNA polymerase (New England Biolabs, Ipswich, MA), 5 µL GC Enhancer (New England Biolabs, Ipswich, MA), 2 µL DNTP mixture (2.5 mmol/L^{-1}), 1 µL DNA template (2 ng/μL^{-1}), 1 µL of each primer (10µmol/L^{-1}), and 10 µL sterilized distilled water. The conditions used for amplification in the thermocycler were as follows: 98°C for 2 min; 15 cycles of 98°C for 30 s; 62°C for 30 s; and 72°C for 90 s; and a final extension time of 5 min at 72°C.

A PacBio library was constructed using the Template Prep Kit 1.0 (Pacific Biosciences, Menlo Park, CA, USA) containing a pool of barcoded amplicons from the bacterial sample. The full-length 16S rRNA gene was sequenced using P6-C4 chemistry on a PacBio RS II instrument (Pacific Biosciences, Menlo Park, CA, USA).

Sequence data analysis and microbial population identification

Raw data were processed by the SMRT Portal, Version 2.7 (PacBio) to obtain effective sequences with a minimum of 3 full

Figure 1. A mass of green pus was found between the abdominal fat layer and muscle layer in a dead East Asian finless porpoise in Ningbo, East China Sea, China.

passes and a minimum predicted accuracy of 90 (Hou et al., 2015). The sequencing data were processed using the software package QIIME version 1.8.0 (Caporaso et al., 2010). Sequences shorter than 500 bp were removed prior to downstream analyses. Chimeric sequences were detected and removed using USEARCH version 5.2.236 (Edgar, 2010). The remaining sequences were clustered into OTUs (Blaxter et al., 2005) based on an "open-reference" OTU-selecting method at 97% identity using UCLUST (Edgar, 2010) (Figure 2). Taxonomy was assigned to the representative sequence of each OTU using the Greengenes database version 13.8 (Desantis et al., 2006), Ribosomal Database Project (RDP) database version 11.1 (Cole et al., 2009) and Silva database version 115 (Quast et al., 2013). The OTUs occurring only once or twice were discarded. After the sequencing data were rarefied, the following alpha diversity measures were calculated: Number of OTUs, Chao1 estimate of species richness (Chao, 1984), ACE estimate of species richness (Dawid, 1993), Shannon diversity (Shannon, 1948), and Simpson diversity (Simpson, 1949).

GraPhlAn is a new tool for compact and publication-quality representation of circular taxonomic and phylogenetic trees with potentially rich sets of associated metadata (Asnicar et al., 2015), and it was used to quickly detect the dominant microbe groups from the complex community data in this study. Then, KEGG PATHWAY Database (http://www.genome.jp/kegg/pathway.html) in PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille et al., 2013) was applied to predict the functional composition of the metagenome of the microbial community from its 16S profile.

RESULTS

Bacterial diversity

Following the removal of chimeras and circular consensus sequencing reads shorter than 500 bp, a total of 30768 reads of processed full-length 16S rRNA sequences were obtained for their samples. In total, 101 bacterial species were identified, and they belonged to six phyla: Actinobacteria (50.62%), Proteobacteria (42.24%), Bacteroidetes (5.06%), Firmicutes (1.82%), Cyanobacteria (0.22%) and Acidobacteria (0.05%). Four alpha diversity indices were calculated; the Chao1 estimator (307) was similar to the ACE estimator (307) while the Shannon diversity index and Simpson index were 4.2 and 0.86, respectively. Various colours on the GraPhlAn circular taxonomic and phylogenetic trees were used to distinguish among different taxa and their abundance were reflected through the node size (Figure 3).

Among the bacterial species, *Rhodococcus qingshengii* (26.25%), *Rhodococcus jialingiae* (22.43%), *Ralstonia pickettii* (16.03%) and *Moraxella osloensis* (9.97%) were the most prevalent (Table 2). Of these bacteria, two bacteria, *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*, can produce green pigmentations consistent with the green pus in the two dead finless porpoises.

Prediction of microbial metabolic function

The KEGG analysis indicated that 12 metabolic functions were associated with the microbial samples isolated from the infected finless porpoise. The major functions were amino acid metabolism (12.47%), carbohydrate metabolism (11.58%), xenobiotics biodegradation and

Table 1. The body measurements of infected finless porpoises and non-infected individuals and T-Test results.

Sample ID	Infected porpoises			Non-infected porpoises						*T* Test
	200924	200935	Average	200931	2010055	2011005	2015039	2016011	Average	P value
Sex	M	M	-	M	M	M	M	M	-	
Body length (cm)	114	118	116	117.5	114.2	119	114	113	115.54	0.710
Dorsal blubber thickness (cm)	1.6	1	1.3	1.7	1.6	1.4	2.8	2.5	2	**0.063**
Lateral blubber thickness (cm)	1.4	1.6	1.5	1.6	1.7	1.9	3	2.5	2.14	**0.074**
Ventral blubber thickness (cm)	1.8	1.6	1.7	1.6	2	1.7	3.1	2	2.08	0.228
Cervical blubber thickness (cm)	1.4	1	1.2	1.9	1.5	2.2	2.2	2.5	2.06	**0.007***
Axilla girth(cm)	64.5	68	66.3	70	62.1	69	67	72	68.02	0.353
Maximum girth (cm)	68	73	70.5	72	66.1	72	72	76	71.62	0.518

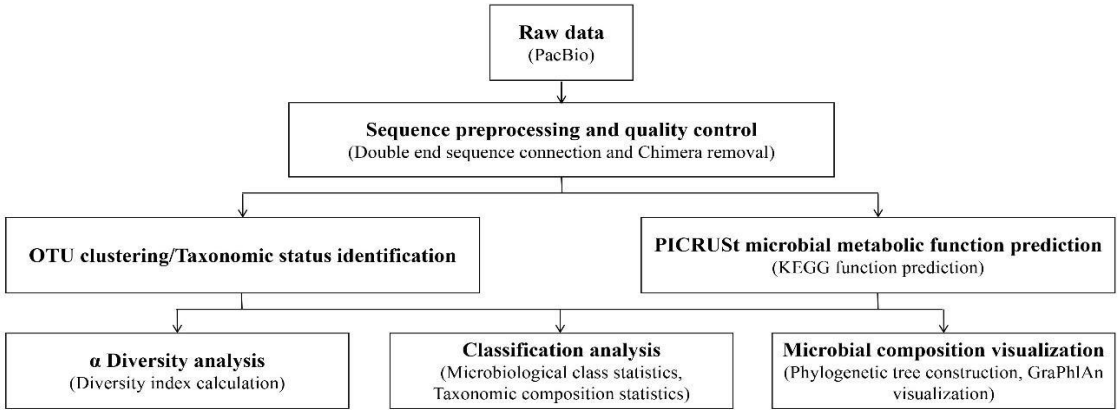

Figure 2. The framework of sequences data analysis of the bacterial diversity in an infected East Asian finless porpoise from the East China Sea.

metabolism (7.81%), lipid metabolism (6.01%) and energy metabolism (4.98%) (Figure 4).

Nutritional status comparison

The mean value of the six bodies indices (dorsal blubber thickness, lateral blubber thickness, ventral blubber thickness, cervical blubber thickness, axilla girth and maximum girth) of the infected porpoises were less than in the non-infected porpoises (Figure 5). One sample t-test was performed using IBM SPSS Statistics version 21 (SPSS, Inc., Chicago, Ill., USA), and it showed significantly different cervical blubber thickness (p=0.007) and nearly significant lateral blubber thickness (p=0.074) and dorsal blubber thickness (p=0.063) (Table 1).

This indicated that the bacteria-infected individuals were more emaciated than the non-infected individuals.

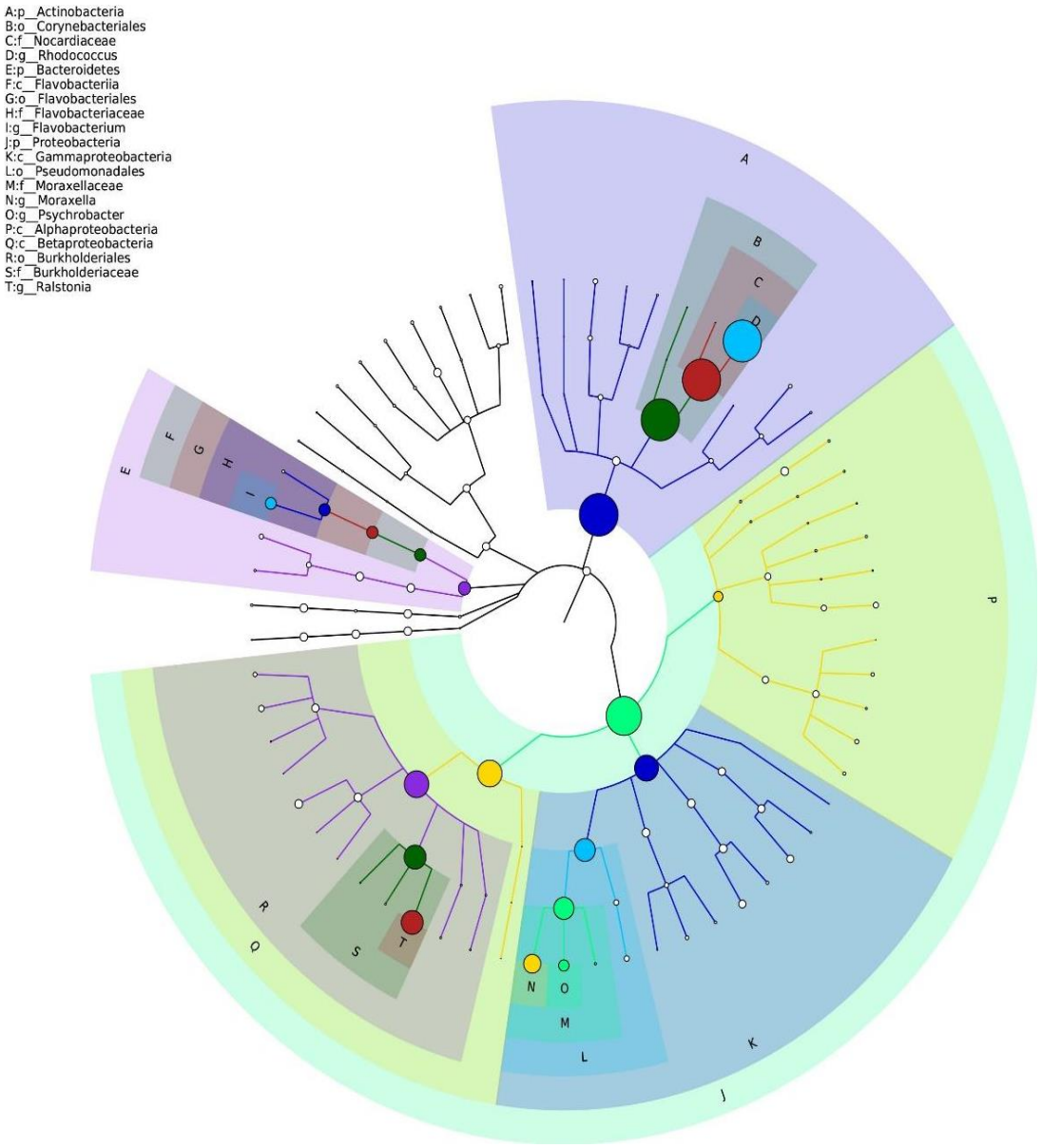

Figure 3. The hierarchical tree displays the relationships among all taxa, from phylum to species (from the inner circle to the outer circle arranged in turn). Average relative abundance is represented with node size, and the top 20 taxa are numbered with letters (A-T). p: Phylum, c: Class, o: Class, f: Family, g: Genus, s: Species.

DISCUSSION

In this paper, single molecule real-time (SMRT) sequencing, developed by Pacific BioSciences (PacBio) were used to overcome the limitations of first- and second-generation sequencing (Rhoads and Au, 2015), which were hardly enough to capture the complete diversity of the bacteria (Pootakham et al., 2017). Therefore, this paper provided a more precise and reliable sequences than first- and second-generation technology.

The infected porpoises generally showed clear notable marasmus unlike the non-infected porpoises. This indicated that the porpoises might have been suffering from infections, which resulted in consumption of fats and body marasmus. Although these assumed diseases have not been determined, the analysis of the pathogenicity of the bacteria provides us with some insights.

A total of 101 bacteria were identified, among them, observations of *R. qingshengii* (26.25%) and *Rhodococcus jialingiae* (22.43%) have been reported in

Table 2. Bacterial species and pathogenicity from the sticky sample isolated from an infected finless porpoise from Ningbo, China, in 2009.

Scientific name	Percentage	Possible source	Possible disease caused	Reference
Rhodococcus qingshengii	26.25	Carbendazim-contaminated soil	Pale liver, splenomegaly, melanosis in abdominal cavity, internal haemorrhaging	1, 2
Rhodococcus jialingiae	22.43	Carbendazim-contaminated water		1, 3
Ralstonia pickettii	16.03	Industrial wastewater	Endocarditis, meningitis, bacteraemia, septicaemia, pneumonia, osteomyelitis, septic arthritis, spondylitis	4, 5
Moraxella osloensis	9.97	Unknown	Endocarditis, osteomyelitis, septic arthritis, bacteraemia, meningitis, pneumonia, endophthalmitis, respiratory failure	6, 7, 8, 9
Psychrobacter cibarius	2.97	Refrigeration	Unknown	10
Flavobacterium johnsoniae	2.11	Soil	Skin disease	11, 12
Flavobacterium chungbukense	1.80	Soil	Unknown	13
Stenotrophomonas maltophilia	1.73	Soil, water, air	Acral necrosis, endocarditis, meningitis, bacteraemia, osteochondritis, sinusitis, diffuse bronchitis, chronic enteritis, septic arthritis, otitis externa, spondylodiscitis, pyogenic liver abscess pyelonephritis, respiratory infection, pneumonia	14, 15, 16, 17
Serratia marcescens	1.62	Unknown	Respiratory tract infection, urinary tract infection, septicaemia, meningitis, endocarditis	18
Aquabacterium commune	1.49	Unknown	Unknown	
Pelomonas saccharophila	1.08	Unknown	Unknown	
Pseudomonas aeruginosa	0.92	Water, soil	Ulcerative keratitis, otitis extema, skin and soft tissue infections, pneumonia.	18, 19, 20, 21

Reference Number: 1, Xu et al., 2007; 2, Avendaño-Herrera et al., 2011; 3, Wang et al., 2010; 4, Kulakov et al., 2002; 5, Ryan et al., 2006; 6, Shah et al., 2000; 7, Vuori-Holopainen and Pehola, 2001; 8, Berrocal et al., 2002; 9, Gargiulo et al., 2015; 10, Bowman, 2006; 11, Flemming et al., 2007; 12, Carson et al., 2010; 13, Lim et al., 2011; 14, Grimont and Grimont, 1984; 15, Fujita et al., 1996; 16, Hanes et al., 2002; 17, Falagas et al., 2009; 18, Kiska and Gilligan, 2003; 19, Paterson, 2006; 20, Driscoll et al. , 2007; 21, Avalos-Te´llez et al., 2010.

the sludge of a carbendazim wastewater treatment facility and carbendazim-contaminated soil (Wang et al., 2010). *Ralstonia pickettii* has been identified in biofilm formation in ultrapure water in industrial systems (Kulakov et al., 2002). The pathogenicity of these species has been reported in some aquatic species, e.g., genus *Rhodococcus* and *Flavobacterium johnsoniae* for freshwater and marine fish (Olsen et al., 2006; Flemming et al., 2007) and a West African dwarf crocodile that died from acute *Stenotrophomonas maltophilia* septicaemia (Harris and Rogers, 2001). The finless porpoise as a top predator in marine environments very likely to contacted these bacteria along the marine food cycle.

Meanwhile, *Moraxella osloensis* (9.97%), *Flavobacterium johnsoniae* (2.11%), *Stenotrophomonas maltophilia* (1.73%), *Serratia marcescens* (1.62%) and *Pseudomonas aeruginosa* (0.92%) were conditioned pathogens. Most of them are widely distributed in nature and exist in animals as normal flora. When this symbiotic relationship turns into parasitism caused by bacterial translocation, as in dysbacteriosis or host-lowered immunity, these normal floras might damage their hosts (Sun et al., 2011).

Additionally, species of the genus *Psychrobacter* have been isolated from various low-temperature habitats or sources, including sea ice, fish, chilled meat and blood products, krill stomach, Antarctic

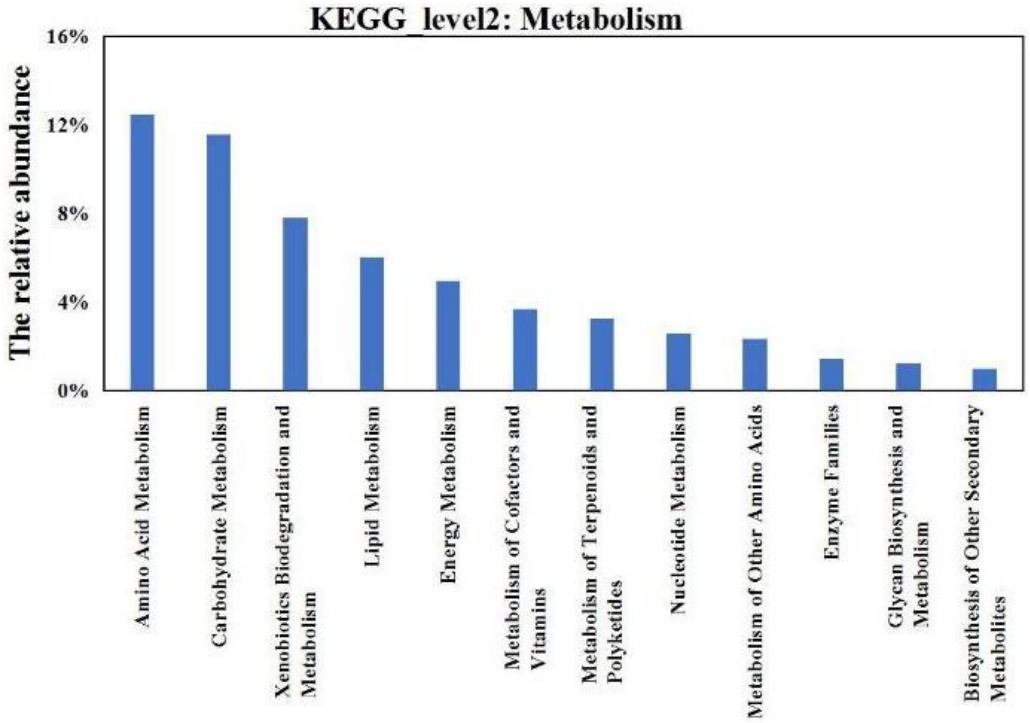

Figure 4. Histogram of prediction of microbial metabolic functions plotted with KEGG functional composition on the horizontal axis and the relative abundance on the vertical axis.

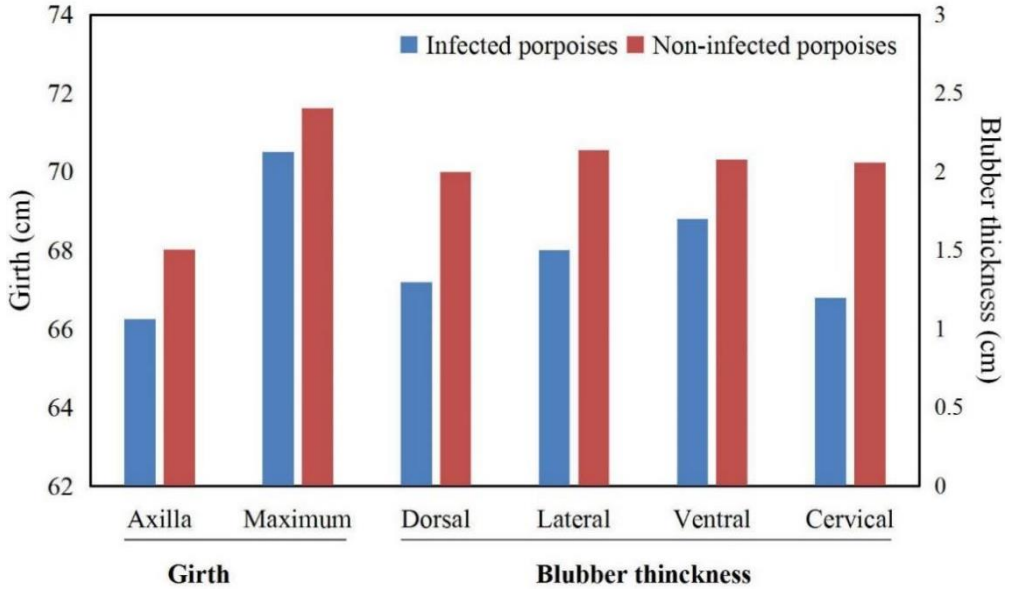

Figure 5. Comparison of the mean value of six body indices between infected porpoises and non-infected porpoises. Blubber thickness: Dorsal blubber thickness, lateral blubber thickness, ventral blubber thickness and cervical blubber thickness; Girth: Axilla girth and maximum girth.

ornithogenic soil and contaminants on lab media (Bowman, 2006), and *Psychrobacter cibarius* (2.97%) has never been detected as an animal pathogen. It can be presumed that the possible origin in the sample was refrigeration in the refrigerator.

Microorganisms and their hosts share the same

environment, and the activities of the microbiomes and their metabolic products influence a variety of aspects in metazoans (Lee and Hase, 2014). This was supported by the KEGG analysis in this study, that is, the microbial communities in finless porpoise involved in major metabolism functions which were the most fundamental requirements for survival, and metabolic disorders may lead to various health problems.

In fact, the main bacterial species have been proven to be related to various pathogenicities, skin diseases, respiratory diseases, diverse inflammations, hematologic diseases, and visceral lesions. For example, *Flavobacterium johnsoniae* and *Pseudomonas aeruginosa* were known as opportunistic skin pathogens (Driscoll et al., 2007; Carson et al., 2010), and they cause soft tissues infections in fish species (Carson et al., 2010). *Moraxella osloensis* was a pathogen that causes respiratory failure (Gargiulo et al., 2015). *Stenotrophomonas maltophilia* could cause endocarditis, meningitis, osteochondritis, sinusitis, diffuse bronchitis, chronic enteritis, septic arthritis, otitis externa, spondylodiscitis, pyogenic liver abscess pyelonephritis (Falagas et al., 2009) and pneumonia (Hanes et al., 2002). *Pseudomonas aeruginosa* has been reported to cause chronic bronchopneumonia in bottlenose dolphins (Avalos-Te´llez et al., 2010). *Ralstonia pickettii* has been associated with unusual clinical situations such as bacteraemia and septicaemia (Ryan et al., 2006). Infection from *Rhodococcus qingshengii* could cause severe visceral lesions, e.g., inducing the presence of the pseudo-membranes on the spleen, liver and heart. Histopathology revealed perivascular leucocytic infiltration, marked granulomas and increased fibroblastic reticular cells in spleen (Avendaño-Herrera et al., 2011).

Based on the analysis above, it was speculated that bacterial infections cause some diseases and metabolic disorders, which might impact the health of finless porpoises to some extent. Regardless of the type of diseases and metabolic disorders would first cause of body weight loss. Further study on pathogenicity experiment was needed in the future.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Assessment of drugs pressure on *Escherichia coli* and *Klebsiella* spp. uropathogens in patients attending Abobo-Avocatier Hospital, North of Abidjan (Côte d'Ivoire)

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The problem of antibiotic resistance of uropathogens appears in Abobo municipality to be worsening because of the overuse and misuse of antibiotics. This study aims to assess the impact of drugs pressure on uropathogenic *Escherichia coli* and *Klebsiella* spp. strains isolated from patients attending Abobo-Avocatier Hospital. The study was conducted in patients suffering from urinary tract infection. Urine samples of patients were collected; culture and antibiogram using the Kirby-Bauer disc diffusion method were performed. The overall prevalence was 31.1% with a significant difference between males and females (p = 0.01). The highest susceptible age group of patients to UTI was 21-45 years (33%). *E. coli* and *Klebsiella* spp were the predominant bacteria among isolated Gram negative. Up to 70% of the isolates of both uropathogens were resistant to Penicillins, Tetracyclines and Ampicillin-Sulbactam. Imipenem was the most active antibiotic on these uropathogens. Quinolones showed a better activity on *Klebisiella* spp. strains than those of *E. coli*. The high value of the Multiple Antibiotic Resistance Index and the rate of multi-resistance from this site suggest the need for continuous monitoring of antibiotic susceptibility profile of bacteria implicated in UTI prior to antibiotic prescription in order to ensure optimal and desired treatment.

Key words: Urinary tract infections, uropathogens, multidrug resistance, Abidjan, Côte d'Ivoire.

INTRODUCTION

Urinary tract infection (UTI) is defined as a multiplication of microorganisms in the urinary tract with or without symptoms (Prakash and Saxena, 2013). This pathology usually starts by a bladder infection and can reach the kidneys to cause renal dysfunction or dissemination in the blood (Vejborg et al., 2011). Depending on the organ

and/or severity, UTI is classified as bacteriuria (urine), cystitis (bladder), pyelonephritis (kidneys) or urosepsy (blood) (Vejborg et al., 2011). UTI is a major public health problem in developing countries with a high morbidity rate and important financial cost (Prakash and Saxena, 2013; Eshetie et al., 2015). Worldwide, about 150 million patients are diagnosed each year for an estimated economic weight of over 6 billion dollars (Prakash and Saxena, 2013). In Africa, the burden of UTI is difficult to be evaluated because diseases notification is scarce. The most affected people are school age children, sexually active women and older adults of both gender (Foxman, 2014). The causative agents belong to Enterobacteriaceae family and *Escherichia coli* and *Klebsiella* spp species are the most commonly isolated (Indu and Deepjyoti, 2012; Melaku et al., 2012). Antibiotic therapy is the standard method for the treatment of bacterial infections. However, in recent years, a significant increase of antibiotic resistance in *Enterobacteriaceae* has been documented worldwide. In Côte d'Ivoire, few studies address the incidence of urinary tract infection and selection pressure induced by antibiotics on uropathogens, while UTI is common in daily practice (Boni et al., 2014; Cisse et al., 2017). This is due to the fact that in Côte d'Ivoire and even in Africa, urine culture and antimicrobial susceptibility testing are unavailable in most of hospital centers (Dosso et al., 2000; Eshetie et al., 2015; Cisse et al., 2017). In addition, cases of urinary tract infection are often treated empirically based on information determined from resistance profile of urinary pathogens (Ekwealor et al., 2016). This situation leads to wrong diagnosis and irrational antibiotic use in the treatment of urinary infection, promoting the emergence and spread of multidrug-resistant strains (Ouédraogo et al., 2017). In Abobo municipality, the antibiotic resistance appears to be worsening and no local database on the urinary infection incidence and resistance profile of strains involved is available. To effectively monitor antibiotic resistance, it is important to know the level of antibiotic resistance and to have a local database. This prospective study aims to investigate and to assess the drugs pressure on uropathogenic *E. coli* and *Klebsiella* spp. isolated from patients attending Abobo-Avocatier Hospital in the North of Abidjan.

MATERIALS AND METHODS

Study area

This study was carried out at Abobo-Avocatier Hospital located in the municipality of Abobo, in the North of Abidjan (Côte d'Ivoire). The Abobo municipality is one of the most populated areas of Abidjan with about 1,030,658 million inhabitants (RGPH, 2014). The hospital has a biomedical laboratory, but only the parasitology unit is functional. This research project opened the microbiology unit in order to implement bacterial diagnosis and optimize the antibiotic treatment of urinary tract infections.

Ethics statement

The study protocol was reviewed and approved by the national ethics committee of Life Sciences and Health in Côte d'Ivoire with the number: N/Ref:106-18/MSHP/CNESVS-KM, US DPT OF HHS REGISTRATION #: IORG00075 on 31st July 2018. This study is part of the ESTHER project which aims to a better diagnosis of urinary tract infections in outpatients in addition to microbiological surveillance of uropathogens and antimicrobial resistance. Consent was obtained from patients and/or guardians after explaining the objective of the study. The laboratory results were communicated to patients via physicians for better antibiotic prescription.

Study design, participants and data collection

It is a cross-sectional prospective study carried out between October 2018 and April 2019 in patients with symptomatic UTI. Only patients who met clinical criteria (algae, pollakuirie, urge urination, suprapubic pain) and urinary strip criteria (leukocyturia, positive nitrite and hematuria) were included in this study. These patients received good instructions on how to collect the urine sample aseptically. About 20 ml of midstream urine specimen was collected from each patient in the morning using a sterile bottle and labeled with the unique sample code, date and time of collection. Demographic characteristics such as gender and age, and clinical characteristics such as previous antibiotic treatment, symptoms, and pregnancy status for women were collected from patients.

Analysis procedure

Urine dipstick technique and microscopic examination

The biochemical test of the urine specimen using the urine dipstick technique was performed. It allowed to reveal metabolic, hepatic and renal disorders, such as urogenital infections by the significant presence of leukocytes and nitrite (Hay et al., 2016). Microscopic examination of the fresh urine was performed to confirm the results of the urine dipstick test by counting leukocytes, erythrocytes, urothelial cells and crystals (Ikhlas et al., 2018). The urine culture was systematically performed if at least a criterion of the dipstick test was positive or significant leucocyturia was observed (10 leucocytes / field) (Ekwealor et al., 2016).

Isolation and identification of uropathogens

Each urine sample was inoculated on the CHROMAgar medium using plastic loops for 10 µl (Ohkusu, 2000). The culture was incubated under aerobic conditions at 37°C for 24 h. After incubation, the growing *Escherichia coli* isolates were recognizable by large colonies dark pink to reddish with or without halo. Klebsiella, Enterobacter, Serratia and Citrobacter (KESC) group

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Table 1. Risk factors associated with urinary tract infections.

Factor	Status of urinary infection			Bivariate analysis	
	N	Positive (%)	Negative (%)	*p*	OR (CI 95 %)
Gender					
Males	57	11 (19.3)	48 (80.7)		1
Females	91	35 (38.5)	56 (61.5)	0.01	2.73 (1.25; 5.95)
Age groups (years)					
14 - 20	16	4 (25)	12 (75)		
21 - 45	91	30 (33)	61 (67)	0.528	1.48 (0.44; 4.96)
> 45	41	12 (29.3)	29 (70.7)	0.747	1.24 (0.33; 4.63)
Total	148	46 (31.1)	102 (68.9)		

N: total number; *p:* probability associated at χ^2 test; OR: odds ratio; $CI_{95\%:}$ 95% Confidence Interval.

colonies were colored in metallic blue and medium in size (Ohkusu, 2000). Identification of bacterial species was made based on reactions of Gram, morphology and biochemical characteristics using the gallery of the reduced rack of LEMINOR (Urea; Indol; Simmons' citrate agar; Kligler Hajna agar; iron Lysine agar), oxidase and catalase tests (Tahou et al., 2017). Clean catch urine samples were collected from patients and bacteriological analyses were performed using standard microbiological procedures.

Antibiotic susceptibility testing and drug resistance

Antibiotic susceptibility testing was done using the Kirby-Bauer disk diffusion method on Mueller Hinton (Oxoid) agar according to the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2017). *E. coli* and *Klebsiella* spp strains were tested on 14 antibiotics (Oxoid) such as: Amoxicilin-Clavulanic acid (AMC; 20/10 µg), Ampicillin (AMP; 10 µg), Penicillin G (P; 1 µg), Tetracycline (TE; 30 µg); Ceftazidime (CAZ; 30 µg), Ceftriaxone (CRO; 30 µg), Cefuroxime (CXM; 30 µg), Nitrofurantoin (F; 300 µg), Gentamicin (CN; 10 µg), Imipenem (IMP; 10 µg), Nalidixic Acid (NA; 30 µg), Ampicillin-Sulbactam (SAM; 20 µg), Chloramphenicol (C; 30 µg) and Ciprofloxacin (CIP; 5 µg). Bacterial colonies from a pure overnight culture were suspended in 2 ml of 0.85% NaCl in order to maintain the bacterial strains in osmotic equilibrium and the bacterial suspension was standardized to 0.5 McFarland (10^8 UFC / ml). The suspension was inoculated on Mueller Hinton agar by striation using a sterile swab and antimicrobial agents are placed onto the surface of the agar and incubated at 37°C for 24 h. After incubation, the diameter of the inhibition zone of bacterial growth formed around the disc was measured and compared to the critical values d and D of each antibiotic disc according to CLSI. The target bacteria were qualified as sensitive (diameter of the inhibition > D) or resistant (diameter of the inhibition < d) or intermediate (d < diameter of the inhibition < D) (Moroh et al., 2014). In this study, isolates of intermediate phenotypes were considered resistant. Standard strains of *E. coli* ® ATCC 25922, *S. aureus* ATCC ® 25923 and *Klebsiella pneumoniae* ® ATCC 35657 (BBL) were used as quality controls for identification and antimicrobial susceptibility testing. Multi-resistant strains were divided into MDR (Multiple Drug-Resistant), XDR (Extensively Drug-Resistant) and PDR (Pandrug-resistant) according to the European Center for Disease prevention and Control (Magiorakos et al., 2012). MDR bacteria are defined as resistant at least to three different classes of antibiotics. XDR bacteria are characterized by their sensitivity to only one class of antibiotics and the PDR bacteria are resistant to all classes of antibiotics tested (Magiorakos et al., 2012).

Statistical analysis

The rate of resistant and multi-resistant isolates to antimicrobial reagents was calculated. The Multiple Antibiotic Resistance Index (MARI) calculation was done (Krumperman, 1983). Comparison between the rate of antibiotic resistance for *E. coli* and *Klebsiella* spp isolates was performed by Chi-Square or Fisher's exact tests according to the Cochran rule. Odds ratio (OR) was estimated to evaluate the occurrence of UTI from female. MAR Index mean of *E. coli* and *Klebsiella* spp were compared using Student's t-test for independent Samples. Heatmaps was carried out to evaluate the similarity between the strains of each bacterial population based on their resistance profiles. All statistics tests and graphs were performed using R software version 3.3.1 and differences were considered to be significant when *p -value* ≤ 0.05.

RESULTS

A total of 148 patients comprising 91 females and 57 males were included in this study. The overall prevalence of urinary infection was 31.1% with 19.3% for males and 38.5% for females (Table 1). Females were around 3 times more likely to be infected than males (OR: 2.73, 95% CI (1.25; 5.95); p = 0.01). No significant differential infection was observed (p > 0.05) among age groups despite the highest susceptible age group of patients was 21 - 45 years (33%) followed by ≥ 45 years (29.3%) and 14 -20 years (25%) in both Female and Male (Table 1).

Figure 1 shows the gender susceptibility to UTI regarding age groups. The results show that the significant differential infection observed between male and female was expressed among 21 - 45 years' age groups, with females 3 times more likely to be infected than males (OR = 3.18; 95% IC [1.14 - 8.87], p = 0.024). However, among the age groups of 14 to 20 and over 45 years of age, the difference in UTI prevalence between male and female was not statistically significant.

Distribution of uropathogenic bacteria isolated

The bacterial pathogens isolated were *E. coli*: 14

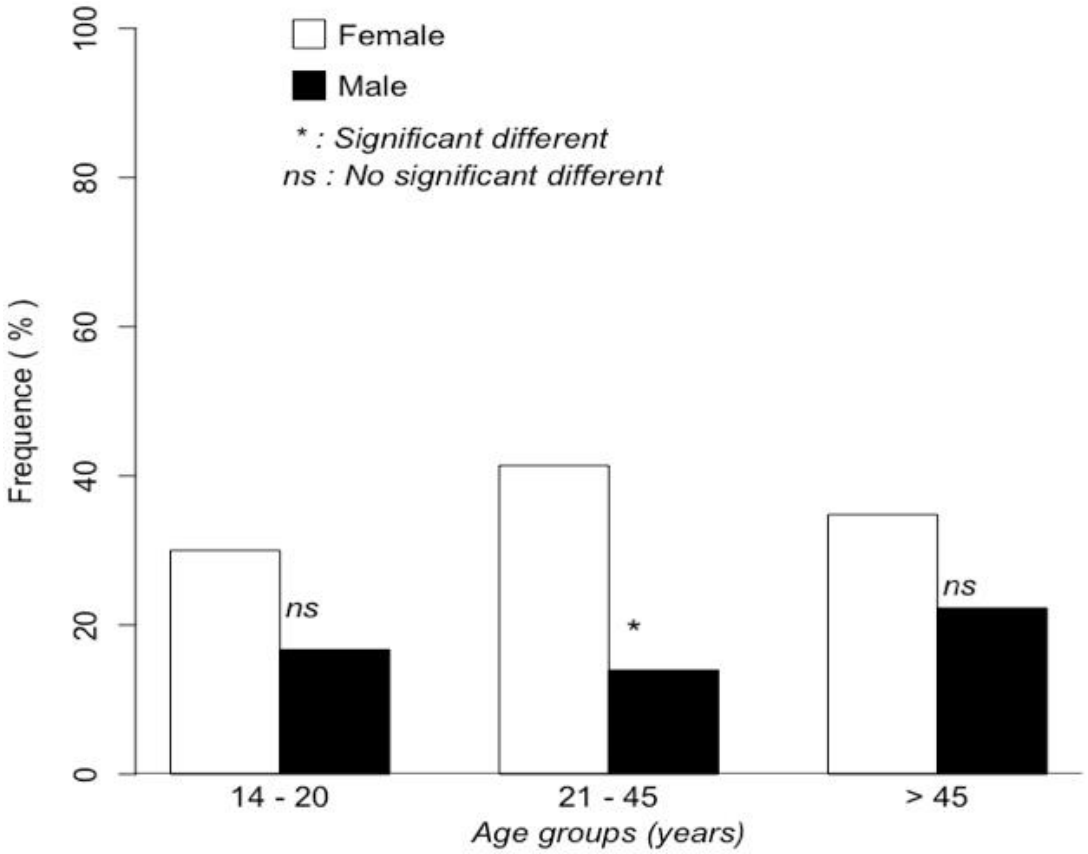

Figure 1. Gender susceptibility to UTI regarding age group.

Table 2. Frequency of uropathogenic bacteria isolated.

Gram	Bacteria isolate	Number of isolates	Prevalence (%)
		25	**54.3**
	Escherichia coli	14	30.4
Negative Gram	*Klebsiella* spp.	8	17.4
	Enterobacter spp	2	4.3
	Proteus spp.	1	2.2
		21	**45.7**
Positive Gram	*Staphylococcus* spp.	14	30.4
	Enterococcus spp	7	15.2
Total		**46**	**100**

(30.4%), *Staphylococcus* spp: 14 (30.4%), *Klebsiella* spp: 8 (17.4%), *Enterococcus* spp: 7 (15.2%), *Enterobacter* spp: 2 (4.3%) and *Proteus* spp.: 1 (2.2%). The most common uropathogenic bacteria were Gram-negative bacteria belonging to *Enterobacteriacea* family with a cumulative prevalence of 54.3%. *E. coli* and *Klebsiella* spp were the most dominant species from this family and each accounted for 56 and 32% respectively (Table 2).

Antibiotic resistance of *E. coli* and *Klebsiella* spp. strains

The assessment of antimicrobial resistance (AMR) was focused on *E. coli* and *Klebsiella* spp because they are the most common for antibiotic resistance monitoring regarding their high frequency and pathogenicity. The results showed strains from both bacteria species were

Table 3. Antibiotic resistance rates of *E. coli* and *Klebsiella* spp isolates.

Antibiotics class	Antibiotics	Bacteria species			Fisher's exact test
		Dose (µg)	*E. coli* (n=14)	*Klebsiella* spp. (n= 8)	*p*-value
Penicillins	Amoxicillin-clavulanic acid	20/10	13 (92.9)	6 (75)	0.527
	Ampicillin	10	14 (100)	8 (100)	1
	Penicillin G	1	14 (100)	8 (100)	1
Cephalosporin II (C2G)	Cefuroxime	30	7 (50)	3 (37.5)	0.675
Cephalosporin III (C3G)	Ceftriaxone	30	6 (42.9)	4 (50)	1
	Ceftazidime	30	6 (42.9)	2 (25)	0.649
Carbapenem	Imipenem	10	1(7.1)	1 (12.5)	1
Quinolones	Ciprofloxacin	5	8 (57.1)	0 (0.0)	**0.017**
	Nalidixic acid	30	12 (85.7)	3 (37.5)	**0.05**
Aminoglycosides	Gentamicin	10	3 (21.4)	2 (25)	1
Tetracyclines	Tetracyclin	30	13 (92.9)	6 (75)	0.527
Phenicols	Chloramphenicol	30	6 (42.9)	4 (50)	1
furantoins	Nitrofurantoin	300	2 (14.3)	3 (37.5)	0.309
Betalactam/betalactamase inhibitor	Ampicillin-Sulbactam	20	12 (85.7)	6 (75)	0.602

100% resistant to Ampicillin and Penicillin G. 92.9% of *E. coli* strains were resistant to Amoxicillin-Clavulanic acid and Tetracycline and the lowest resistance was obtained with Imipenem (7.1%) (Table 3). 75% of *Klebsiella* spp strains were resistant to Amoxicillin-Clavulanic acid and Tetracycline. In addition, all strains were susceptible to Ciprofloxacin and only 12.5% of strains were resistant to Imipenem (Table 3). The Fisher's exact test showed that *E. coli* and *Klebsiella* spp. strains present statistically identical resistance rates against commonly used antibiotics (Penicillins, Cephalosporins, Aminoglycosids, Tetracyclins, Phenicols, Furantoins, Betalactam / Betalactamase inhibitor) and to Carbapenems. However, the resistance rate against quinolones was statistically different between *E. coli* and *Klebsiella* spp strains ($p \leq 0.05$). Quinolones, especially Ciprofloxacin showed very good activity on *Klebisiella* spp strains than *E. coli*, in which resistance rate reached 85.7 and 57.1% for Nalidixic acid and Ciprofloxacin respectively (Table 3). The antibiotic resistance profile of *E. coli* and *Klebsiella* spp strains is indicated in Table 4. The result obtained suggests that the strains might be genetically different from a patient to another. The least multi-resistant strains of *E. coli* were resistant to 3 classes of antibiotic while the most multi-resistant strains were resistant to 9 classes of antibiotic with an average resistance to 6 classes of antibiotic for all isolated strains. Regarding *Klebsiella* spp, strains were resistant to an average of 5 different classes of antibiotic with a strain resistant to only one class of antibiotic (Table 4).

Regarding the heterogeneity of the strains presented by in Figure 2, two clusters were observed for each species of bacteria (*Klebsiella* spp and *E. coli*), demonstrating an intraspecific diversity among patients infected. Each cluster comprised about 50% of the isolates. In both bacterial populations, isolates from cluster I are characterized by resistance to a large number of antibiotics and isolates from cluster II characterized by susceptibility to most of the antibiotics tested (Figure 2).

Multi-resistance and multiple antibiotic resistance index

Multi-resistance and Multiple Antibiotic Resistance index (MARI) of *E. coli* and *Klebsiella* spp. strains are shown in Table 5. Based on their observed phenotypes and classes of multi-resistance defined previously, 100% *E. coli* strains and 87.5% *Klebsiella* spp strains were multi-resistant. No isolates in both *E. coli* and *Klebsiella* spp presented the Pan drug-resistant phenotype. Only a strain of *E. coli* (7.14%) presented extensively drug resistant phenotype. The average multiple antibiotic resistance index of *E. coli* and *Klebsiella* was 0.59 ± 0.2 and 0.51 ± 0.2 respectively (Table 5). The Student's t-test showed no statistically significant difference between the MARI of both bacteria species tested ($t = 0.86$; degree; $p = 0.397$).

DISCUSSION

This study provides valuable data for assessing drugs' pressure and monitoring the antimicrobial resistance among uropathogenic *E. coli* and *Klebsiella* spp to

Table 4. Antibiotic resistant profile of *E. coli* and *Klebsiella* spp isolates

Species	Bacteria strains	Antibiotic resistant profile	No. of ATB class
Escherichia coli	*E.coli1*	AMC, AMP, P, TE, CIP, CRO, CXM, NA, SAM, C	7
	E.coli2	AMP, NA, P, TE	3
	E.coli3	AMC, AMP, C, P, SAM,TE	4
	E.coli4	AMC, AMP, TE, NA, P, SAM	5
	E.coli5	AMC, AMP, TE, CAZ, CIP, CXM, NA, P, SAM	6
	E.coli6	C, CAZ, CN, CRO, CXM, F, AMC, AMP, CIP, NA, P, SAM, TE	9
	E.coli7	AMC, AMP, NA, P, SAM, TE	4
	E.coli8	AMC, AMP, C, TE, CAZ, CIP, CRO, CXM, NA, P, SAM	7
	E.coli9	F, AMC, AMP, TE , P	3
	E.coli10	AMC, AMP, TE, NA, P, SAM	4
	E.coli11	CAZ, CN, AMC, AMP, TE, CIP, CRO, CXM, NA, P, SAM	7
	E.coli12	AMC, CIP, SAM, AMP, NA, P, TE	4
	E.coli13	C, CAZ, CN, CRO, CXM, AMC, AMP, CIP, NA, P, SAM	7
	E.coli14	CAZ, CIP, CRO, CXM, IMP, AMC, AMP, TE, NA, P, SAM	7
	Mean (±Sd)		**6 ± 1.9**
Klebsiella spp.	*Klebsiella*1	AMC, AMP, P	1
	*Klebsiella*2	CAZ, CN, CRO, CXM, F, AMC, AMP, C, NA, P, SAM, TE	9
	Klebsiella 3	F, NA, AMP, P, SAM	4
	*Klebsiella*4	C, AMC, AMP, CRO, CXM, P, TE	5
	*Klebsiella*5	NA, AMC, AMP, C, P, SAM, TE	5
	*Klebsiella*6	IMP, AMC, AMP, P, SAM, TE	4
	*Klebsiella*7	CAZ, CN, CXM, AMC, AMP, CRO, P, SAM, TE	6
	Klebsiella8	AMC, C, CRO, F, SAM, TE, AMP, P	6
	Mean (± Sd)		**5 ± 2.3**

Amoxicillin-Clavulanic acid (AMC), Ampicillin (AMP), Penicillin G (P), Tetracycline (TE), Ceftriaxone (CRO), Ceftazidime (CAZ), Cefuroxime (CXM), Nitrofurantoin (F), Gentamicin (CN), Imipenem (IMP), Nalidixic acid (NA), Ampicillin-Sulbactam (SAM); Chloramphenicol (C), Ciprofloxacin (CIP).

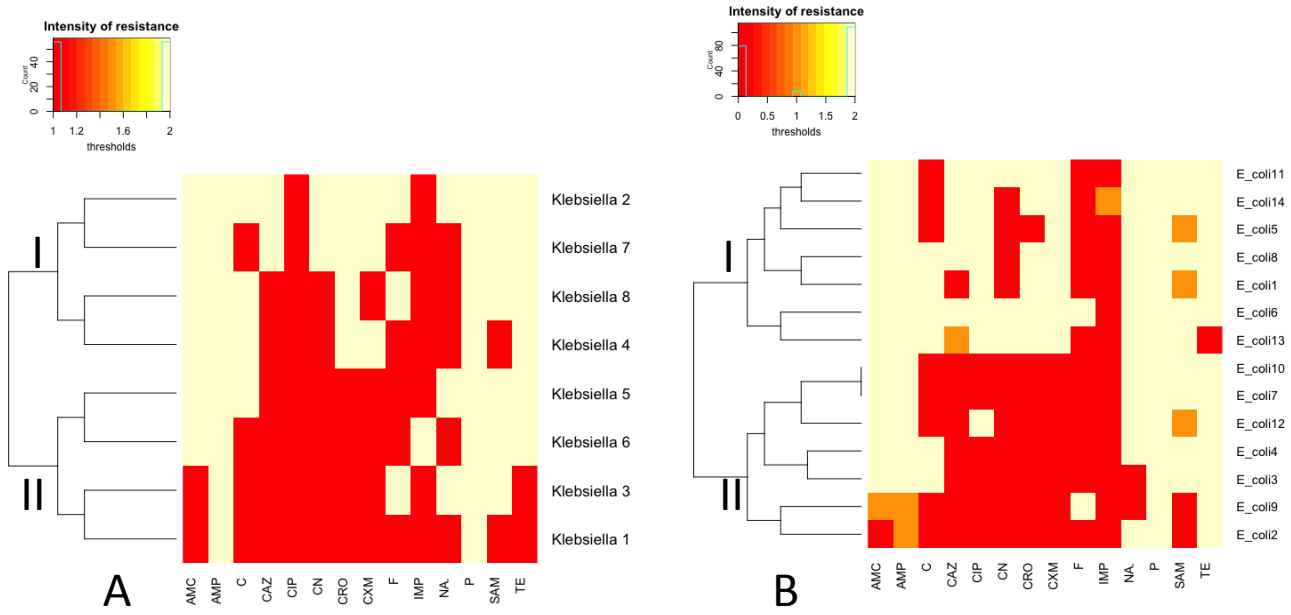

Figure 2. Heatmap showing the relationship and variation of antibiotic resistance profiles between isolates of *Klebsiella* spp. (A) and *E. coli* (B). Light color = area of resistance, red color = area of sensitivity, orange color = intermediate area.

Table 5. Multiresistance rate in *E. coli* and *Klebsiella* spp.

Bacteria species	Total	MDR (%)	XDR (%)	PDR (%)	Mean MARI
E. coli	14	14 (100)	1 (7.14)	0 (0.0)	0.59 ± 0.2
Klebsiella spp	08	7 (87.5)	0 (0.0)	0 (0.0)	0.51 ± 0.2
Student's T-test		--	--	--	t = 0.86; p = 0.397

MDR: Multiple Drug-Resistant; XDR: Extensively Drug-Resistant; PDR: Pandrug-resistant; MARI: Multiple Antibiotic Resistance Index.

improve patients' treatment. It is a first study on urinary infection in the municipality of Abobo. All infected patients had a community-acquired UTI with a high overall prevalence up to 31.1% but not significantly different than reported elsewhere in Côte d'Ivoire, at Treichville Teaching Hospital (25%) (Moroh et al., 2014). However, the obtained UTI prevalence was higher than those recorded in some West African countries: 9.5% in Ghana (Obirikorang et al., 2012) and 13.1% in Nigeria (Iregbu and Princewill, 2013). Contrary to those countries, our prevalence was lower than other sub-Saharan countries, 58.3 % in Cameroon (Prakash and Saxena, 2013), 51% in South Africa (Habte et al., 2009) and 41.4% in Ethiopia (Akoachere et al., 2014). Differences in UTI rates regarding areas could be explained by the disparities in location and health facilities in addition to patients' status (acute UTI or post-antibiotic treatment) (Shatalov, 2015). This study showed a significantly higher prevalence of urinary tract infection in females (38.5%) than in males (19.3%) with a 2.6 times higher risk compared to males. These results, is in agreement with those of Prakash and Saxena (2013), might be due to the close proximity of females' urethra to the anus, shorter urethra, incontinence, and bad female toilets (Orrett and Davis, 2006). No significant association between age and UTIs was observed in this study. But the highest rate of UTI was reported in patients aged 21 to 45 years with females three times more likely to be infected than males. Similar results were obtained by Eshetie et al. (2015) in northwestern Ethiopia, in which age was not associated with the development of urinary tract infection. The reason for the significant increase in urinary tract infection risk observed in females aged 21 to 45 years might be associated with high sexual activity in this age group. This factor is important in the exchange of microbial strains between individuals (Foxman, 2014; Vasudevan, 2014).

Gram negative bacilli accounted for 54.3% of the total number of bacterial isolated. *E. coli* and *Klebsiella* spp were the most predominant (Habte et al., 2009; Bao et al., 2013; Moroh et al., 2014; Ebongue et al., 2015). This high prevalence of *E. coli* and *Klebsiella* spp. might be due to their virulence and pathogenicity (Prakash and Saxena, 2013). In addition, these uropathogens are frequently difficult to treat because of their intrinsic and acquired resistance to multiple antibiotic families (Billy, 2003).

Antimicrobial susceptibility testing showed high resistance rates to penicillins, tetracyclines, ampicillin-sulbactam and to third generation cephalosporins (ceftriaxone) among *E. coli* and *Klebsiella* spp strains. This high level of expressed resistance could be attributed to the irrational use of these antibiotics and the lack of proper UTI diagnosis and antibiotic susceptibility testing in the municipality of Abobo. As reported by Ekwealor et al. (2016), the misuse of antibiotics in a society where people indulge in self-medication and the intensification of empirical and probabilistic prescriptions are practiced is an important way to promote antibiotic resistance. Resistance to penicillins and cephalosporins classes of antibiotic might be due to the production of beta-lactamases (penicillinases and cephalosporinases) encoded in the bacterial genome (Mpelle et al., 2019). Several studies worldwide have reported the emergence and dissemination of *E. coli* and *Klebsiella* spp strains producing extended spectrum of beta-lactamases (Moyen et al., 2014; Abujnah et al., 2015; Mpelle et al., 2019). The drug pressure induced might change the bacterial ecology, which favors an adaptation of the microbial agent to its new environment, developing resistance mechanisms transmissible by mobile genetic elements such as plasmids (Sbiti et al., 2017). Quinolones (ciprofloxacin and nalidixic acid) have shown an excellent activity on more than 60% of *Klebsiella* spp isolates, whereas high resistance to this antibiotic class was observed among *E. coli* isolates. The significant difference observed in quinolones resistance between *E. coli* and *Klebsiella* spp could be related to the frequency of isolation of uropathogenic *E. coli* and the intensive use of quinolones (e.g. Ciprofloxacin) in the treatment of infections (Dosso et al., 2000; Kim and Hooper, 2014) involving this pathogen at Abobo-Avocatier Hospital. Imipenem has shown excellent activity against both *E. coli* and *Klebsiella* spp isolates. This high susceptibility to imipenem could be explained by the fact that carbapenems are new broad-spectrum antibiotics, and their use is not yet widespread in developing countries such as Côte d'Ivoire (Kattan et al., 2008). Also, the high cost of this antibiotic might limit its use in self-medication treatment of bacterial infections. The prevalence of MDR strains in our study is higher than the one observed at the teaching hospital of Treichville in the southern Abidjan, where MDR prevalence for *E. coli* and *Klebsiella* were

14.4 and 23.1% respectively (Moroh et al., 2014). Multiple Antibiotic Resistance Index values are greater than 0.2 (0.59 for *E. coli* and 0.51 for *Klebsiella* spp.), showing that strains of *Klebsiella* spp. and *E. coli* are originating from an environment where the selection pressure due to excessive prescribing or abusive use of broad-spectrum antibiotics is alarming (Tambekar et al., 2006; Sbiti et al., 2017). This observed multi-resistance phenotype could be linked to an accumulation of several resistance mechanisms encoded in bacterial genome (Ekwealor et al., 2016). Otherwise, the variability of the antibiotic resistance profile between isolates of bacterial species could be due to the fact that patients from our study might have acquired UTI involving strains from diverse origin comprising domestic animals, where the pressure of the drugs was applied differently (Manges et al., 2001; Vincent et al., 2010; Jakobsen et al., 2011). Also, the medication behavior of the population in UTI treatment with under-dosage, non-respect of the treatment plan, use of the remaining drugs, excessive use of antibiotics without medical advice and taking antibiotics for viral infections (Alhomoud et al., 2017) could be some of the reasons behind variability of antibiotic resistance profiles observed.

Conclusion

In this study, the culture-positive rate of uropathogens was high with the majority coming from female patients. *E. coli* and *Klebsiella* spp were the most common Gram negative bacilli isolated. The antibiotic resistance profile of *E. coli* and *Klebsiella* spp. strains is alarming in patients attending Abobo-Avocatier Hospital with high levels of antibiotic resistance. The high value of Multiple Antibiotic Resistance Index and the rate of multi-resistance (MDR) from this site suggest the need for continuous monitoring of antibiotic susceptibility profile of bacteria implicated in UTI prior to antibiotic prescription in order to ensure optimal and desired treatment. Based on variations in antibiotic resistance profiles observed in the bacterial species studied, we plan to study the genetic relationships between bacterial strains and to characterize the different resistance mechanisms involved using molecular biology and bioinformatics tools.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Antimicrobial activity of cotton and silk fabrics dyed *with Datura stramonium* (Jimson weed) plant leaf extracts

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Functional finishes are agents that are applied to fabrics to make them suitable for specific uses and contribute a specific attribute to a fabric without altering its appearance. In this context, the present study reveals the importance of dyed fabrics with *Datura stramonium* plant leaf extracts to control antibiotic-resistant bacteria, which have been a threat to human health and posed noticeable challenges confronted with medical physicians in the treatment of many infectious diseases. More still, the importance of using natural dye is not limited to its antimicrobial and other medicine values but also to its wide range of advantages via elimination of environmental pollution due to the usage of synthetic dyes. 100% plain woven, desized, scoured and bleached cotton fabric samples and 100% degummed and bleached hand knitted silk fabric samples were dyed with aqueous extracts from *D. stramonium* plant leaves in combination with Alum and Iron Sulphate as mordants using post-mordanting method and then tested against two strains of *Staphylococus aureus* (Gram-positive bacteria) and *Pseudomonas aeruginosa* (Gram-negative bacteria) using Agar diffusion method as per SN195920-1992 standard. The fastness properties (wash fastness, light fastness and rubbing fastness) of the dyed fabrics were assessed following guidelines from The American Association of Textile Chemists and Colorists (AATCC) and International Organization for Standardisation (ISO) testing methods. Spectrophotometric experiments were employed to evaluate the colour strength (K/S) of the dyed samples. The antimicrobial activity results showed that for both cotton and silk dyed fabric samples; there was an interruption of the microbial growth beneath the fabric and a clear zone of inhibition around the fabric. Dye ability and fastness results showed that dyed fabric samples with *D. stramonium* extracts using mordants had relatively high colour strength (K/S) values as compared to the control fabrics which were dyed without a mordant and their colorant was more stable to light, rubbing and washing, respectively.

Key words: Antimicrobial activity, silk fabric, cotton fabric, *Datura stramonium* extract, mordants.

INTRODUCTION

With the presence of a huge number of synthetic drugs, natural bioactive agents within the plant kingdom continue to be part of the health care either in developed or developing economies. (Resmi, 2014). Healthy and active lifestyles have led to a rapidly increasing market for a wide range of antimicrobial textiles, which intern,

have stimulated intensive research and development efforts (Sasmita et al., 2013). *Datura stramonium* (Jimson weed) is grown in many areas across the world annually. It belongs to the Solanaceae family and is known to be a medicinal herb. Several findings show that *D. stramonium* plant contains compounds like alkaloids, saponins, steroids, tannins, and glycosides which exhibit antimicrobial properties (Soni et al., 2012; Alapati and Sulthan, 2015; Manikandan and Ananth, 2016). Due to its bitterness and poisonous nature, it is traditionally used in many drugs for the treatment of skin disorders, ear pains, coughs, fever, gastric pains, asthmatic attacks, sprains, muscle pains, cramps contusions, snakebites, piles, convulsions, gums during toothache and applied on dog bite wounds (Alapati and Sulthan, 2015; Aqib Sayyed, 2015).

Recently, various research have shown that indeed textile fibres provide an excellent substrate and conditions for the growth of micro-organisms (Morais and Guedes, 2016; Bhuyan et al., 2016). These micro-organisms can transfer infectious diseases as well as cause discolouration, bad odor, and garment degradation. In a bid to overcome these pathogenic organisms, antimicrobial and self-cleaning properties were imparted onto textile materials (Oh and Na, 2014). Also, as a result of the growing concern for the environment, the use of natural products to impart various functions to textiles has attracted increasing attention. This has been well examined by the use of *Citrus grandis osbeck* for its antimicrobial effect on fabrics (Yi and Yoo, 2010) and use of *Aloe vera* extract and its natural antibacterial finishing for textile applications (Nadiger and Shukla, 2017). Several reports have confirmed that natural dyes can be used not only to provide colour but also reduce microbial growth (Narayanaswamy et al., 2013; Ali, 2015; Rajendran, 2011; Bhuyan et al., 2016). Like these, many natural resources that impart antimicrobial properties to textiles were found and investigated deeply.

The leaf extracts of *D. stramonium* were studied and approved to have excellent antimicrobial activity against micro-organisms based on the availability of large amounts of bio-active compounds such as tannins, flavonoids and alkaloids, which are effective against bacterial and fungal infections (Bhuyan et al., 2016). Also, it has been proved that these antimicrobial agents which have varying composition and concentration in different plant parts are usually accumulated as secondary metabolites in plant cells (Tawiah et al., 2016) and that leaves possess the highest antimicrobial activity levels thus opted for in therapeutic applications (Gutarowska et al., 2013). In respect to that, the use of *D. stramonium* leaf extract as a textile finish on 100% cotton and silk fabrics and its dyeing potential has not been examined and reported in any form.

In the current study, the interaction of extracted natural dyes from the plant species, *D. stramonium* with cotton and silk fabrics using selected mordants and their potential antimicrobial efficacy against *Pseudomonas aeruginosa* and *Staphylococcus aureus* bacterial species which are known to cause cross infections in the hospitals as well as being responsible for causing unpleasant odors in textiles were investigated. A comparative study of the colour strength and fastness properties with respect to Alum and Iron Sulphate used as mordants is also reported.

MATERIALS AND METHODS

Collection of materials

Desized, scoured and bleached plain weave 100% cotton fabric samples were purchased from Southern Range Nyanza Textiles Limited and 100% degummed and bleached mulberry silk hand knitted fabric samples from Uganda National Sericulture Centre, Kawanda, Wakiso district, Uganda.

The leaves of *D. stramonium* plant were collected from nature around Busitema University campus, Busia district, Uganda.

Distilled water, Sodium Sulphate for dye exhaustion, Potassium Aluminium Sulphate (Alum) and Iron Sulphate used as mordants, analytical balance, mortar, pestle and Nutrition agar (MHA) were all obtained from Textile labs, Moi University.

Preparation of the sample

Jimson weed (*D. stramonium)* leaves were washed thoroughly with running tap water followed by distilled water to remove soil particles, shed dried at room temperature for 4-5 days, ground using a mortar and a pestle into powder form and sieved to remove any large residues.

Dye extraction

The obtained powder sample of 150 g was added to 1000 ml of distilled water at the boil for 1 h to extract the dye. The obtained mixture was cooled at room temperature and filtered through Whatman No. 1 filter paper and used for dyeing immediately.

Dyeing of fabric samples

Some of the cotton and silk fabric samples were dyed using post-mordanting method with Alum and Iron Sulphate used as mordants according to Miah et al. (2017) while others were dyed without using a mordant.

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Dyeing of cotton fabric samples without a mordant

With slight modifications, cotton fabrics were dyed according to Mohan (2012) whereby bleached cotton fabric samples (8×10 cm) were dipped in a dye bath (150 cm^3) and after 10 min, 20% on weight of fabric (O.W.F) of sodium sulphate was added. With intermittent stirring, the dyeing was done for an hour at 50°C. Thereafter, dyed samples were squeezed to remove excess dye and then using a non-ionic soap solution (2 g/l) at 50°C for 10 min, the fabric samples were washed, rinsed with running tap water and dried at room temperature.

Dyeing of cotton fabric samples with mordants (Alum and Iron Sulphate)

The same dyeing procedures were followed as mentioned earlier, with the exception that a mordant solution (100 cm^3) of 10% OWF at 60°C was used to soak the dyed samples for 30 min at a material to liquor ratio of 1:20. Then non-ionic soap solution (2 g/l) was used in washing the dyed fabric samples at 50°C for 10 min prior to rinsing with running tap water and finally drying at room temperatures.

Dyeing of silk fabric samples without a mordant

Silk samples dyeing was done according to Janani and Winifred (2013) method with slight modifications. During dyeing, the beakers were covered to minimize the addition of oxygen to the solution. Intermittent stirring was done after every 10 min. Pieces of degummed and bleached silk fabrics measuring (8×10 cm) were soaked in distilled water and thereafter transferred to dye bath liquor (700 cm^3) and the mixture heated gradually to 60°C while stirring for 30 min. Then they were removed from the dye bath and rinsed repeatedly in clean water until no more colour change was observed in the cleaning water. Finally, the dyed samples were dried at room temperature.

Dyeing of silk fabric samples with mordants (Alum and Iron Sulphate)

The same procedures were followed as mentioned earlier. Except that after the samples were dyed, they were removed from the dye bath and immediately soaked in a solution of a mordant. Different fabric samples were soaked separately in mordanting solution which was made to stand for 15 min. The dyed samples were then rinsed repeatedly in clean water until there was no more colour change observed in the cleaning water. The samples were thereafter dried at room temperature.

Colour shade measurements

The dyed fabric samples using selected mordants and others without a mordant were characterised using SF 600 Spectra flash reflectance spectrophotometer to evaluate the colour shades produced during dyeing process.

Colour strength of the dyed cotton and silk fabric samples

The colour strength (K/S) values of cotton and silk dyed fabric samples with and without using mordants were determined using the Kubelka-Munk equation as follows:

$$K/S=(1-R)^2/2R$$

where R is the reflectance value at the maximum absorbance wavelength, S is the coefficient of light scattering and K is the coefficient of light absorption. The SP60 series X-rite type spectrophotometer with wavelengths ranging from 390 to 710 nm were used to measure the dyed samples whereby five measurements were taken for each and average results were recorded.

Fastness properties

The fastness properties of dyed cotton and silk fabric samples with and without the use of mordants to light, washing, and rubbing were evaluated based on the AATCC test method 16-2004, ISO 105 CO_2 test method and AATCC test method 8-2007, respectively (American Association of Textile Chemists and Colorists, 2010).

Antimicrobial activity of the dyed fabric samples against *P. aeruginosa* and *S. aureus* strains

In determining the antimicrobial activity of cotton and silk fabric samples dyed with *D. stramonium* leaf extract, *P. aeruginosa* (Gram-negative) was considered due to its popularity of being used as a test organism and its resistance to common antimicrobial agents and *S. aureus* (Gram-positive) bacterial species was used since it is well known for causing cross infections in the hospitals as well as responsible for unpleasant odors in textiles. The strains were cultured on nutrient agar and incubated at 37°C for 24 h and examined for the zone of inhibition.

Assessment of antimicrobial activity of dyed cotton and silk fabric samples

Using the Agar Diffusion method as per SN 195920-1992, the dyed and undyed fabric samples with *D. stramonium* extracts were qualitatively assessed for antimicrobial activity by placing both samples in intimate contact with AATCC bacteriostasis Mueller Hinton agar which has been previously inoculated with an inoculum of test organisms in duplicate Petri-dishes. They were then incubated at 37°C for 24 h and examined for the interruption of the growth along the streaks of inoculum directly beneath the fabric samples and for a clear zone of inhibition beyond its edge using visible observation and a compound microscope.

RESULTS

Colour shades produced by using different mordants

The application of *D. stramonium* dye extracts onto cotton and silk fabric samples with Alum and Iron Sulphate as mordants under post-mordanting method produced variable colour shades as shown in Table 1. Alum mordant produced yellow-green shades on both cotton and silk dyed fabrics, Iron Sulphate gave dark green shades and control experiment (without a mordant) produced slightly dark green shades.

Effect of different mordants on colour strength of the dyed fabrics

With the help of SP60 series X-rite type spectrophotometer,

Table 1. Colour shades produced on cotton and silk fabric samples post-mordanted dyed.

Mordant	Alum	Iron Sulphate	Without a mordant
Cotton fabric dyed samples			
Silk fabric dyed samples			

Table 2. Colour strength (K/S) values of Cotton and silk fabric samples dyed with *Datura stramonium* leaf extract.

Mordant	Method	Cotton fabric (K/S Values)	Silk fabric (K/S Values)
Control	-	1.2	1.3
A	Post	1.5	2.5
B	Post	1.8	5.3

A-Potassium Aluminium Sulphate (Alum), B-Iron Sulphate, Control-Cotton and silk fabric samples dyed without a mordant, Post-Post mordanting.

reflectance values at maximum wavelengths were obtained thus colour strength (K/S) values of cotton and silk fabric samples dyed with *D. stramonium* extracts computed and recorded as shown in Table 2. Cotton and silk fabrics dyed using Iron Sulphate mordant showed the highest colour strength (K/S) values of 1.8 and 5.3, respectively followed by 1.5 and 2.5, respectively for using Potassium Aluminium Sulphate (Alum) as mordant and finally 1.2 and 1.3, respectively for control fabrics. This was further clearly demonstrated as shown in Figure 1 for both cotton and silk fabrics. Also, it was observed that silk dyed fabrics showed better colour strength values as compared to cotton dyed fabrics when using both mordants as well as without the mordant.

Fastness properties

Tables 3 and 4 show fastness properties of the various samples dyed with and without mordants added to the leaf extracts of *D. stramonium*. The control sample exhibited moderate to good (3 - 4) fastness properties in most aspects for both fabrics though, for the case of silk fabrics, fastness ratings were more satisfactory. The mordanted fabric samples all showed good to excellent (4 - 5) fastness properties to light, washing, and rubbing.

Assessment of antimicrobial activity of dyed cotton and silk fabric samples

The results of the agar diffusion method against the test organisms *P. aeruginosa* and *S. aureus* are as shown in Figures 2 and 3. The zone of bacterial inhibition is indicated by diffused colour around the dyed fabric samples and a weak growth towards them. This was further clarified with the help of Table 5 where dyed fabric samples for cotton (D_2c) and silk (D_2s) showed maximum antimicrobial activity on both *S. aureus* and *P. aeruginosa* tests with a weak microbial growth towards them as

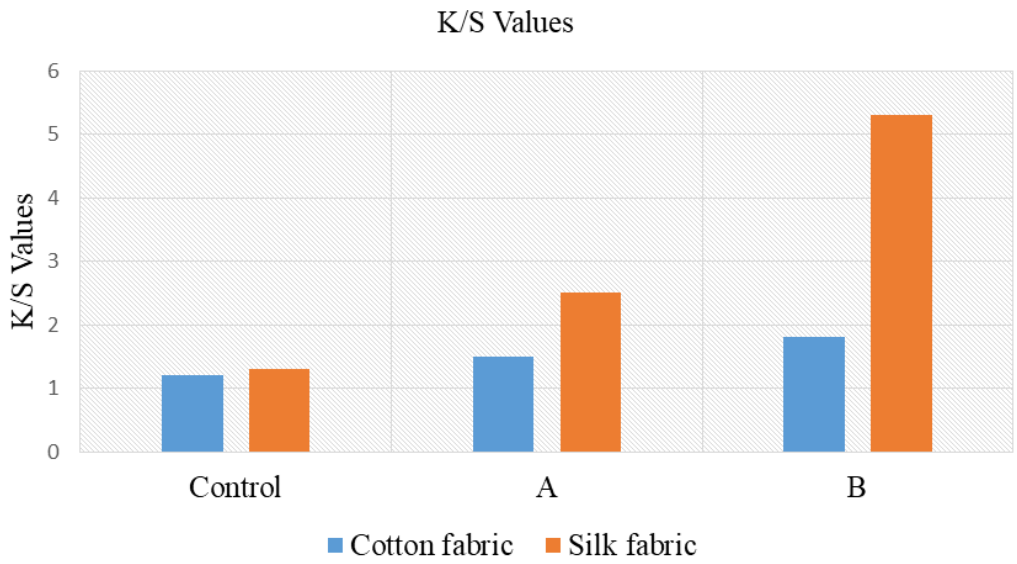

Figure 1. Showing the colour strength (K/S) values of dyed cotton and silk fabric samples.

Table 3. Fastness properties of cotton fabric samples dyed with *Datura stramonium* leaf extract.

Mordant	Method	Light fastness	Wash fastness		Rubbing fastness	
			CC	CS	Dry	Wet
Control		4	3	4	4-5	3-5
A	Post	4-5	4	4-5	5	4-5
B	Post	4-5	4	4-5	4-5	4

A-Potassium Aluminium Sulphate (Alum), B-Iron Sulphate, Post-Post mordanting, Control-Cotton fabric samples dyed without a mordant, CC-Colour change, CS-Colour staining, 3-Moderate, 4-Good and 5-Excellent.

Table 4. Fastness properties of Silk fabric samples dyed with *Datura stramonium* leaf extract.

Mordant	Method	Light fastness	Wash fastness		Rubbing fastness	
			CC	CS	Dry	Wet
Control		4-5	3-5	4	4-5	4
A	Post	5	4-5	5	5	4-5
B	Post	5	4	5	5	4

A-Potassium Aluminium Sulphate (Alum), B-Iron Sulphate, Post-Post mordanting, Control-Silk fabric samples dyed without a mordant, CC-Colour change, CS-Colour staining, 3-Moderate, 4-Good, 5-Excellent.

shown in Table 6. Then dyed fabric samples for cotton (D_1c), (Dc) and Silk (D_1s), (Ds) showed moderate antimicrobial activity on both *S. aureus* and *P. aeruginosa* tests with heavy growth towards silk fabrics and weak growth towards cotton fabrics.

DISCUSSION

Basing on the results presented in this study, dyeing of cotton and silk fabric samples using leaf extracts from *D. stramonium* was done using the post-mordanting method in order to obtain better colour shades. This is in line with findings done by Janani et al. (2014) on mordanting methods. The variations in the colour shades of the dyed fabrics were attributed to the use of different mordants forming characteristic dye-fibre complexes of different shades whereby Potassium Aluminium Sulphate (Alum) produced a yellow-green shade compared to that produced by using Iron Sulphate which was more dark

Figure 2. Agar plate pictures of Cotton fabric samples dyed with *Datura stramonium* against *Staphylococcus aureus* (Staph) and *Pseudomonas aeruginosa* (Pseud) on Mueller Hinton agar. D_1c-Cotton dyed fabric with Alum, D_2c-Cotton dyed fabric with Iron Sulphate, Dc-Cotton dyed fabric without a mordant, and P-Control.

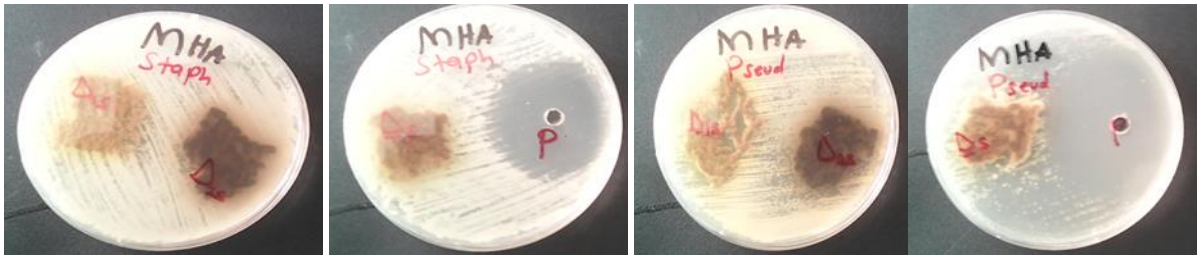

Figure 3. Agar plate pictures of Silk samples dyed with *Datura stramonium* against s *staphylococcus aureus* (Staph) and *Pseudomonas aeruginosa* (Pseud) on Mueller Hinton agar. D_1s-Silk dyed fabric with Alum, D_2s-Silk dyed fabric with Iron sulphate, Ds-Silk dyed fabric without a mordant, and P-Control.

Table 5. Test results of antimicrobial activity of cotton and silk dyed fabric samples.

Organisms	Cotton dyed fabric samples			Silk dyed fabric samples		
	D_1c	D_2c	Dc	D_1s	D_2s	Ds
S. aureus	+	++	+	+	++	+
P. aeruginosa	+	++	+	+	++	+

D_1c-Cotton dyed fabric with Alum, D_2c-Cotton dyed fabric with Iron sulphate and Dc-Cotton dyed fabric without a mordant. D_1s-Silk dyed fabric with Alum, D_2s-Silk dyed fabric with Iron sulphate, Ds-Silk dyed fabric without a mordant. ++ = Maximum antimicrobial activity (interruption of the microbial growth beneath the fabric and a clear zone of inhibition around the fabric); + = Moderate antimicrobial activity (interruption of the microbial growth beneath the fabric but no clear zone of inhibition around the fabric).

Table 6. The microbial growth rate towards the cotton and silk dyed fabric samples.

Organisms	Cotton dyed fabric samples			Silk dyed fabric samples		
	D_1c	D_2c	Dc	D_1s	D_2s	Ds
S. aureus	W	W	W	H	W	H
P. aeruginosa	W	W	W	H	W	H

W-Weak growth rate, H-High growth rate.

green. This was because mordants are known for forming an additional linkage with dye molecules as confirmed by the study done by Narayanaswamy et al. (2013).

The colour strength (K/S) values for dyed fabric samples increased considerably with the use of mordants (Alum and Iron Sulphate) as compared to the control fabric samples (Figure 1) but with Iron Sulphate mordanted fabric samples, their colour strength values

were extremely high emphasizing better-linking properties with the extracted dye. This was attributed by the formation of strong complexes between the mordants and dye molecules as it is in line with findings reported by Wanyama et al. (2014) on characterisation and application of natural dyes.

Based on the colour fastness results presented, the control sample exhibited moderate to good (3 - 4) fastness properties in most aspects for both fabrics though, for the case of silk fabrics, fastness ratings were more improved. The mordanted fabric samples all showed good to excellent (4 - 5) fastness properties to light, washing, and rubbing. This may have been attributed to the presence of tannins in the dye extract (Bhuyan et al., 2016) as well as mordants used which helped the dye to bond well with the fibre matrix. In general, cotton and silk fabrics dyed with the *D. stramonium* extracts using mordants (Alum and Iron Sulphate) gave very good acceptable fastness properties having in mind that natural dyes normally exhibit low substantivity to fibres and fabrics.

In the Agar Diffusion Test results for antimicrobial activity against standard test cultures (Figures 2 and 3), the diffused colour around the dyed fabric samples and a weak growth towards them was an indication of bacterial inhibition. Dyed cotton and silk fabric samples using Iron Sulphate mordant showed maximum antimicrobial activity on both *S. aureus* and *P. aeruginosa* tests as compared to those fabric samples dyed using Alum mordant. This is true since the dye fixation levels vary with different mordants used under suitable conditions thus forming different sizes of inhibition zones (Gupta and Laha, 2007). On the other hand, results showed that there was a high microbial growth rate towards silk fabrics as opposed to the low microbial growth rate towards cotton fabrics. This disparity in the rate of microbial growth is expected to have been caused by the slow release of the active substances from the silk fabric surface to the organisms streaked on the incubated plates containing the culture media.

Conclusions

The study mainly focuses on the antimicrobial activity of cotton and silk fabrics dyed with *D. stramonium* crude plant leaf extracts. The antimicrobial tests demonstrate an exciting opportunity for the naturally dyed fabrics in developing protective clothing against common infections in hospitals and hotels. Two test organisms namely *P. aeruginosa* and *S. aureus* were used in the study and from the results, it can be concluded that both cotton and silk dyed fabrics showed very good inhibitory effects against the two test organisms. A high inhibition zone was recorded with the cotton and silk fabrics dyed with *D. stramonium* leaf extract mordanted with Iron Sulphate in comparison to the fabrics dyed with Alum mordant. The dye ability tests carried out confirmed that the natural herbal extract has a good depth of dyeing towards both fabrics (cotton and silk) but much more with silk fabrics as compared to cotton. The selected mordants used also contributed to significant K/S values that were realized (K/S value = 5.3 for silk mordanted with iron sulphate in comparison with K/S value = 1.8 for cotton fabric mordanted with iron sulphate). It was also noted that the use of both mordants gave results which were significantly higher for silk fabrics than for cotton fabrics. Fastness properties (washing, light, and rubbing) for cotton and silk dyed fabrics ranged from good to excellent. These results clearly show that utilizing extracted natural dyes from *D. stramonium* as dyeing materials significantly facilitate obtaining quality fabrics having both dye ability and antimicrobial properties. However, more research can be done to determine the bioactive principles at various stage of plant growth since their composition varies with the age of the plant and in different species of the same plants.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Screening of fungal contaminants in banana tissue cultures in Jkuat, Kenya

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Tissue culture is prone to high costs of production arising from losses incurred from fungal contamination. The aim of the study was to characterise fungal contaminants and elucidate the exhibited mode of resistance to most preferred sterilants. Twenty nine fungal samples were collected at the different stages of tissue culture growth, using purposive sampling technique. Morphology results were confirmed by molecular characterization using fungal 18S rRNA sequences. Biochemical and antibiosis tests, identification of genes for capsulation and ATP binding Cassete (ABC) transporters, were performed to show the relationship between the fungi and sterilants resistance. Amylases and proteases were highly expressed by all isolates while xylanases and lipases were moderately expressed and esterases were lowly expressed. Only fourteen isolates had antagonistic activity for *Candida albicans* while nine of them had antagonistic activity for *Pseudomonas aeruginosa.* Three isolates were both antagonistic for *Staphylococcus aureus* and for *Escherichia coli*. *Cunninghamella bainieri* (10R) recorded a unique antibiosis and extra cellular enzymatic activity ($p<0.05$). All the isolates were positive for mdr1 gene and three isolates had CAP64 capsule genes. *Aspergillus* sp., *Penincillium* sp., *Cladosporium* sp., *Cunninghamella* sp. and *Fusarium* sp. were identified to be the major fungal contaminants of tissue culture banana cultures in JKUAT laboratories.

Key words: Tissue culture, 18S rRNA, fungal contaminants, banana cultures.

INTRODUCTION

Bananas (*Musa* spp.) are ranked fourth as the world's most valuable crop after rice, wheat and are staple food in Uganda, Rwanda, Burundi and parts of Kenya and Tanzania (Rubaihayo, 2003; AHBFI, 2013). They are rich source of energy, with a banana fruit weighing 100 g containing approximately 350 Kilo Joules of energy (Hanumantharaya et al., 2009). Apart from containing carbohydrates, the banana fruit is rich in phosphorous, calcium, iron, potassium and magnesium and traces of zinc, copper, chlorine, cobalt and iodine (Rubaihayo, 2003). Most of the vitamins especially vitamin A, vitamin C and riboflavin are present in fair quantities (Rubaihayo,

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2003). Thus from the nutritive point of view, bananas can be considered as fruits that contains many nutrients and are highly nourishing (Mbaka et al., 2008).

Despite it being a valuable crop, its production worldwide has been decreasing. In Latin America, Caribbean and Asia the decline has been attributed to an increase in banana pests and diseases, climate change, rising cost of fertilizers among others (FAO, 2006; Dita et al., 2013). In Africa, its production has declined due to an increase in pests and diseases, reduced soil fertility and an increase of moisture stress (Nyombi, 2013).

The use of tissue culture (TC) to produce clean and disease free planting material has been adopted to address some of these problems. Tissue culture has been used to clean diseased planting materials obtained from infected soils and farms (Kahangi et al., 2004). Despite the technology being very profitable, it is prone to high costs arising from losses incurred due to bacterial and/or fungal contamination (Msogoya et al., 2012). Bacterial contamination is easily controlled by the available sterilants. However the available sterilants have proven to be ineffective in controlling fungal contamination. This might be because fungi have ABC transporters for active efflux of toxic substances from the fungal system (Del Sorbo et al., 2000).

Fungi have also been reported to form capsules around their spores that help them avoid stressful conditions including hostile host immunological reactions (Zaragoza et al., 2009). Fungi may also produce extra cellular enzymes or exhibit antibiosis for self-defence (Compant et al., 2012). *Fusarium* sp. for instance, produce lipases that enable the fungi to infect the host (Feng et al., 2005), while the *Aspergillus* species like *A. flavus* produce amylases, proteases, lipases that enable the fungi to attack and cause disease to the host (Amaike and Keller, 2011). Extracellular enzymes like lignases, esterases, glutathione 5-transferases, cytochrome are also involved in degradation of fungicides (Hernndez et al., 2013), providing another reason why fungi are resistance to most preferred sterilants. Fungi have enormous potential to produce metabolites (Zhao et al., 2014). Most microbes produce bioactive metabolites that act on the host by either depriving it of nutrients, space, lysing cells or blocking specific functions that are related to the hosts growth and metabolism, including shutting down hosts defence mechanisms and thus allowing pathogen to attack without any resistance (Habiba et al., 2002; Zhao et al., 2014).

In many TC laboratories, a large number of explants, estimated at between 40 to 60% are destroyed in the cultures due to both exogenous and endogenous microbial contaminants (Msogoya et al., 2012; Helaly et al., 2014). Aseptic techniques employed in tissue culture laboratories include surface sterilization using 0.3% Redomil® (fungicide) plus Tween® 20, 70% ethanol and 7% bleach, sterilizing the media and culture bottles, flaming the inoculating tools used until red hot among others (Odutayo et al., 2007). Surface sterilization eliminates the exogenous microbial contaminants only while endogenous ones emerge on nutrient-rich culture medium (Mng'omba et al., 2007). An application of systemic fungicides before collection of explants can eliminate the endophytic contaminants (Habiba et al., 2002). Despite following these aseptic procedures, microbial contamination still remains a major problem affecting banana *In vitro* propagation in tissue culture laboratories.

Limited research has been conducted on fungal contaminants in TC laboratories. The objective of this study was to identify the fungal contaminants in TC laboratories and whether produced extracellular enzymes, exhibit antibiosis, have ABC transporters or capsule genes for self-defence.

MATERIALS AND METHODS

Study site

Samples of contaminated banana cultures were obtained from banana tissue culture laboratories in the Jomo Kenyatta University of Agriculture and Technology (JKUAT), Nairobi, Kenya.

Sample collection and isolation

Purposive sampling technique (Bernard, 2002) was used to collect twenty-nine samples of contaminated cultures at three developmental stages of tissue culture namely initiation, multiplication and rooting stages in 1st of October to 30th of November in 2017. Collected fungal cultures were assigned code names for easy traceability of their origin. To achieve this, samples from initiation, multiplication and rooting stage were labelled I, M and R respectively. The fungal contaminants were grown on sterile Potato Dextrose Agar (PDA) media. The medium was supplemented with 25 mg/l Streptomycin to prevent bacterial contamination (Neondo, 2017). Each of the fungal contaminant was cultured on this medium and incubated for seven days at 30°C. Pure fungal isolates were obtained after repeated subcultures in fresh PDA media (Msogoya et al., 2012) which were then used for subsequent studies.

Morphological characterization

Colony and cell morphology

The isolates were grown on fresh PDA media, descriptors such as colony colour, margin and elevation was observed and recorded. For cell morphology, wet mount slides of the isolates were prepared and covered carefully using a cover slip to avoid air bubbles. They were stained with lactophenol cotton blue and observed under Olympus BX43 light microscope at ×100 for identification of the appearance of conidiophores and conidia (Solano, 2011). The Saccardian System was then described and used to presumptively classify the fungal isolates morphologically (Barnett, 1962; Barnett and Hunter, 1998).

Biochemical characterization

Based on morphological characterization some isolates resembled

one another, thus in subsequent experiments these isolates were treated as one sample and the sample size was narrowed to 18.

Determination of amylolytic activity

Determination of amyolytic activity was done using the amylases test as described by Hankin and Anagnostakis (1975). The isolates were inoculated in nutrient agar containing 0.2% of soluble starch in grams per litre, pH 8.0 and incubated for seven days at 30°C. The plates were divided into four quadrants for replication purposes. The cultures were treated with 1 % Lugol's iodine. A positive results was recorded when brown iodine colour turned blue black while in negative isolates the brown colour of iodine remained (Castro et al*.,* 1993).

Determination of the esterase activity

Esterase activity was determined using media described by Sierra (1957), which was composed of peptone 10.0 g/l, NaCl 5.0 g/l, $CaCl_2$ $2H_2O$ 0.1 g/l, agar 18.0 g/l, pH 8.0. Sterilized Tween 80 in a final concentration of 1 % (v/v) was added to sterilized culture media. The plates were divided into four quadrants and incubated for seven days at 30°C. Positive results showed presence of a precipitation of calcium crystals around the colonies and no crystals for negative results (Solano, 2011).

Determination of the lipolytic activity

Lipolytic activity was determined using methodology described by Sierra (1957) with the exception that sterilized Tween 80 was replaced with Tween 20. The plates were marked into four quadrants and incubated for seven days at 30°C. Precipitation of calcium salts around the colonies was observed as an indication of the presence of lipase production while colonies without crystals for negative results (Solano, 2011).

Determination of the proteolytic activity

Proteolytic activity was determined using the protocol described by Zilda et al. (2012). The isolates were cultured on media comprising Nutrient Broth 8.0 g/l, glucose 1.0 g/l, agar 18.0 g/l, pH 8.0. Separately autoclaved 15 ml of skimmed milk was added to the autoclaved culture media. The plates were divided into four quadrants and incubated at 30°C for seven days. After the incubation period, 2.0 ml of HCl 0.1 Molar (M) was added to the plates. Positive isolates for protease production had clear halos around the colonies while the negative isolates did not have clear halos (Neondo, 2017).

Determination of the cellulolytic activity

Cellulolytic activity was determined using media that comprised of 7.0 g KH_2PO_4, 2.0 g K_2HPO_4, 0.1 g $MgSO_4.7H_20$, 1.0 g $(NH_4)_2SO_4$, 0.6 g yeast extract, 10 g microcrystalline cellulose and 15 g agar per litre (Stamford et al., 1998). The plates were marked into four quadrants and incubated at 30°C for seven days. The plates were then incubated at 50°C for an overnight after the incubation period. Clear zones around the colonies indicated presence of cellulase production while negative results did not have clear halos (Solano, 2011).

Determination of the xylanolytic activity

The fungal isolates were grown on Czapek's agar medium containing xylan as the sole carbon source to determine their ability to produce xylanase (Ruijassenaars and Hartmans, 2001). The medium contained Birch wood xylan 5.0 g/l, Peptone 5.0 g/l, Yeast extract 5.0 g/l, K_2HPO_4 1.0 g/l, $MgSO_4.7H_2O$ 0.2 g/l and agar 20 g/l. The plates were divided into four quadrants, inoculated with the fungal isolates and then incubated for seven days at 30°C. The plates were then flooded with 0.1% (w/v) Congo red dye. For positive results, there was colour change from red to orange but for the negative results the red colour remained (Solano, 2011).

Antibiosis test

The test organisms used were *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25922), *Escherichia coli* (ATCC 25923) and fungi *Candida albicans* (ATCC 90028). They were obtained from Kenya Medical Research Institute-Centre for Microbiology research. Fungal spore paste was prepared by mixing 10^6-spores per ml suspension with one drop of triton X-100 and used to saturate fungal inoculant discs (Sterile What man No.1 filter paper discs-1 cm diameter) following Kirby-Bauer disc diffusion method (Neondo, 2017). The discs were placed on the surface of the Mueller Hinton agar plates which had been freshly swabbed with 100 µl of each test organisms. The plates were sealed with parafilm and incubated at 30°C for a period of four days. Zones of inhibition around the colonies were measured and recorded (Neondo, 2017).

Molecular characterization

DNA extraction

The 18 fungal isolates were studied further using molecular tools. Fungal DNA was done using protocal reported by Gontia et al. (2014). Fresh fungus was put in sterile 1.5 ml eppendorf tubes that contained 0.5 g sterile sieved sand. The cultures were crushed using sterile micro pestles. A total of 600 µl of extraction buffer (0.1 M Tris HCL pH 8, 10 mM EDTA pH 8, 2.5M NaCl, and 3.5 % CTAB, and 150 µL of 20 mg/mL of proteinase k) was added. Incubation of the cells was at 65°C for one hour in a water bath with shaking after every 20 min. The samples were centrifuged for 10 minutes at 10,000 Xg. The supernatant was transferred to sterile 1.5 ml eppendorf tubes and equal volume of phenol chloroform-isoamylalcohol (25:24:1) was added to remove the proteins. The samples were centrifuged again at 10,000 Xg for 10 min. The supernatant was transferred to clean 1.5 ml eppendorf tubes.

To the supernatant, equal volume of chloroform isoamyl was added to wash the fatty tissues and phenol by gentle shaking. The sample was centrifuged at 8944 Xg[1] (times gravity) for 10 min. The supernatant was transferred in 1.5 ml eppendorf tubes and equal volume of isoamyl was added to completely remove fatty tissues and phenol from the DNA. The aqueous phase was transferred to a new 1.5 ml eppendorf tube and DNA precipitated with equal volume of absolute ethanol. Precipitation was done for overnight at -20°C. The precipitates were centrifuged for 15 min at 15115 Xg and the supernatant discarded. The pellet that contained the genomic DNA was rinsed with 70% ethanol twice to remove traces of salts. The DNA was dried in an incubator at 40°C for thirty minutes to completely remove ethanol and then dissolved in 100 µl TE buffer pH8. The genomic DNA was confirmed by gel electrophoresis using 0.8% agarose in 1x TAE buffer stained with 0.5 mg/L ethidium bromide. Three microliter of DNA and 1 µl loading dye was mixed and put on the agarose gel. The gel was run at 80 V for one hour

[1] Times gravity calculated using $g = (1.118 \times 10^{-5})\ R\ S^2$ Where g is the relative centrifugal force, R is the radius of the rotor in centimeters, and S is the speed of the centrifuge in revolutions per minute

and DNA visualized under ultraviolet light. The DNA was then stored at -20°C for subsequent us.

Amplification of 18S rRNA gene

In order to identify 18S rRNA gene in isolates, polymer chain reaction was done using forward primer (566F) 5'-CAGCAGCCGCGGTAATTCC-3'and reverse primer (1200R) 5'CCGTGTTGAGTCAAATTAAGC-3' (Nayarisseri et al., 2013; Hadziavdic et al., 2014). Amplification was performed using Peqlab primus 96 PCR machine and the expected fragment size was 650 bp. Amplification was carried out in a 40 µl mixture containing 23.2 µl PCR water, 8 µl reaction buffer, 2 µl forward primer, 2 µl reverse primer, 0.4 µl BIOLINE Taq polymerase, 0.4 µl bovine serum and 2 µl template DNA. The DNA template was not added to the negative control (Solano, 2011). PCR conditions for 18s rRNA were; Initial denaturation (95°C for 5 min), 40 cycles of each denaturation (95°C for 30 s), annealing (58°C for 45 s), extension (72°C for 40 s) and final extension step (72°C for 5 min). After successful amplification, they were stored at 4°C (White et al., 1990). The PCR amplicons were confirmed using a 2% agarose gel. The unpurified PCR product was divided into two portions and 20 µl was put in 1.5 ml eppendorf tubes and sealed using parafilm. The tubes were placed in a box with interior holders and cotton wool was used to prevent the tubes from moving around inside the box ready for shipping to Macrogen in South Korea for purification and Sanger dideoxy sequencing. The other portion of PCR product was stored at -20°C for subsequent use.

Phylogenetic data analysis

After sequencing, the sequences were visually checked and edited using ChromasPro 2.6.6 software (Technelysium, 2018) to obtain consensus sequences. In this analysis, because of the inclusion of divergent sequences and the differences in length among the aligned DNA sequences, a large number of gaps (that is, insertions and deletions) were introduced in the aligned dataset. As a result, the gaps were treated as missing data. The consensus sequences were then compared with other DNA sequences in GenBank using basic local alignment search tool (BLAST) in National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Identification of the isolates was based on the highest similarity of the BLAST search (Shayne et al., 2003). The 18s rDNA gene sequences with the closest neighbour strains were aligned using MUSCLE software (Edgar, 2004). Phylogenetic analysis was performed using neighbor-joining method and maximum composite likelihood (MCL) methods (Tamura et al., 2004) of MEGA version 7.0 software, with 1,000 bootstrap replicates

Screening for ABC (ATP binding cassete) transporter and capsules genes

Amplification of ABC transporter was done using ABC1, mdr1 and CDR2 genes. Capsules genes were screened using CAP64 and CAP10 genes. They were designed using Primer3 Plus software version 0.4.0 (Untergasser et al., 2007). The primer sizes were set between 18 and 22b with optimum sizes of 20b. The melting temperatures of primers were set between 50 and 60°C with optimum temperatures of 55°C. The PCR was performed on a GeneAmp PCR system 9600 (Applied Biosystems). Amplification was carried out using 1 µl reverse primer, 0.2 µl BIOLINE Taq polymerase, 0.2 µl bovine serum and 1 µl template DNA. A total of 40 cycles of amplification was performed with template DNA and denaturation at 95°C for 5 min; primer annealing was between 44 and 54°C for 45 s and primer extension at 72°C for 40 s. The PCR amplicons were visualized through gel electrophoresis on a 2% agarose gel with 0.5 mg/L ethidium bromide added directly. The negative control did not have DNA template.

Statistical analysis

Data on antibiosis and extra cellular enzymatic experiments was noted and recorded. MINITAB software version 9.1 (Groebner et al., 2008) was used to perform analysis of variance (ANOVA) for all the measured data. Tukey's honest significant difference (HSD) test (Montgomery and Douglas, 2013) was used to compare and separate the means of diameter of zones of inhibition and clearance (presented in the form of alphabet letters in the tables). Correlation profiles of zones of inhibition, antibiosis with respect to selected isolates were visualized as heat maps generated by a hierarchical clustering R script using R version 3.3.1 software (Fox and Andersen, 2005). Genetic affiliation of the screened isolates was deduced from phylogenetic tree generated using Mega 7 (Kumar et al., 2016).

RESULTS

Colony and cell morphology

Most of the isolates top colour was grey with few having reddish white colour. The bottom colour of most of the isolates were cream with few been orange and white. Most of the isolates had entire margins with few having curled margin. The elevation differed with most of the isolates having risen and few having convex one (Table 1). On cell morphology, most of them had aseptate (Plate 1f) and septate hyphae (Plate 1c) and few of them had intertwined mycelia (Plate 1m). A dendrogram was drawn based on the colony and cell morphology characters giving 5 clusters. It was drawn using Ward D method and the distance between characters measured using Euclidean metric. This clustering shows that contaminants at given stage of culture are similar in that cluster 2 is comprised of contaminants from multiplication, cluster 3 rooting and one initiation (Figure 1).

Biochemical characterization

The ability of fungal isolates to produce extracellular enzymes was studied. The isolates were able to produce different enzymes, with amylases and proteases highly produced. The zones of clearance values were considered as indicators of enzyme activities in the isolates, which may play role in sterilants degradation. Amylases and protease were produced by all fungal isolates with 10R recording the highest (6.100±0.1915) and 11 lowest (3.650±0.0957) amylase activity (Figure 2 and Plate 2e). Isolate 10R recorded highest (7.550±0.0957) and 14 lowest (2.275±0.1797) protease activity (Table 2 and Plate 2a). Most of the isolates were able to produce lipases and xylanases. For lipolytic test, isolates 10R and I1 recorded high and low zones of

Table 1. Morphological characteristics of fungal isolates from JKUAT Banana Tissue Culture laboratory.

Isolate	Colour (top)	Colour (bottom)	Margin	Elevation
I1	Brown	Cream	Entire	Raised
I2	Grey, white	Cream	Entire	Flat
I3	Grey, white	Cream	Entire	Raised
I4	Grey	Cream	Entire	Convex
I5	Greenish/yellow	Cream	Undulated	Raised
I6	grey, white	Cream	Entire	Flat
I7	greenish/yellow	Cream	Undulated	Raised
I8	white(hairy)	Orange/white	Undulated	Raised
I9	grey/white	Cream	Undulated	Raised
M1	Greenish	Cream	Entire	Raised
M2	grey /white	Cream	Entire	Raised
M3	brown/white	Cream	Entire	Raised
M4	yellow/white	Cream	Curled	Raised
M5	green/yellow	Cream	Undulated	Raised
M6	grey/white	Cream	Undulated	Raised
M7	white/yellow	Cream	Undulated	Flat
M8	white/yellow	Cream	Undulated	Flat
M9	white/yellow	Cream	Entire	Raised
M10	white/yellow	Cream	Entire	Raised
R1	Grey	Yellowish	Entire	Convex
R2	white/yellow	Cream	Entire	Raised
R3	White	Cream	Entire	Raised
R4	reddish/white	Cream	Entire	Raised
R5	white/yellow	Cream	Entire	Raised
R6	white/yellow	Cream	Curled	Raised
R7	white/yellow	Cream	Curled	Raised
R8	grey/white	Yellow	Undulated	Raised
R9	white/yellow	Cream	Entire	Raised
R10	white(hairy)	Orange/white	Undulated	Raised

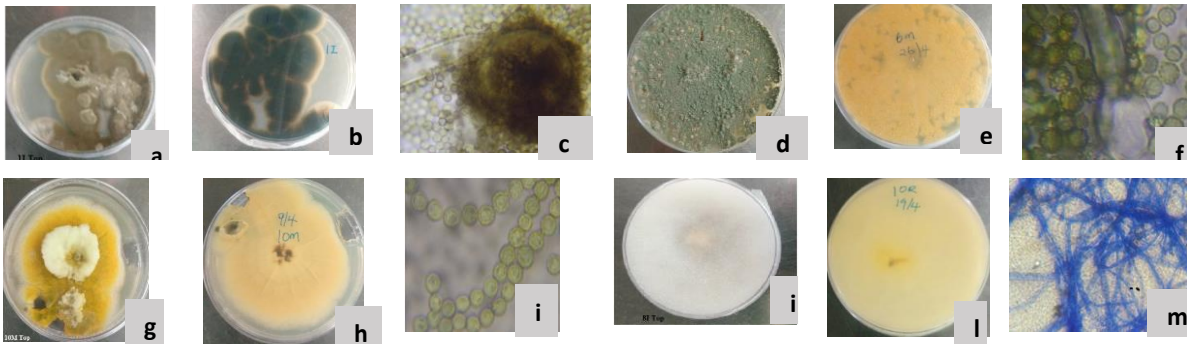

Plate 1. (a) Brown with cream margin; **(b)** Black with cream margin; **(c)** Brush like hyphae terminal with septate hyphae; **(d)** Grey granular; **(e)** Cream; **(f)** Aseptate hyphae; **(g)** Yellow white with white margin; **(h)** Cream bottom and margin; **(i)** Spores arranged in chain; **(k)** Hairy cotton; (**i**) Cream white; **(m)** Long intertwined branched mycelia.

clearance with average diameters of 5.050 ± 0.2223 and 4.850 ± 0.2217 mm, respectively (Table 2, Figure 2 and Plate 2d). Analysis of xylanase enzymatic test showed that isolates 10R and I4 recorded highest and least with a diameter of 5.95 ± 0.05 and 3.250 ± 1.2500 mm, respectively (Table 2, Figure 2 and Plate 1c). For

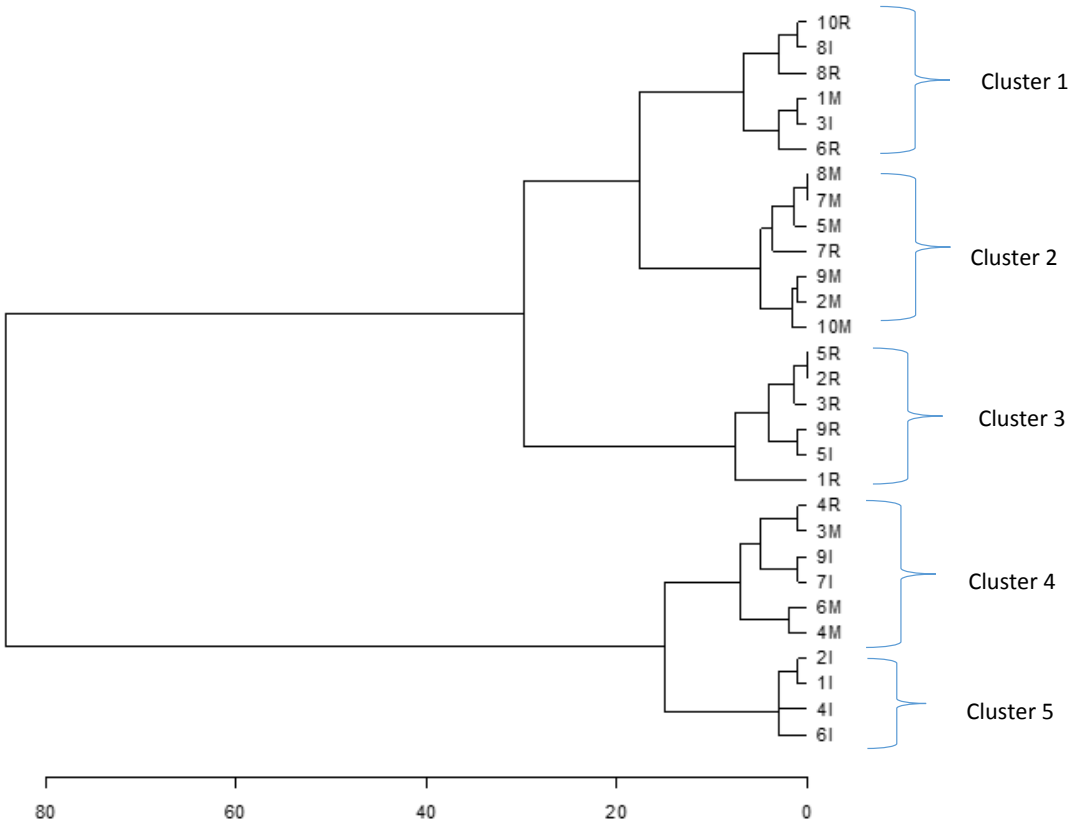

Figure 1. A dendrogram showing how the fungal isolates clustered morphologically.

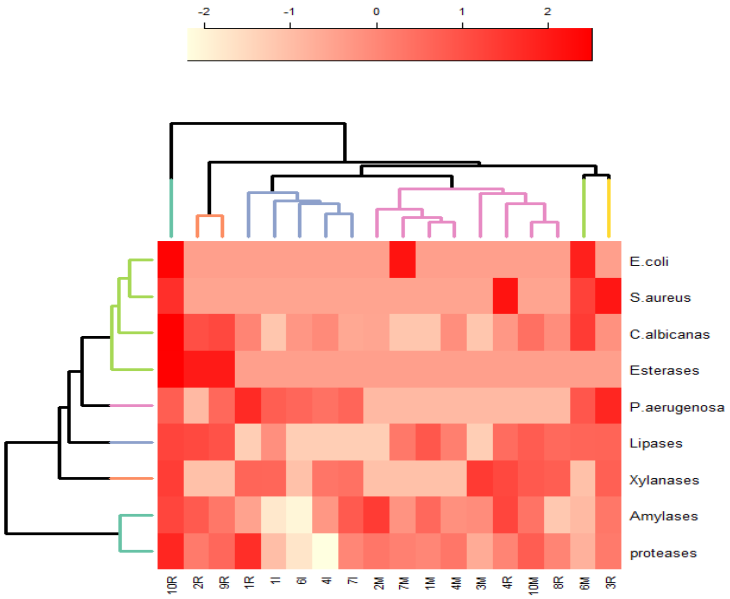

Figure 2. Hierarchical cluster gram of assayed fungal isolates. The heat map (based on Manhattan metric) shows relationship between fungal isolates and the measured morphometric descriptors. The coloured scale bar indicates the quantified strength of the assayed morphometric descriptor. Red and light red colours in the heat map indicate the highest and the least recorded significant mean values respectively at P≤0.05 for the assayed treatments.

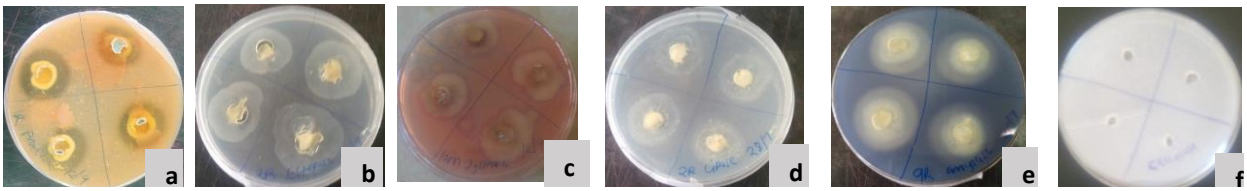

Plate 2. (a) R1 Positive results for protease **(b)** Positive results for esterase activity(precipitates of calcium salts around the colony) **(c)** Isolate M10 showing positive results for xylan utilization **(d)** Positive results for lipase tests (precipitates of calcium salts around the colony) **(e)** Positive results for amylase **(f)** Negative results of cellulose tests

Table 2. Mean diameters (mm) of inhibition zones of fungal growth by enzymes.

Isolate	Esterases (diameter in mm)	Proteases	Lipases	Amylases	Cellulases	Xylanases
11	-	3.850±0.0957^{ef}	3.850±0.4500^{bc}	5.650±0.1708^{abc}	-	4.300±0.1^{bcd}
I4	-	7.550±0.0957^{a}	-	4.800±0.1414^{cde}	-	5.700±0.1291^{a}
I6	-	5.600±0.0816^{bcd}	-	4.30±0.1915^{defg}	-	-
I7	-	5.200±0.2^{d}	-	3.650±0.0957^{fg}	-	3.450±0.4193^{de}
M1		5.200±0.2^{gh}	4.350±0.1258^{ab}	5.450±0.3202^{abc}	-	-
M2	-	2.900±0.1915^{h}	-	6.100±0.1915^{a}	-	-
M3		7.350±0.0957^{a}	-	4.950±0.1893^{bcde}	-	5.850±0.0957^{a}
M4	-	5.600±0.5354^{bcd}	2.3±0.2041^{de}	4.9±0.3^{bcde}	-	-
M6	-	4.150±0.6702^{e}	2.9±0.3873^{de}	3.450±1.1701^{g}	-	-
M7	-	5.350±0.15^{cd}	3.150±1.05^{cd}	4.850±0.5188^{bcde}	-	-
M10	-	6.2±0.0816^{b}	4.15±0.2250^{ab}	5.300±0.1915^{abcd}	-	4.5±0.3317^{bc}
R1	-	4.3±0.2646^{e}	-	4.650±0.3775^{cdef}	-	4±0.1633^{cde}
R2	5.35±0.2363^{a}	5.5±0.1291^{cd}	4.850±0.2217^{a}	5.650±0.3304^{abc}	-	-
R3	-	5.500±0.0577^{cd}	3.900+0.1291^{bc}	5.250+0.5560^{abcd}	-	4.2±0.1896^{cde}
R4	-	5.250±0.1258^{d}	3.600±0.0645^{e}	5.950±0.05^{ab}	-	5.250±0.3862^{ab}
R8	-	5.250±0.15^{fg}	3.700±0.2287^{de}	4.1±0.4796^{efg}	-	3.950±0.0500^{cde}
R9	-	5.950±0.1258^{bc}	4.550+0.2217^{ab}	5.250±0.35^{bcd}	-	-
R10	4.5±0.1732^{c}	2.275±0.1797^{h}	5.050+0.2223^{de}	5.95±0.05^{ab}	-	3.250±1.2500^{e}

esterase production, only isolates R2 and R10 were positive (Table 2, Figure 2 and Plate 2b). All isolates did not produce cellulose enzymes.

Analysis of extra cellular enzymatic activity for individual fungal isolate revealed that, isolate 10R recorded high extra cellular activity for esterases, protease, lipase amylase and xylanases tests of average clearance diameter of 5.35±0.2363, 7.550 ± 0.0957, 5.050 ± 0.2223, 5.95 ± 0.05 and 5.700 ± 0.1291 mm, respectively, p value = 0.000 (Table 2 and Figure 2).

Antibiosis

The effect of fungal isolates on different test organisms was studied. When the isolates were cultured in media containing *C. albicans,* antagonistic activity was observed in 14 isolates namely I4, I6, I7, M2, M4, M6, M10, R1, R2, R3, R4, R8, R9 and R10, with R1 recording the highest (3.0±0.2356 mm) and I1 lowest (0.5±0.8923 mm) diameters of zones of clearance (Table 3, Figure 2 and Plate 3d). The values of zones of clearance shows how efficient the fungal isolates are, in producing metabolites that may play role in sterilants resistance. About 11 isolates had antagonistic activity against *P. aeruginosa* that is I1, I4, I6, I7, M3, M6, M7, R1, R3, R9 and R10 with R3 recording the highest (5.800±0.4773) and I1 lowest (2.250±0.0500) antagonistic activity (Table 3, Figure 2 and Plate 3a). Few isolates had antagonistic activity against *S. aureus* and *E. coli.* Isolate R4 and M6 recorded highest and lowest antibiosis property of *S. aureus* of 3.700±0.1915 and 2.500±0.1315, respectively (Table 3, Figure 2 and Plate 3b). Isolate R10 and M6 recorded highest and lowest antagonistic activity against *E. coli* of 2.660±0.1314 and 2.175±0.0479, respectively (Table 3, Figure 2 and Plate 3c).

Correlation profile between morphometric descriptors (antibiosis and enzymatic activity estimates) and fungal isolates revealed that there are five functional clades (Figure 2). Isolate 10R formed solitary clade and the

Table 3. Antibiotic activity (mean of zone of inhibition) of the selected fungal isolates against four test organisms.

Isolate	*P. aeruginosa*	*S. aureus*	*E. coli*	*C. albicanas*
I1	3.600±0.2160^{ab}	-	-	-
I4	2.850±0.2363^{bc}	-	-	1.900±0.3456^{c}
I6	3.250±0.1258^{bc}	-	-	2.100±0.500^{b}
I7	3.300±0.3873^{abc}	-	-	1.300±0.4255^{e}
M1	-	-	-	-
M2	-	-	-	1.000±0.1256^{f}
M3	-			
M4	-	-	-	0.900±0.5678^{g}
M6	5.800±0.4773^{a}	2.500±0.1315^{c}	2.175±0.0479^{b}	3.000±0.2356^{a}
M7	-	-	2.400±0.1414^{a}	-
M10	-	-	-	0.460±0.7656^{i}
R1	5.600±0.1291^{ab}	-	-	0.500±0.8975^{i}
R2	-	-	-	1.800+0.9823^{d}
R3	3.870±0.1000^{ab}	3.600±0.0000^{a}	-	0.800±1.0500^{gh}
R4	-	3.700±0.1915^{a}	-	0.700±0.9367^{h}
R8	-	-	-	0.860±0.8923^{g}
R9	3.200±0.1414^{bc}	-	-	0.840±0.7345^{g}
R10	2.250±0.0500^{c}	2.960±0.2215^{ab}	2.660±0.1314^{a}	0.720±0.6500^{h}

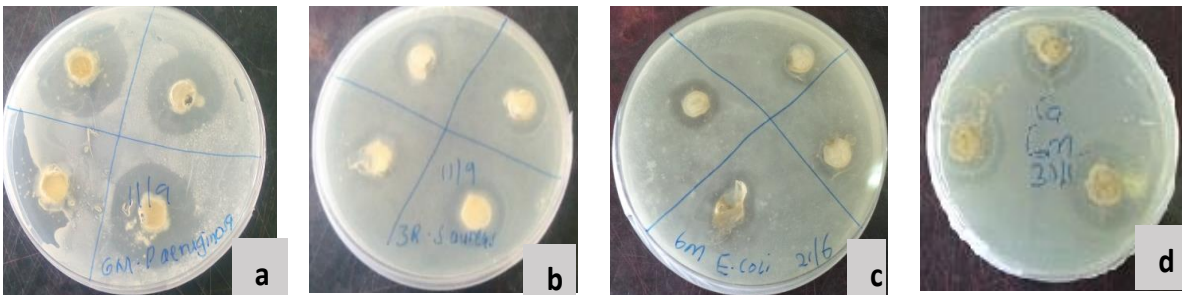

Plate 3. (a) Antagonistic activity of M6 on *P. aeruginosa*. **(b)** Antagonistic activity of R3 on *S. aureus* **(c)** Antagonistic activity of M6 on *E. coli* **(d)** Antagonistic activity of M6 on C. *albicans.*

remaining isolates formed four clades as shown in Figure 2. Isolate 10R recorded a unique antibiosis and extra cellular enzymatic correlation profile (the only member in this functional clade) as shown by colours dominating its column. On the contrary, isolates from initiation stage recorded relatively low expression values for most of the assayed tests among all isolates evaluated (Figure 2).

Phylogenetic analysis of the fungal isolates

After amplification, all the fungal isolates had 18S rRNA gene (Figure 3). After which phylogenetic analysis generated neighbour joining phylogenetic tree using 18s rRNA gene sequences with the closest neighbour strains which shows the phylogenetic relationships among the various fungal isolates (Figure 4).

Screening for ABC transporter genes

Only one set of the degenerate primer was able to amplify the ABC transporter gene producing a fragment of size 172 bps. All the isolates had this gene (Figure 5).

Screening for capsule genes

CAP64 was able to amplify only three isolates with an amplicon size of 260 bp, while CAP10 did not amplify any isolate. These isolates are *Penincillium citrinum* (R1), *Aspergillus fumigatus* (R2) and *Pencillium* sp. (R8) (Figure 6).

DISCUSSION

Based on morphological characterization, some isolates

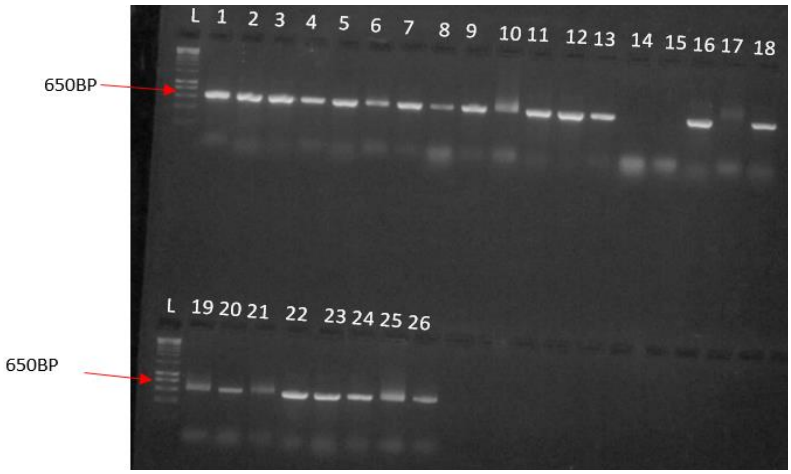

Figure 3. PCR products for the amplification of 18S rDNA for the fungal isolates using 1200R and 566F universal primers. The expected band size amplified is 650 bps. 2% agarose was used. L-Molecular marker (company name and catalogue number (1 kb plus ladder ThermoFisher scientific, Catalog number:10787018) 1-1I, 2-4I, 3-6I 4-7I, 5- MI, 6- M2, 7-M3, 8-M4, 9-M6, 10-M7, 11-M10, 12-R1, 13-R3, 14-Control, 15- Control, 16-R3, 17-R4, 18-R4, 19-R8, 20-R9, 21-R10, 22-I8, 23-M5, 24-I5, 25-I9, 26-R6, 27-R2.

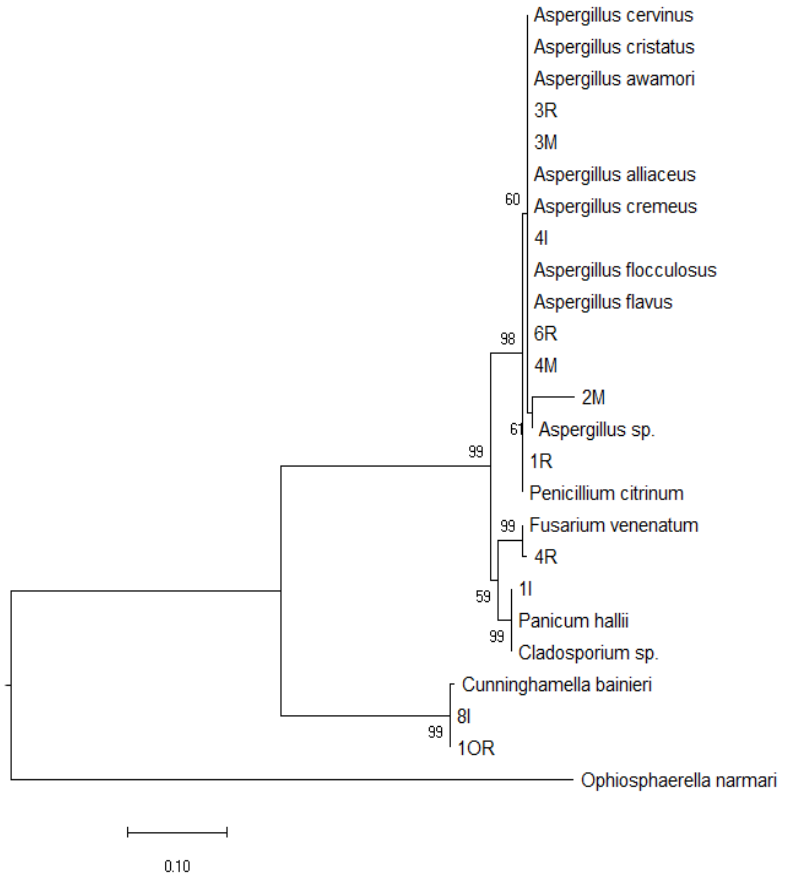

Figure 4. Neighbor joining phylogenetic tree displaying relationship between18s rRNA gene sequences and the closest neighbour strains. The scale bar refers to 0.1 substitutions per nucleotide position. Only boostrap values above 50 are shown.

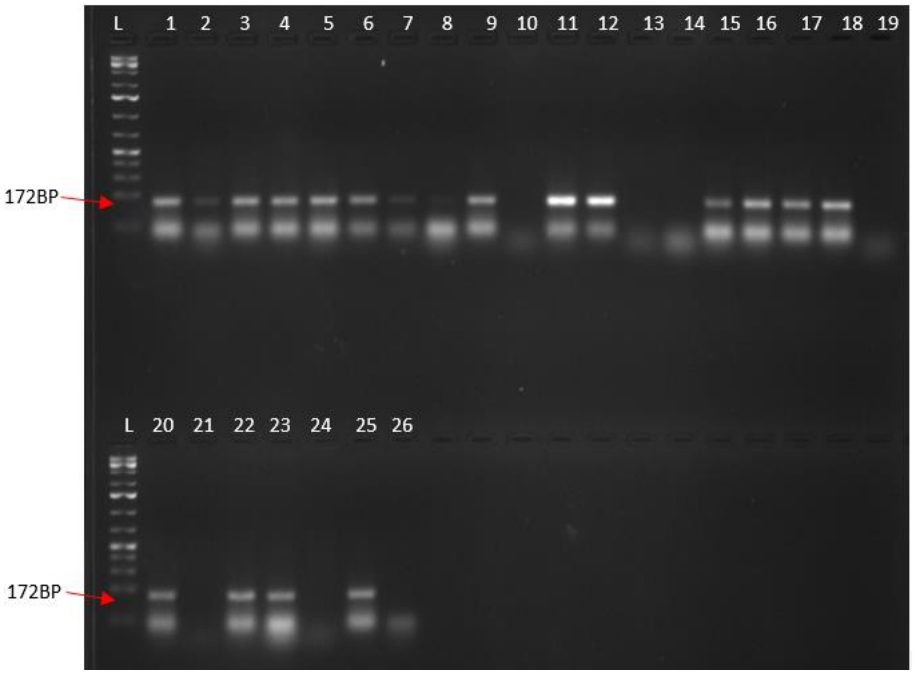

Figure 5. PCR products for the amplification of ABC transporter genes for the fungal isolates using degenerate primers set 1 primers. Lane 1 represents a 1 kb plus ladder. The expected band size amplified is 172 bps. L-Molecular marker (1 kb plus ladder, ThermoFisher scientific, Catalog number:10787018) 1-14, 2-M4, 3-R1, 4-R4, 5-M1, 6-I7, 7-M6, 8-R4, 9-R2, 10-Control, 11-M2, 12-I6, 13-Control, 14-Control, 15-R3, 16-R8, 17-M3, 18-R9, 19-Control, 20-M10, 21-Control, 22-10R, 23-M7, 24-M7, 25-I1, 26-Control.

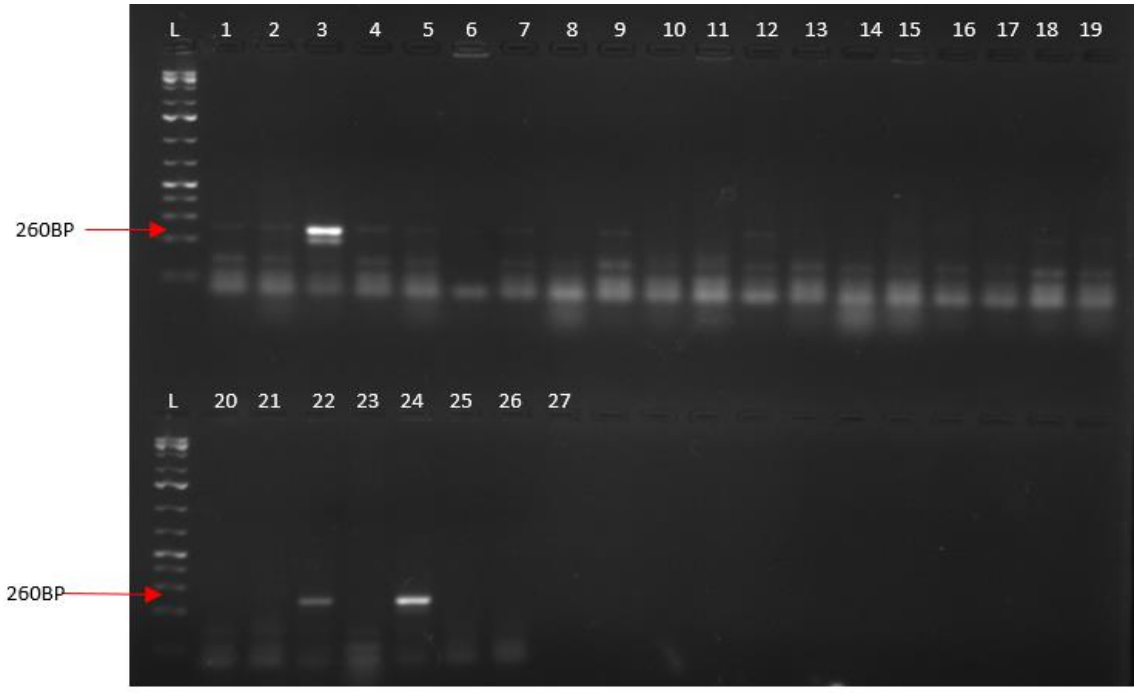

Figure 6. PCR products for the amplification of capsules genes for the fungal isolates using CAP64 primers. Lane 1 represents a 1 kb plus ladder. The expected band size amplified is 260 bps. L-Molecular marker(1 kb plus ladder); 1-14, 2-M10, 3-R1, 4-R4, 5-M1, 6-I9, 7-M6, 8-I7, 9-2, 10-M2, 11-I6, 12-I2, 13-R7, 14-R3, 15-10R, 16-M4, 17-I1, 18-M3, 19-R9, 20-R9, 21-M10, 22-R2, 23-M7, 24-R8, 25-I9, 26-I1, 27-Control.

resembled one another, thus in subsequent experiments these isolates were treated as one sample and the sample size was narrowed to 18. These fungal isolates belonged to genera; *Aspergillus*, *Penincillium*, *Cladosporium* and *Fusarium.* Other studies discovered the same isolates in TC laboratories (Odutayo et al., 2007; Msogoya et al*.,* 2012; Ankur et al., 2014). However*, Cunninghamella* sp. was the only fungus that was found in JKUAT Tissue laboratory occurred in both initiation and rooting stage which could be as a result of resistance mechanism. Morphological characterization was only possible up to genus level and therefore molecular characterization identified up to the species level and confirmed morphological results.

Production of extracellular enzymes plays an important role in degradation of sterilants through catalytic metabolic reactions like hydrolysis, oxidation, addition of an oxygen to a double bound, amino group oxidation to a nitro group, hydroxyl group added to a benzene ring, dehalogenation, reduction of a nitro group (NO_2) to an amino group, replacement of a sulphur with an oxygen, metabolism of side chains, ring cleavage (Hernndez et al., 2013). Other studies shows that metabolism of sterlilants may involve a three-phase process. In the first phase of metabolism, the initial properties of a parent compound are transformed through oxidation, reduction, or hydrolysis to generally produce a more water-soluble and usually a less toxic product than the parent (Hernndez et al., 2013). The second phase involves conjugation of a pesticide or pesticide metabolite to a sugar or amino acid, which increases the water solubility and reduces toxicity compared with the parent pesticide (Van et al., 2003). The third phase involves conversion of Phase II metabolites into secondary conjugates, which are also non-toxic (Ramakrishnan et al., 2011). In these processes fungal isolates are involved in producing extra cellular enzymes including hydrolytic enzymes, peroxidases, oxygenases, esterases, lipases, xylanases among others (Hernndez et al., 2013; Singh et al., 2016). In this study, production of extracellular enzymes, amylases, proteases, xylanases, cellulases and esterases were investigated. Isolate 10R recorded high extracellular activity and was both in initiation and rooting stage of *In vitro* culture. This could be attributed to its ability to produce higher amounts of extracellular enzymes which are involved in detoxification of sterilants.

Previous studies show that fungi mostly produce metabolites when under stress as a self-defence mechanism and some of them are produced to scavenge oxidizing agents like fungicides (Bertrand et al., 2014). Fungal isolates in this study were scrutinized for their ability to exhibit antibiosis and isolates in the rooting stage had the highest diameter of clearance due to antibiotic production. This could be attributed to their ability to produce higher amounts of antibiotics which are believed to play a role in shutting the immunity of the host for fungi to attack without any resistance (Compant et al., 2012). This resonates with earlier studies that showed that fungi produce metabolites that act on the host depriving it nutrients, space, lysing cells and block specific functions related to host growth (Bertrand et al*.,* 2014) hence their resistance to sterilants.

The tree displays three clades in which the isolates have been clustered*. Aspergillus* species clustered with *Pencillium citrinum* in one major clade with a score of 98% since they are from the same family Aspergillaceae (Hussain et al., 2017) while *Fusarium venenatum* and *Cladosporium* sp*.* were grouped in another clade with a score of 59% as they are not such closely related but are only from the same subphylum Pezizomycotina (Ashfaq et al., 2017). *Cunninghamella bainieri* formed separate cluster since it was from a different phylum from the rest of clades called Mucoromycota (Karim et al., 2007). Like in morphological characterization, the main clusters revealed by molecular characterization of fungi belonged to the genera *Aspergillus*, *Penincillium*, *Cladosporium* and *Fusarium* (Table 4)*.* The genus *Aspergillus* separated into well supported eight sub groups belonging to eight species; *Aspergillus cervinus, Aspergillus cristatus, Aspergillus awamori, Aspergillus alliaceus, Aspergillus cremeus, Aspergillus flavus* and *Aspergillus* sp. (Table 4). In this study, morphological characterization was only possible up to generic level and it was difficult identifying *Aspergillus* species and *Penincillium* species since they are highly speciose lineages of fungi. Raja et al. (2017) had made similar proposition that morphological characterization may not perform well in lower level classification especially in highly speciose lineages of fungi since morphological characters may not always provide accurate groupings within an evolutionary framework, mainly at the species level. Due to the morphological characters presented, there was suspicion that 2M was *Penicillium* sp*.* but after molecular work, it was found to be *A. allieus.* Such morphological ambiguities have been experienced, as characters used in identification are often too variable and dependent on colony and cell morphology (Foltz et al., 2013). Therefore, using the two techniques (morphology and molecular) necessitates close to accurate identification of species. Most of the fungal contaminants were found to belong to the *Aspergillus* species and according to Cassells (1991) they are exogenous and their presence in this study may be due to inadequate surface sterilization. *Aspergillus* species are the main cause of aspergillosis in immunocompromised individuals and symptoms of infection by this species include is fever and coughing (Baysan et al., 2010). These species could have been introduced into the laboratory by insufficient asepsis among workers during tissue culture operations. *Fusarium* sp. have been reported as an endophytic fungus in banana (Suryanarayanan et al., 2000). The sterilization protocol used in the JKUAT tissue culture laboratory involves only surface sterilization using 7% bleach and 70% ethanol.

Table 4. Blast results of fungal isolates from IBR laboratory and their close relatives.

Sample ID	Length (BP)	Accession numbers	Affliated to	Closest match in BLAST	% identity	Reference
I1	650	MN549412	LT860211.1	*Cladosporium* sp. NS1 partial 18S rRNA gene, isolate NS1	99	Ashfaq et al. (2017)
I4	641	MN549413	MN326853	*Aspergillus* sp. isolate *7S6*	99	Hamed (2019)
I6	657	MN549414	JF895924.1	*Aspergillus* sp. *I16-3.*	99	Iniya et al. (2011)
I7	644	MN549415	MH539639.1	*Aspergillus orzae* isolate *RIB40*	99	Ramesh and Josephin (2019)
M1	628	MN549416	MN453363	*Aspergillus niger* strain *BA2*	99	Al-Talhi (2019)
M2	670	MN549417	KF018469.1	*Aspergillus alliaceus* strain *21.1*	96	Demirel et al. (2013)
M3	628	MN549418	KY307867.1	*Aspergillus cervinus* strain *JAPC9*	99	Abraham and Chakraborty (2016)
M4	641	MN549419	AB008399.1	*Aspergillus cremeus*	95	Nikkuni et al. (1998)
M6	657	MN549420	KU711809.1	*Aspergillus sojae* isolate *LF2_CPD_NRRI*	96	Pattanayak et al. (2019)
R1	639	MN549421	MK615877	*Penicillium citrinum*	99	Liu (2019)
R2	649	MN549422	MF563964	*Aspergillus fumigatus* strain *T3*	99	Liu (2017)
R3	632	MN549423	JN604547.1	*Aspergillus flocculosus* strain *OUCMDZ-730*	99	Wang and Zhu (2011)
R4	612	MN549424	XR003150050.1	*Fusarium vinenatum*	99	King et al. (2014)
R8	640	MN549425	KP872503.1	*Penicillium* sp. Y28	100	Li et al. (2015)
R9	631	MN549426	KY233188.1	*Aspergillus flavus Ya1*	99	Hussain et al. (2015)
R10	682	MN549427	EF562534.1	*Cunninghamella bainieri*	99	Karim et al. (2007)

This explains why *Fusarium* sp. was found in rooting stage in this study since it cannot be eliminated by surface sterilization. Earlier studies had made similar proposition (Cassells, 1991) that *Fusarium* sp. contaminates after many subculture or transfer since it cannot be destroyed by surface sterilization. Systemic sterilants can be used to get rid of endogenous contaminants (Omamor et al., 2007; Mng'omba et al., 2007).

ABC transporter genes are responsible for the production ABC transporter pumps that fungi use for efflux of toxic substances from the fungal system (Park and Williamson, 2015; Neelab and Singh, 2018) hence the resistance of fungicides. This explains why the current sterilization protocol in banana tissue culture laboratory is ineffective.

Capsule genes are responsible for formation of a capsule which provide resistance to stressful conditions (Zaragoza et al., 2009) and are very rare in eukaryotes but ubiquitous in prokaryotes (Park and Williamson, 2015). This isolates in this study with capsules genes were collected from rooting stage meaning they resisted sterilization at initiation and multiplication stages. This could be as a result capsule gene. This gene was found in *Aspergillus fumigatus* (2R). This supports earlier studies that *A. fumigatus* is capable of growing in temperatures over 50˚C, which is in contrast to other fungi that are mesophilic (Bhabhra and Askew, 2005). This might be attributed to protective coverage on the fungal system.

Conclusion

This study also indicates that JKUAT tissue culture laboratory harbour's diverse fungal species. *C. bainieri* (10R) was found both in initiation and rooting stage. This might be because of its ability to produce extracellular enzymes, ABC transporter genes and capsule gene which are responsible for sterilants resistance. It was ascertained that fungal isolates are capable of producing enzymes which have been confirmed to play a role in degradation of fungicides. They produce antibiotics which shut the immunity of the host for the fungi to attack without any resistance hence the sterilants resistance. They also have mdr1 genes that form ABC transporter pumps that are involved in efflux of any toxic substances from the fungal system. Some of the fungal isolates have capsules genes which are responsible for formation of capsules that protects fungi from stressful conditions. The main reason why fungi

contaminants in JKUAT laboratories are difficult to eliminate is because of their resistance mechanism. Therefore sterilants that inhibit production of extra cellular enzymes, antibiotics, ABC transporters and capsules should be introduced in tissue culture laboratories.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Utilization potential of quality protein maize in spiced ogi

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The high incidence of protein energy malnutrition among children under 5 years in Nigeria informed this study. Quality protein maize (QPM) produces more lysine and tryptophan than most modern varieties of maize. It has been reported to be economically superior to traditional maize. This research focuses on QPM ogi fortified with indigenous food spices in order to improve its health benefits and organoleptic properties. The yellow variety QPM ART98/SW1 was processed into ogi with inclusion of natural spices (garlic, ginger and clove) each at 1 and 3% concentration. Spiced ogi paste samples were assessed for proximate, minerals, functional and sensory properties on day zero (0). The other portions of the spiced ogi samples were stored for 15 days for microbial analysis and pH. For all parameters studied, plain QPM ogi served as control. Total viable count increased with days of storage in all the ogi samples but the least counts were recorded in QPM ogi spiced with 3% cloves. Crude protein was highest (6.21%) in QPM ogi spiced with 3% clove whereas the control contained 5.57%. The pH of all the ogi samples decreased with days of storage. The most abundant mineral in all the ogi samples is magnesium (mg) which ranges from 1213 to 1495 mg/kg. The result of functional properties shows that the ogi samples had good gelatinization properties. In conclusion, addition of spices improved the nutrient contents of ogi made from QPM. Quality protein maize ogi with 1% ginger was preferred to all the other spiced QPM samples in terms of sensory attributes. Shelf life of QPM ogi could be improved by fortifying with clove at a 3% level.

Key words: Quality protein maize, spices, ogi.

INTRODUCTION

Quality protein maize (QPM) contains almost twice as much usable protein as other maize grown in the tropics. It yields 10% more grain than traditional varieties of maize (Omolaran et al., 2014). In Central and South America, Africa and Asia, several hundred million people rely on maize as their principal daily food, for weaning babies and for feeding livestock (Hilary, 2014).

Nutritionally, QPM grains contain approximately 55 and 30% more tryptophan and lysine, respectively compared to normal maize varieties. This has been confirmed to be true even in QPM varieties developed in Institute of Agricultural Research and Training (IAR&T) Ibadan (Omolaran et al., 2014; Lawal et al., 2014). Lysine and tryptophan allow the body to manufacture complete proteins, thereby eliminating wet malnutrition. In addition, tryptophan can be converted in the body to niacin, which

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theoretically reduces the incidence of pellagra (Mamatha et al., 2017). Maize is known to be the primary provider of calories supplying 20% of the world's food calories (Olakojo et al., 2007). The development of high-protein foods of plant origin is essential in developing countries because of the high cost of animal protein. Consumption of such products may play a major role in combating malnutrition, which is a serious problem in developing countries (Aminigo and Akingbala, 2004).

Ogi is either consumed as porridge (pap) or as a gel-like product (agidi) by a very large number of Nigerians. Pap however is the most important traditional food for weaning infants and the major breakfast cereal for adults especially those low income earners that cannot afford imported baby foods (Ijabadeniyi, 2007; Eke-Ejiofor and Beleya, 2017). Ogi is a popular breakfast cereal gruel in West Africa with high acceptability, low cost and shelf life stability characteristics. Normal maize proteins have poor nutritional value for monogastric animals such as humans and pigs because of lesser content of essential amino acids such as lysine and tryptophan (Akande and Lamidi, 2006). Ogi made from QPM varieties do have a significant impact on the nutritional status of vulnerable groups (Lawal et al., 2014). Its fermentation involves lactic acid bacteria converting the carbohydrates in the cereals during ogi production to organic acids which contribute to softness in the product and the characteristic flavor and sour taste (Banigo and Muller, 1972). Conventionally, ogi (pap) is processed, prepared and consumed without addition of spices. In the recent time, local consumption pattern has moved towards the inclusion of different single or combined spices by the local processors with the view to improving the nutritional value and taste of the products. Spices are culinary herbs which have aromatic or pungent flavor. They are dried seeds, fruit, root or vegetable substances used in preparation of soups to enhance the flavor of such food (Farinde, 2015). Spices do not only excite taste, they are composed of high quality phytonutrients, essential oils, antioxidants, minerals and vitamins that are essential for overall health sustenance. Fortification of traditionally fermented food products is a vital process of increasing the concentration and bioavailability of the nutritional content of the edible part of the plant food especially cereals to the levels that consistently exceed the inherent content (John et al., 2017). Studies carried out to show the interrelationship between microorganisms found in Ogi and the nutritional benefits reveal that some of the microorganisms used in fermentations result in the addition of the nutritive value of ogi. There is an increase in the lysine content during fermentation (Odunfa et al., 1994). The isolated microorganisms that were characterized using conventional methods in fermenting varieties of maize ogi included fungi (molds and yeasts) and bacteria species such as *Lactobacillus* species and

Saccharomyces cerevisiae that has played an important role during fermentation (Ijabadeniyi, 2007; Falana et al., 2011). Hence, this work focuses on the evaluation of microbiological, nutritional and sensory properties of QPM spiced product (ogi).

METHODOLOGY

QPM yellow variety ART 98/SW1was processed into ogi paste (QPM ogi) with the addition of three different spices (clove, ginger and garlic) at 1 and 3% concentration. Microbiological properties were determined (total bacteria count, total fungi count and lactic acid bacteria count) and the shelf life of spiced QPM ogi paste were assessed over 15 days at room temperature. The nutritional properties of spiced QPM ogi was assessed (proximate analysis, minerals and functional properties) only at day 0. The ogi samples were also subjected to sensory evaluation. Data obtained from the analyses were subjected to analysis of variance (ANOVA) and means were separated by using Duncan Multiple Range Test at $p < 0.05$.

Source of material used

QPM variety ART 98/SW1 was obtained from the seed store of the Institute of Agricultural Research and Training, Ibadan. The spices were bought from Aleshinloye market in Ibadan, and processed in the IAR&T food processing laboratory.

Preparation of spices

Fresh ginger and garlic (500 g each) obtained were peeled, washed and mashed manually with a mortar. Cloves in its dry form were also ground to powdery form and they were separately kept in airtight containers. The spices were weighed, and added to the ogi paste mixed together at a 1 and 3% concentration.

Preparation of ogi with spices

QPM grains were sorted from the shaft and dirts. A 2 kg of maize grains were added to 5L of water and steeped for 3 days (72 h) at room temperature. The maize grains were then washed and was wet milled into paste using a local ATLAS grinder. Water was added and sieved with muslin cloth, the slurry was allowed to settle for one day, the supernatant was decanted and the ogi paste recovered. The solidified ogi paste was portioned into seven parts. One part served as control, which had no spice. The other six parts had 1 and 3% of ginger, garlic and cloves, respectively. A 500 g of ogi paste was added to 5 g of each spice at 1% concentration; also 500 g of ogi paste was added to 15 g of each spice at 3% concentration.

Preparation of pap from spiced ogi

Each sample of spiced ogi paste (30 g) was transferred into a bowl and evenly mixed with 15 ml of cold water to avoid lumps. Boiling water was then added with continuous stirring to gelatinize the ogi to make pap. This was repeated with all seven samples before it was served for sensory evaluation.

Microbial analysis

Microbial load of the spiced QPM ogi samples were determined using the method of Ntuli et al. (2013). Nutrient agar (NA- BIOTEC

Medical Market UK Limited, Stanmore, United KIngdom) was used for bacterial count, Potato Dextrose Agar (PDA- A Neogen Company, LAB M Limited, Heywood Lancashire, United Kingdom) was used for fungal count and Man-De Rosa and Sharp medium (MRS- A Neogen Company, Heywood, Lancashire, United Kingdom) was used for lactic acid bacterial counts, respectively. One litre of each of NA, PDA and MRS was prepared and boiled to dissolve the media, and autoclaved at 121°C for 15 min. One gram of each sample was weighed into a test tube containing 9 ml of sterile distilled water and serially diluted until a dilution factor of 10^{-5} was reached. One millilitre of the last dilution factor was seeded aseptically into sterile plates (streptomycin was added to PDA to inhibit bacterial growth). The media were poured individually in triplicates. After solidifying, the plates were incubated in an incubator at 37°C for 24 h for NA and MRS and 25°C for 3 to 5 days for PDA, all the plates were incubated invertedly (upside down). Isolation and identification of bacteria in the ogi samples were based on selective media (Ntuli et al., 2013), while fungi were isolated following the method of Alkenz et al. (2015).

Chemical analysis

Proximate and mineral composition

Proximate composition of the ogi samples was determined using the standard method of AOAC (2005). Each sample was analysed in triplicate for crude protein, crude fat, crude fibre, total ash, moisture and carbohydrate. Nitrogen was converted to crude protein by multiplying with a factor of 6.25. The carbohydrate content was calculated based on the difference.

Mineral content of the ogi samples (Ca, Mg, Fe and Zn) were determined using the digestion method of AOAC (2005). Atomic absorption spectrophotometry (Accusys 211, Buck Scientific, USA) was used.

*Hydrogen ion concentration (*pH)

Hydrogen ion concentration or pH of each sample was measured with a standard pH meter (Mettler Toledo FG2/EL2 produced by Mettler – Toledo GmbH Analytical, CH-8603 Schwerzenbach, Switzerland) according to the method of Bolade et al. (2018). The pH was determined by dipping the electrode of the pH meter in the sample. The pH meter was calibrated using pH 4 and 7 buffers.

Functional properties

Water absorption capacity, swelling power and solubility power were determined following the methods described by Adepeju et al. (2014). Bulk density was determined following the method described by Bolaji et al. (2014).

Sensory evaluation

Freshly prepared spiced quality protein maize ogi (pap) samples were presented to a panel of 20 trained judges who are regular consumers of ogi. The panellists were given water for mouth rinsing after each tasting and they were asked to score the ogi samples for colour, appearance, flavour, texture, taste, and overall acceptability using a 9 point hedonic scale (where 9 = like extremely and 1= dislike extremely) (Farinde, 2015).

Statistical analysis

All determinations were measured in triplicate and the data obtained from the analyses were subjected to an analysis of variance (ANOVA). Means of values were separated using the Duncan Multiple Range Test. Significance was accepted at $p < 0.05$ (SAS, 1995).

RESULTS

Total bacteria count of spiced QPM ogi paste at day zero ranged from 0.4×10^5 to 2.2×10^5 $cfug^{-1}$, while at day fifteen, the total bacteria count ranged from 0.8×10^5 to 3.8×10^5 $cfug^{-1}$. Total bacterial count, in spiced QPM ogi increases gradually in growth as the number of days for storage increased (Figure 1). Control (Con) had the highest total bacterial count whereas ogi with 3% cloves (Clo3) recorded the lowest count. There was no fungal count on Days 0 and 5 during storage of spiced QPM ogi meanwhile scanty growth was recorded on day 10 (Figure 2). Lactic acid bacteria count increased with days of storage in all the ogi samples and the least counts were also recorded in QPM ogi spiced with 3% cloves (Figure 3).

The crude protein was highest (6.21%) in QPM ogi spiced with 3% cloves and QPM ogi without spice (control) had the lowest value (5.57). QPM ogi spiced with 3% garlic had the highest crude fat, crude fibre and ash contents. Moisture content ranged from 13.65 to 14.39% and carbohydrate ranged from 79.34 to 80.54% (Table 1). Plain QPM ogi (control) was significantly higher ($p < 0.05$) in calcium and iron when compared with all the other ogi samples. QPM ogi spiced with clove at 3% level recorded the highest zinc content (292 mg/kg) (Table 2).

The pH of all the ogi samples decreased with days of storage. In control (Con) the pH value is 3.5 at day 0, whereas at day 15 the value recorded 3.4. Also in Clo3 (3% cloves), the pH at day 0 is 3.7 and at day 15 of storage it is 3.5 (Table 3).

Bulk density was the highest in plain QPM ogi while Ogi spiced with garlic at 3% level recorded the highest water absorption capacity. Swelling power was the highest in QPM ogi spiced with 1% cloves, and solubility power ranged from 2.5 to 7.8% (Table 4). Plain QPM ogi was most accepted in all the sensory attributes tested (8.0) followed by QPM ogi spiced with ginger at 1% level, while the clove preparation had the lowest value (3.5) (Table 5).

DISCUSSION

Total bacterial count, in spiced QPM ogi increases gradually in growth as the number of days for storage increase, this agrees with the work of John et al. (2017) who reported that total bacteria count recorded for the untreated samples of ogi were found to be higher in ogi than the treated samples. He further explained that the higher microbial load in ogi may be due to accumulation of microorganisms in the water that was retained in the ogi. There was no fungal count on Days 0 and 5 during storage of spiced QPM ogi meanwhile scanty growth was

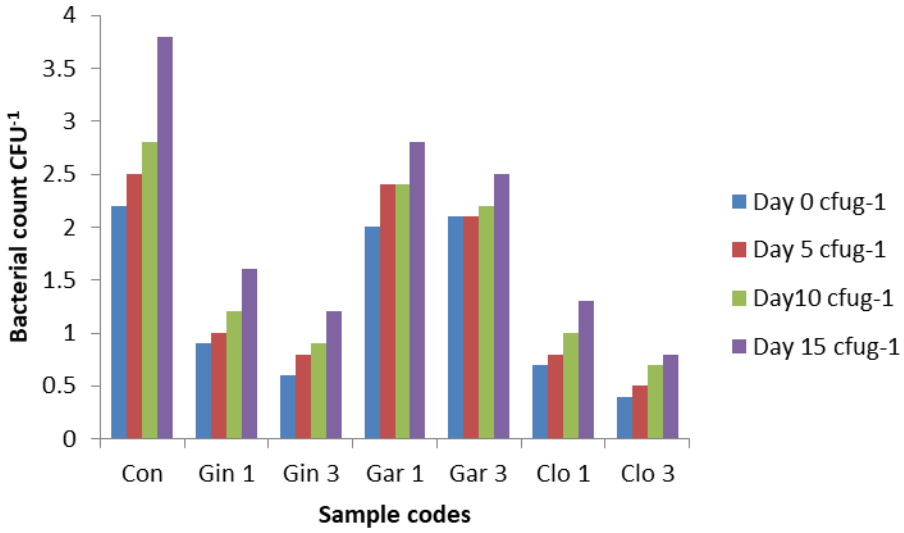

Figure 1. Total bacterial count of spiced QPM ogi paste.

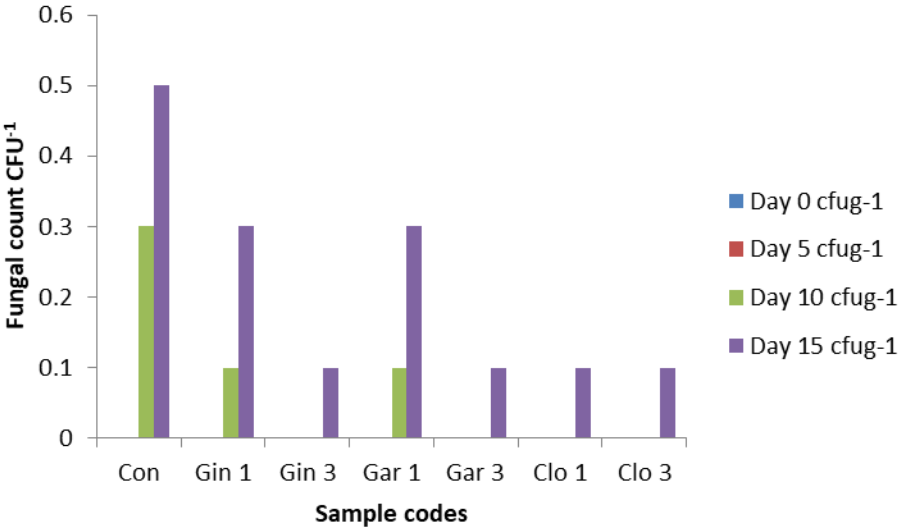

Figure 2. Total fungal count of spiced QPM ogi paste.

recorded on day 10. This is in agreement with the report of Omemu et al. (2007). He reported that moulds were not isolated until days 10 and 12 in the corn steep liquor and the ogi samples, respectively.

Lactic acid bacteria count increased with days of storage in all the ogi samples, and this agrees with the work of Adesokan et al. (2010). Many studies have reported that lactic acid bacteria (LAB) in the fermentation of ogi contribute to the flavor and aroma development of ogi and inhibit the growth of other organisms (Oyewole, 1997).

John et al. (2017) reported that the value of crude protein in spiced ogi was higher than the value of crude protein in the control which correlates with this study. The fortification of ogi with spices improved significantly ($p< 0.05$) the nutrient composition of ogi samples (protein). Spices are very important both as food and to contribute to the overall wellbeing of people. Ginger, garlic and clove contain high level of antioxidants which help in preventing cell damage caused by free radicals and also contain essential oils (Farinde, 2015). The QPM spiced ogi samples provide a good amount of calcium, magnesium, iron and zinc. Calcium helps bone to grow rapidly especially the growing children and to build strong bones. Magnesium helps to maintain normal nerve and muscle function and supports a healthy immune system (Soetan et al., 2010). QPM spiced ogi provides a good amount of iron needed in the production of haemoglobin

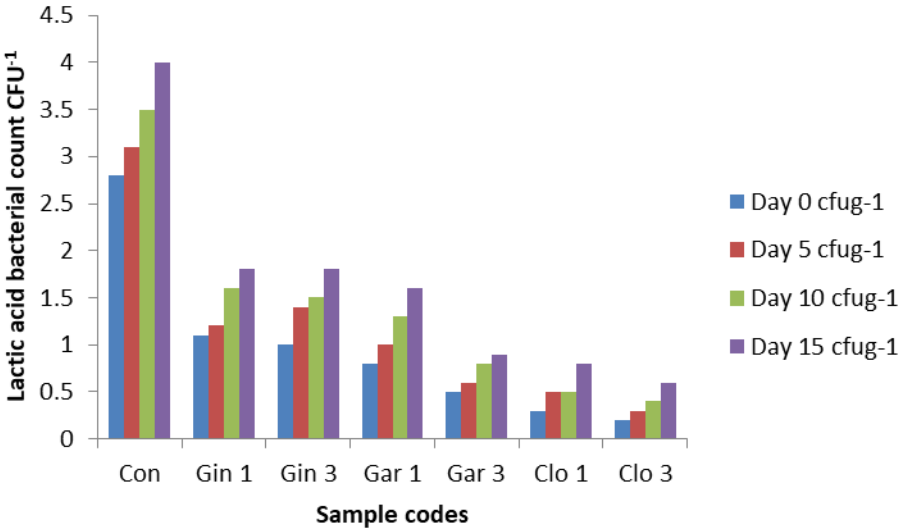

Figure 3. Total lactic acid bacterial count of spiced QPM ogi paste.

Table 1. Proximate composition of spiced QPM ogi.

Sample code	Crude protein (%)	Crude fat (%)	Crude fibre (%)	Total ash (%)	Moisture content (%)	Carbohydrate (%)
Con	5.57^{g}	0.66^{a}	0.18^{g}	0.79^{g}	14.28^{c}	79.34^{g}
Gin 3	5.89^{e}	0.48^{e}	0.23^{c}	0.82^{f}	13.76^{f}	80.54^{a}
Gin 1	5.68^{f}	0.55^{c}	0.21^{e}	0.85^{e}	14.33^{b}	80.04^{b}
Gar 3	6.03^{c}	0.62^{b}	0.26^{a}	0.96^{a}	13.81^{e}	79.64^{e}
Gar 1	5.97^{d}	0.43^{g}	0.22^{d}	0.88^{c}	13.65^{g}	79.97^{c}
Clo 3	6.21^{a}	0.52^{d}	0.20^{f}	0.93^{b}	14.39^{a}	79.38^{f}
Clo 1	6.11^{b}	0.45^{f}	0.24^{b}	0.86^{d}	13.82^{d}	79.79^{d}

Means in the same column followed by the same letter are not significantly different from each other at $p < 0.05$. Con- QPM ogi without spices Gin 1- QPM ogi spiced with ginger at 1%; Gin 3- QPM ogi spiced with ginger at 3%; Gar 1- QPM ogi spiced with garlic at 1%; Gar 3-QPM ogi spiced with garlic at 3%; Clo 1- QPM ogi spiced with clove at 1%; Clo 3- QPM ogi spiced with clove at 3%.

Table 2. Mineral composition of spiced QPM ogi.

Sample code	Ca (mg/kg)	Mg (mg/kg)	Fe (mg/kg)	Zn (mg/kg)
Con	576^{a}	1442^{c}	611^{a}	109^{b}
Gin 3	401^{c}	1315^{e}	168^{g}	18^{f}
Gin 1	275^{d}	1495^{a}	401^{b}	76^{c}
Gar 3	411^{b}	1465^{b}	302^{d}	34^{e}
Gar 1	63^{g}	1365^{d}	196^{f}	17^{g}
Clo 3	94^{e}	1213^{g}	374^{c}	292^{a}
Clo 1	70^{f}	1215^{f}	255^{e}	39^{d}

Means in the same column followed by the same letter are not significantly different from each other at $p < 0.05$

which carries oxygen in the blood (Ojo and Enujiugha, 2016). Zinc helps with hormone production and proper growth and repair (Soetan et al., 2010).

The pH of all the ogi samples decreased with days of storage, this might be as a result of lactic acid production by fermentative organisms responsible for the fermentation of ogi. This observation is in agreement with the previous report of Adesokan et al. (2010) and Okwute and Olafiaji (2013). The decrease in bulk density may help in reduction of transportation and packaging cost

Table 3. pH of Spiced QPM Ogi.

Sample code	Day 0	Day 5	Day 10	Day 15
Con	3.5^{d}	3.5^{b}	3.4^{b}	3.4^{b}
Gin 1	3.6^{c}	3.4^{c}	3.4^{b}	3.3^{c}
Gin 3	3.5^{d}	3.4^{c}	3.4^{b}	3.3^{c}
Gar 3	3.6^{c}	3.4^{c}	3.4^{b}	3.3^{c}
Gar 1	3.8^{a}	3.4^{c}	3.4^{b}	3.3^{c}
Clo 1	3.7^{b}	3.5^{b}	3.4^{b}	3.4^{b}
Clo 3	3.7^{b}	3.6^{a}	3.5^{a}	3.5^{a}

Means in the same column followed by the same letter are not significantly different from each other at $p < 0.05$

Table 4. Functional properties of spiced QPM ogi.

Sample code	Bulk density (g/cm^{3})	Water absorption capacity (gs^{-1})	Swelling power (%)	Solubility power (%)
Con	0.8^{a}	2.3^{b}	7.7^{c}	4.7^{bc}
Gin 1	0.6^{b}	2.3^{b}	7.8^{c}	6.1^{ab}
Gin 3	0.7^{ab}	1.3^{c}	7.6^{c}	4.2^{c}
Gar 3	0.7^{ab}	4.5^{a}	7^{cd}	2.5^{d}
Gar 1	0.7^{ab}	2.3^{b}	9.6^{b}	7.8^{a}
Clo 1	0.6^{b}	2.3^{b}	11.9^{a}	5.4^{b}
Clo 3	0.6^{b}	2.1^{bc}	6.3^{d}	4.4^{c}

Means in the same column followed by the same letter are not significantly different from each other at $p < 0.05$

Table 5. Sensory analysis of spiced QPM ogi.

Sample code	Colour	Appearance	Flavour	Texture	Taste	Overall acceptability
Con	8.0^{a}	7.8^{a}	7.5^{a}	7.4^{a}	8.4^{a}	8.0^{a}
Gin 1	7.3^{c}	6.5^{c}	5.9^{d}	5.8^{d}	6.2^{b}	7.1^{b}
Gin3	7.4^{b}	7.6^{b}	6.9^{b}	6.6^{b}	6.2^{b}	6.8^{c}
Gar 1	7.3^{c}	6.3^{d}	6.5^{c}	6.3^{c}	5.3^{c}	6.0^{d}
Gar 3	4.5^{e}	6.3^{d}	4.5^{e}	6.3^{c}	3.8^{d}	5.6^{e}
Clo 1	4.5^{e}	5.2^{e}	3.4^{g}	3.8^{f}	3.0^{e}	3.5^{g}
Clo 3	4.6^{d}	5.2^{e}	4.0^{f}	4.0^{e}	2.8^{f}	3.8^{f}

Means in the same column followed by the same letter are not significantly different from each other at $p < 0.05$.

that is the packaging will be economical (Bolaji et al., 2014; Ojo and Enujiugha, 2016). Water absorption capacity is desirable for the improvement of mouthfeel and viscosity reduction in food products (Ojo and Enujiugha, 2016). Swelling power reflects the extents of the association forces within the granules. Higher solubility in any sample implies that it is lesser in leaching than other sample, hence the higher the solubility, the more ogi reconstitute well in water (Bolaji et al., 2014). From time immemorial, spices have been employed for their aromatic, medicinal and flavouring characteristics. Apart from these characteristics, it also acts as an antimicrobial agent in foods. The study of Adesokan et al. (2010) and Okwute and Olafiaji (2013) agrees with this report which reveals that incorporation of spices into ogi led to an improved sensory attributes and a reduction in microbial load during storage and hence an improved shelf life.

Conclusion

Addition of spices improved the nutrient content of ogi made from QPM. QPM ogi with 1% ginger was preferred to all the other spiced QPM samples in terms of sensory attributes. Spices had a significant reduction effect on the microbial count of the ogi samples. Shelf life of QPM ogi could be improved by fortification with cloves at 3% level.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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