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African Journal of Microbiology Research

Full Length Research Paper

Microbial quality of Nile perch (*Lates niloticus*) and physico-chemical properties of salted sun-dried products sold at regional markets, Tanzania

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This study was conducted to investigate the microbial and physico-chemical qualities of salted sun-dried Nile perch (*Lates niloticus*) products and frozen fish from various storage facilities in Mwanza, Tanzania. The bacterial flora, moisture contents (MCs), and water activity (A_w) were investigated using standard methods. A total of 120 samples were collected for microbiological analysis with 90 of the samples additionally analysed for MC and A_w. Findings showed that the mean total viable counts (TVCs) in salted sun-dried products sampled during rainy season was 4.5 log cfu/g in fish heads with MCs of 38.0% and A_w of 0.682. This was significantly higher ($P<0.05$) than what was recorded during the dry season with mean TVCs of 3.0 log cfu/g at MCs of 24.6% and A_w of 0.625. Fish chests had TVCs of 3.3 log cfu/g and MCs of 27.6% and A_w of 0.659 in rainy season with no significant seasonal difference ($P>0.05$). Fish belly flaps had TVCs of 3.3 log cfu/g at 26.4% MCs and 0.669 A_w in rainy season which were comparable ($P>0.05$) to those dried in the dry season. The microbial species recovered were *Staphylococcus* spp., *Enterobacter* spp., *Psychrobacter* spp., and *Bacillus* spp. Neither *Escherichia coli* nor extended-spectrum beta-lactamase producing *Enterobacteriaceae* were detected. Frozen Nile perch had TVCs of 5.7 log cfu/g on skin, 5.4 log cfu/g in gills and 2.9 log cfu/g in flesh and were within acceptable limit set by Tanzanian standards. These results reveal that dried Nile perch products are generally safe for human consumption; however, the recovered bacteria indicate a need of implementing hygienic procedures during processing of products for improved quality and safety.

Key words: Salted sun-dried fish, microbial quality, food safety, physico-chemical parameters.

INTRODUCTION

Nile perch (*Lates niloticus*) from Lake Victoria is one of the most important fish species for fisher folks in Tanzania as well as for the nation due to its economic and nutritional health benefits (Kirema-Mukasa, 2012). Fish are an important source of animal protein and other essential elements to sustain human health (Ikwebe et al., 2017; Immaculate et al., 2013; Majumdar et al., 2017). Nile perch of good quality are processed as fillets

for export markets especially to the European countries and Asia while other fish parts are processed for domestic and regional African markets (Kabahenda and Hüsken, 2009; Kirema-Mukasa, 2012). Currently, the Nile perch market is growing due to product diversification including salted sun-dried bi-products such as heads, chests, belly-flaps and whole fish which are sold for human consumption. The salted sun-dried Nile perch products are mostly exported to countries such as the Democratic Republic of Congo, Rwanda and Burundi (Kirema-Mukasa, 2012).

Salting and sun-drying is an ancient preservation method which has been applied to different foods such as fish, meat, and vegetables (Immaculate et al., 2013; Nagwekar et al., 2017). Sun-drying of fish is simple, cheap, and affordable, but an adequate dried product requires enough sun (Ikwebe et al., 2017). The method can improve the shelf life of products if post-processing handling is properly done to avoid bacterial contamination (Nagwekar et al., 2017). Although the salted sun-dried Nile perch products have been widely marketed in East and Central African regional markets, limited information is available on their microbiological quality and safety aspects.

Moisture content (MC) and water activity (A_w) are important factors determining food quality, preservation and shelf life of food stuffs. Also, they are used to predict microbial growth and determine the microbiological stability of food products (Bevilacqua et al., 2017; Nielsen, 2010). Previous studies have described how the MCs and A_w can influence microbial growth on salted sun-dried fish and fish products (Nagwekar et al., 2017; Sampels, 2015). This includes the mechanism of products drying process which reduces the MC and Aw to minimise microbial proliferation in food. Although the preservation method is affordable, the drying condition, packaging, and storage may not be hygienically satisfactory to maintain the quality of the dried products.

Nile perch are caught in deep waters usually with low levels of microbial contamination (Immaculate et al., 2013; Koral et al., 2013). However, during subsequent handling along the value chain from capture to market, different bacteria of public health implications may come in contact with the fish, causing a decline in its safety (Immaculate et al., 2013). Therefore, the determination of microbiological quality of frozen Nile perch from cold storage facilities is very important as a strategy for safeguarding consumer's health.

The aim of this study was therefore to investigate the microbial quality and safety of frozen Nile perch and its bi-products in line with physico-chemical qualities of processed sun-dried products marketed in the Lake Victoria region.

MATERIALS AND METHODS

Sampling, laboratory sample preparation and analysis

A total of 120 samples were collected from March to July 2018. The samples included frozen Nile perch from cold storage facilities and salted sun-dried bi-product (heads, chests and belly flaps). Sampling locations were located in Ilemela and Nyamagana districts of Mwanza region, Tanzania. Microbiological and MC analysis were done at the National Fish Quality Control Laboratory (NFQCL), Mwanza and A_w analysis at the Department of Food Technology, Nutrition and Consumer Sciences laboratory, Sokoine University of Agriculture (SUA) in Morogoro. Identification of bacterial isolates was done at the Department of Veterinary and Animal Sciences, University of Copenhagen. Size of each sample was about 2 kg for frozen fish and 400 g of salted sun-dried products.

Ninety processed salted sun-dried Nile perch products were collected from different processors at the Kirumba Market in Mwanza. All samples were analysed for *Salmonella* spp., *Escherichia coli*, total coliform counts (TCCs), total viable counts (TVCs), and Extended Spectrum Beta-Lactamase (ESBL) producing *Enterobacteriaceae* using standard methods as described below. Samples were collected both in the rainy season (March-May) and dry season (June-July). Forty-five dried products (15 samples of each type) were collected during the rainy season and forty-five in the dry season (15 each sample type). Samples were collected using sterile rubber gloves, placed into sterile plastic zip-lock bags and transported to NFQCL for analysis. In the laboratory, each salted sun-dried sample was divided into three portions with the first portion used for microbiological analysis; the second portion was used for MCs analysis, and the third portion was packed and transported to SUA for the A_w analysis. For microbiological analysis, a 25 g sample was chopped and mixed into Buffered Peptone Water (BPW) (Oxoid Ltd, Hampshire, England) in sterile stomacher bags and homogenised in a stomacher (Seward 400, UK) before analysis.

A total of 30 frozen Nile perch were collected using sterile rubber gloves from storage facilities for microbiological analysis. Samples were placed in sterile plastic zip-lock bags, preserved in an insulated box containing cooling elements and transported to NFQCL for analysis. In the laboratory, frozen fish were thawed at room temperature for 2-3 h, and then by using sterile scissors and surgical blades; gills, skin and flesh were removed and chopped into a 25 g sample which was mixed with sterile 225 ml of BPW and analysed as per protocols stated in each method based on parameter analysed.

Detection of *Salmonella* species

Salmonella spp. was detected using the International Organisation Standard (ISO) method (ISO 6579:2002/Amd.1:2007). Briefly, pre-enrichment was done in BPW at 37°C for 24 h followed by enrichment in Rappaport Vassiliadis broth (Oxoid Ltd) at 42°C for 24 h and Mueller-Kauffman Tetrathionate-novobiocin broth (Oxoid

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Ltd) at 37°C for 24 h. Presumptive *Salmonella* colonies were biochemically confirmed on Triple Sugar Iron agar (Oxoid Ltd) and isolates were tested for agglutination with polyvalent *Salmonella* antisera (Rapid Lab Ltd, UK) with strain *S.* Typhimurium ATCC 13311 (Public Health, England) used as a positive control.

Enumeration of *Escherichia coli,* total coliforms (TCCs) and ESBL-producing *Enterobacteriaceae*

Enumeration of *E. coli* and TCCs were done on Brilliance *E. coli*/coliforms selective agar medium (BE/C) (Oxoid Ltd) by the spreading technique following the manufacturer's instructions. Serial dilutions were made according to ISO method (ISO 6887-1, 2017). From each dilution, 0.1 ml was drawn and inoculated onto prepared petri dishes containing sterile BE/C medium; the inoculum was spread, left to solidify, and then incubated at 37°C for 24 h. After incubation, bacteria were counted with the aid of a colony counter where colonies with purple colour were identified as *E. coli* while coliform bacteria were pink. *E. coli* ATCC 25922 was used as positive control. Enumeration of ESBL-producing *Enterobacteriaceae* was done on MacConkey agar (Oxoid Ltd), supplemented with 2 μg/ml of cefotaxime as described by Moremi et al. (2016).

Enumeration of total viable counts (TVCs)

TVCs were enumerated on plate counts agar (PCA) (Oxoid Ltd) at 30°C (ISO 4833-1, 2013). Serial dilutions were made as per ISO 6887-1 (2017), and from each dilution, 1 ml was drawn and inoculated into a sterile petri dish. The molten PCA was poured, mixed, left to solidify and the plate incubated at 30°C for 72 h. Colonies, that is, <300 colony forming unit (cfu) were counted with the aid of a colony counter. Colonies representing different morphological types were selected from the PCA plates and stored in 50% glycerol in liquid nitrogen for further analysis.

Moisture content analysis

The MC in salted sun-dried Nile perch products was determined according to the Association of Official Analytical Chemists Standard (AOAC) method number 950.46 (B) (AOAC, 2006). Briefly, 2 g of the sample (in duplicate) was weighed and evenly distributed into pre-heated petri dishes, then heated in an oven set at 102°C for 16 h parallel to an equal weight of pure pentahydrate copper sulphate ($CuSO_4.5H_2O$) as a control. Afterward, the sample was cooled in desiccator for 30 min and then reweighed. The average MCs (from the duplicates) were calculated and reported in percentage as per AOAC requirements.

Water activity analysis

The A_w was analysed according to the standard method (ISO 21807, 2004) using Novasina water activity meter (Pfaffikon, Switzerland). Briefly, a duplicate 2 g of grinded salted sun-dried sample was measured and placed into the water activity meter and left to stabilise for 20 to 30 min before the reading was recorded. The average of the duplicates was calculated and recorded as the final reading. Figures were reported with three decimals.

Microbial identification on salted sun-dried Nile perch products

The selected isolates from PCA were transported to Denmark for identification using matrix assisted laser desorption ionization-time-of-flight (MALDI-TOF) technology. The isolates were checked for purity following subculture onto blood agar and incubation at 37°C for 24 h, and then a single colony was selected and placed on glass slide for identification in Vitek MS MALDI-TOF Mass Spectrometer (bioMérieux, Inc., France). Identification of isolates was interpreted based on a comparison to SuperSpetra containing sets of genus, species and strains biomarkers characteristic for respective groups of microorganisms as stated in the instructions of the MALDI-TOF. Only isolates with an identification of 80% or more confidence was trusted.

Data analysis

Analysis of data was performed using Stata version 14 (StataCorp LP) descriptive statistics to obtain mean, standard deviation, and to show data variability in different parameters analysed. Also, the frequencies distributions of *Salmonella* spp. in different sample categories were determined. Seasonal variation in the different parameters analysed was analysed using single factor ANOVA. Results were presented in box-plots figures with the significance defined at $P<0.05$.

RESULTS AND DISCUSSION

Overall, high TVCs were reported in salted sun-dried Nile perch products collected during the rainy season with mean counts ranging from 3.3 to 4.5 log cfu/g while those collected during the dry season had lower mean counts ranging from 2.9 to 3.1 log cfu/g ($P<0.05$) (Figure 1a). However, these results are still within the acceptable limit set by the Tanzanian standard, that is, 1.0×10^6 cfu/g (TZS, 1988). The seasonal difference in TVCs was likely attributed to observed unhygienic products handling during, and after processing as well as drying conditions which attracted insects like flies on the dried products as also reported in previous studies (Immaculate et al., 2013; Nagwekar et al., 2017). The study shows the relationship between TVCs, MC, and A_w obtained in salted sun-dried Nile perch products collected in the rainy season and dry season, in that, the increase of MCs and A_w was proportional to the increase of TVCs in samples. The TVCs in Nile perch products were supported by the MC and A_w results obtained. High MC values ranging from 26.4 to 38.0% and A_w of 0.659 to 0.682 were recorded in products sampled during the rainy season when compared to products sampled during the dry season in which, MCs ranged from 18.3 to 24.6% and A_w 0.619 to 0.643, respectively (Figure 1b-1c). The average results of MCs and A_w were within the limits range required in salted dried fish and fish products (MCs 15-30%) and (A_w 0.60-0.75); but, were higher than the minimum limits for prevention of bacterial growth (MCs <15%; A_w <0.6) as specified in standards (IS, 2001; ISO, 1999). High TVCs, MCs and A_w values as seen in our products sampled during the rainy season are normally associated with high humidity, rainy weather conditions and low drying temperature (Logesh et al., 2012; Patterson and Ranjitha, 2009). Thus, the drying time

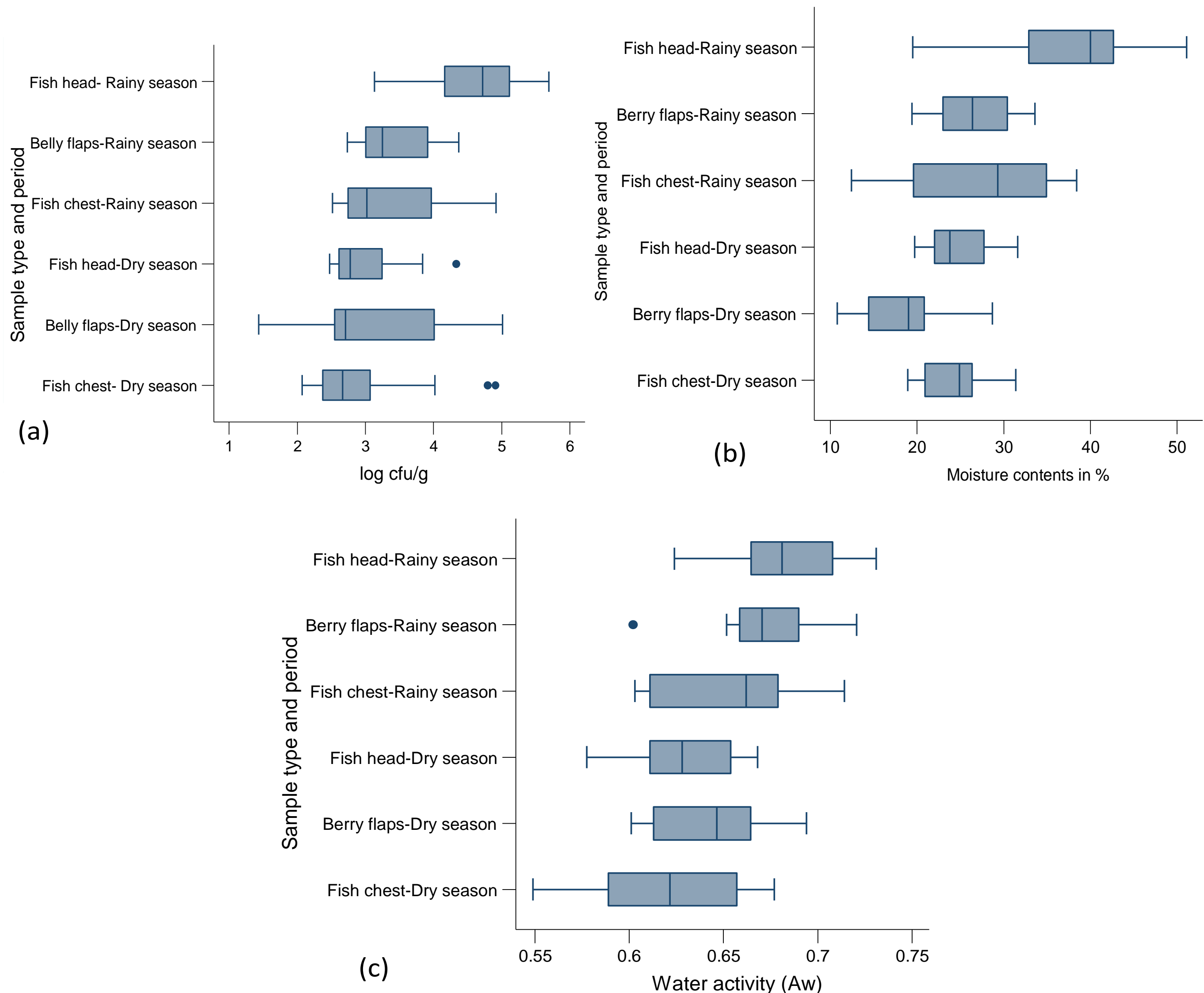

Figure 1. (a) Box plot of total viable bacterial counts in salted sun-dried Nile perch products, **(b)** Box plot of moisture contents (%) in salted sun-dried Nile perch products, (c) Box plot of water activity (A_w) in salted sun-dried Nile perch products.

during the rainy season is longer than in the dry season which together with poor hygienic conditions of processing premises might contribute to the products being more exposed to insect infestation and bacterial contamination (Ikwebe et al., 2017; Sivaraman and Siva, 2015). The study results are in agreement with other studies reporting high TVCs in salted sun-dried fish products (Nagwekar et al., 2017; Saritha et al., 2012; Sulieman and Mustafa, 2012). Also, MC values found in the current study were lower than the ones reported for different salted dried fish (Nuwanthi et al., 2016), but they were in agreement with the studies reported by Majumdar et al. (2017). The A_w values in this study were lower than 0.8 described by Koral et al. (2013), and 0.77 reported by Lin et al. (2012); irrespective of the season of sampling, however, they were higher than 0.5 reported by Geetha et al. (2014). Salt and drying processes are key factors contributing significantly to reduction of TVCs, MCs, and A_w in products (Ginigaddarage et al., 2018; Majumdar et al., 2017). The MCs and A_w values reported in the different salted sun-dried Nile perch products can be expected to support microbial growth during the rainy season so that products undergo microbial spoilage faster than those processed in the dry season.

Although the concentrations of bacteria suggest that the salted sun-dried Nile perch products are safe for human consumption, some of the identified bacteria especially *Staphylococcus xylosus, Bacillus megaterium*, *Klebsiella oxyota,* and *Enterobacter cloacae* might affect the products safety. These bacteria have been reported

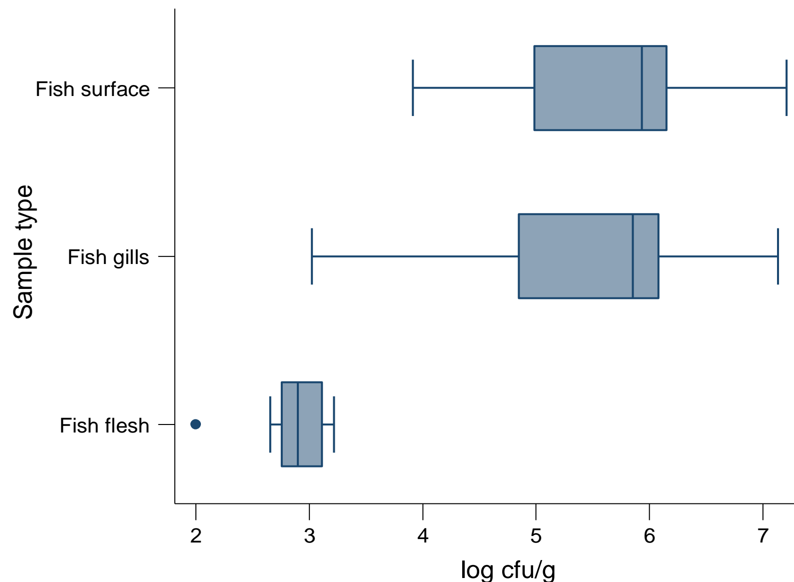

Figure 2. Box plot of total viable counts in frozen Nile perch samples.

in other studies to be responsible for histamine formation in salted fish and therefore pose a risk of histamine toxicity to humans consuming the products (Koral et al., 2013; Lin et al., 2012; Tsai et al., 2005). Histamine is a toxin formed by microbial decarboxylation of histidines as a result of time-temperature irregularity/abuse during storage of salted fish and/or fish products (Koral et al., 2013; Nagwekar et al., 2017; Tsai et al., 2005). The current study did not analyse histamine in salted sun-dried products; however, it is an area worthy of further studies in order to quantify the potential food safety risks to humans.

Our findings also shows that, only three out of 15 samples of belly flaps collected in rainy season had mean TCCs of 4.4×10^1 cfu/g and fish heads (1/15) had 1.4×10^1 cfu/g and *Salmonella* spp. was detected on the fish heads (6.7%, n=15) and, belly flaps (6.7%, n=15) samples collected during the rainy season whereas samples did not contain *Salmonella* spp. in the dry season. These findings are similar to those reported by Gabriel and Alano-Budiao (2015). Presence of TCCs and *Salmonella* spp. in dried products could be an indication of the poor products handling after processing leading to cross-contamination with bacteria from the environment. The absence of *E. coli* and ESBL-producing *Enterobacteriaceae* in the tested samples suggests that salted sun-dried Nile perch products were not contaminated with faecal bacteria.

Microbial load in fresh fish are an important determinant of the storage time of the products. The results of TVCs in frozen Nile perch showed high counts in skin and low in flesh (Figure 2). The high TVCs indicate that the fish can rapidly decompose and undergo spoilage when exposed to ambient temperature, as a result of metabolic activities. Moreover, *E. coli* concentrations were low where; 3/30 of fish gills had mean counts of 2.4 log cfu/g and skin (2/30) with counts of 2.1 log cfu/g while *E. coli* was not detected in flesh samples. The TCCs showed that fish gills (13/30) had mean counts of 2.8 log cfu/g whereas skin (7/30) had mean counts of 3.0 log cfu/g. The presence of *E. coli* and TCCs in samples albeit in low concentrations may imply poor fish handling as also described in other studies (Saritha et al., 2012; Sulieman and Mustafa, 2012) despite the fact that they were within acceptable limits as stated in Tanzania standard (TZS, 1988). The reported *Salmonella* spp. in fish gills (13.3%, n=30) and skin (6.7%, n=30) may suggest that the contamination may have occurred in the aquatic environment where fish were caught rather than from storage facilities.

Conclusions

The results of the present study provide important baseline information on the status of microbial quality in Nile perch products, which is essential for policy decisions geared towards safeguarding the quality and safety of these products to consumers. The different bacteria species recovered from salted sun-dried products provide an indication that there is a need for public authorities in the fisheries sector to recommend hygienic procedures to fit in salted sun-drying processing method.

Adoption of other drying methods that minimise contamination such as solar conduction dryers needs to be considered in order to preserve the quality and safety of Nile perch products in the study area.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Isolation and identification of bacteria from high-temperature compost at temperatures exceeding 90°C

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Conventional composts exhibit temperatures ranging from 50 to 80°C during organic waste degradation by microorganisms. In high-temperature compost, temperatures can reach ≥90°C with appropriate bottom aeration. To elucidate specific characteristics of the bacterial activity in high-temperature compost and to regenerate a high-temperature compost from isolates, bacterial isolation and characterization were performed. Although the isolated taxa varied depending on sample and temperature, the use of gellan gum medium and cultivation at 60°C led to high diversity among the isolated taxa. In addition, combining the use of the compost extract with water-solvent medium led to the isolation of more diverse species. Based on 16S rRNA gene sequencing, the isolates shared ≥99% similarity with *Geobacillus thermodenitrificans*, *Ureibacillus* spp. (*Ureibacillus suwonensis*, *Ureibacillus themosphaericus*) and *Aeribacillus pallidus*, and these isolates were isolated from both steady-state and newly prepared small-scale composts. Thus, these taxa were considered to be frequently observed regardless of the composting process. Although the frequency of isolation of mesophilic bacteria from this high-temperature compost was lower than that from ordinary composts, these bacteria have been isolated from ordinary composts and there was a discrepancy between the *in situ* compost temperature (≥90°C) and their maximum growth temperature (≤70°C).

Key words: High-temperature compost, *Geobacillus thermodenitrificans*, *Ureibacillus suwonensis*, *Ureibacillus thermosphaericus*, *Aeribacillus pallidus.*

INTRODUCTION

Composting is an environmentally friendly process for degradation of organic waste. Organic waste is broken down concomitant with successive changes in the microbiota at various stages of composting. Wastes that have been completely broken down are then used to fertilize soil for plant growth. During ordinary composting, the temperature increases to approximately 50–80°C, at which microbial activity increases (Ryckeboer et al.,

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2003; Kumar, 2011). The heat produced is convenient for fertilization of soil for agricultural purposes because this heat is detrimental to harmful microorganisms and weed seeds. When composts are subjected to appropriate aeration, the temperature can exceed 90°C. Although there have been many studies on the constituent microorganisms in ordinary composts (Strom, 1985; Dees and Ghiorse, 2001; Ryckeboer et al., 2003; Wang et al., 2007; Kumar 2011; Antunes et al., 2016; Cerda et al., 2017), there have been only a few studies on microorganisms in high-temperature composts (≥90°C) (Oshima and Moriya, 2008; Yoshii et al., 2013; Tashiro et al., 2016). Although several examples of high-temperature composts are available, the operational procedures and the types of organic wastes applied might differ. Therefore, a different microbiota may exist in each type of high-temperature compost.

The ratio of microorganisms that can be isolated to preexisting background microorganisms in compost samples is low similar to that in ordinary environmental samples (Amann et al., 1995; Rappé and Giovannoni, 2003). Composting environments are specific environments that fluctuate over relatively large temperature ranges. In addition, many possible microbes can be present in these environments, depending on maintenance procedures and added substrates. Therefore, isolation of microorganisms is of great interest for identification of novel genetic resources. Several microorganisms associated with novel genera have been isolated (Ohno et al., 2000; Hatayama et al., 2005, 2006; Yabe et al., 2010, 2011a, b; Moriya et al., 2011; Wu et al., 2014; Sakai et al., 2015; Siddiqi et al., 2016). Microorganisms that belong to new genera can potentially be isolated from composts by testing new samples and developing new media.

Isolates from high-temperature compost have not been well explored. Application of isolates from compost will allow rapid production of high-temperature compost, which will increase the efficiency of waste decomposition. In this study, to understand the type of bacteria exist in high-temperature compost and to regenerate high-temperature compost by utilizing these isolates in a future study, this study aimed to isolate bacteria from the following high-temperature composts (≥90°C): an already running steady-state compost in several temperature states and a newly prepared small-scale compost without added substrate. Attempts were made to isolate the associated bacteria using various media, and then examined the taxonomic positions of these isolates. Finally, the isolates were compared with identified clones using a culture-independent method.

MATERIALS AND METHODS

Compost samples

Compost samples were obtained from the Chitose Recycling Factory (42° 51' 35'' N, 141° 42' 31'' E) in Chitose, Hokkaido. Various solid wastes or effluents from raw garbage, the food industry and fisheries were added to a pail (approximately 10 × 10 × 3 m, width × length × height) with this compost to degrade the organic compounds and evaporate the water in the waste. Samples from a steady-state compost, which was maintained for 10 years at different temperatures (90-100°C), were obtained at different sampling times (Table 1). In addition to the steady-state compost, samples were obtained from a newly prepared compost (approximately 1 × 1.5 × 1.5 m, width × length × height), which was not affected by loaded wastes. The compost was prepared by adding only water, no substrate, to the original material containing a composted tree-sourced material mixture (mostly root and bark), and this mixture was used as a sample for bacterial isolation.

Bacterial isolation and cultivation

Compost extract was used as an ingredient in the medium to accelerate colony formation by bacterial strains that are difficult to grow in ordinary media. The compost extract was prepared by mixing 100 g of the obtained compost with 1 L of distilled water and autoclaving at 121°C for 20 min. Then, the supernatant was obtained for use. The medium listed in Table 1 for bacterial isolation was used for the corresponding sample described in Table 2. The compost aliquot was first diluted ten-fold (w/w) with sterilized physiological saline. The liquid was serially diluted up to 10^5-fold, and the 10^3–10^5-fold dilutions were used to inoculate the medium, followed by incubation under aerobic conditions at 50–70°C for 2 days. We isolated colonies that were dependent on the variation and re-isolated these colonies at least three times from each medium or culture procedure. The composting process involves degradation introduced wastes. Therefore, it was important to examine whether the isolates could decompose substrates. Hydrolysis of starch (amylase) and casein (protease) and catalase activity were examined according to the methods described in Cowan and Steel's manual (Barrow and Feltham, 1993). Hydrolysis of xylan and cellulose was determined using 1% of each substrate according to the method described by Teather and Wood (1982).

DNA extraction, PCR and clone library construction

Colonies of the isolates from the compost samples were grown on the corresponding agar plates as described in Table 1, and DNA was extracted using the InstaGene matrix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. For the culture-independent approach based on a 16S rRNA gene library, DNA was directly extracted from an aliquot of the compost sample using ISOIL (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. PCR was performed to identify isolates obtained via the culture-dependent approach and to construct the 16S rRNA gene library in the culture-independent approach using the DNA extracted from the isolates and from the compost samples, respectively. The universal primers 9F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1514R (5'-AAGGAGGTGATCCAGCC-3') were used for PCR (Paster et al., 2002). The solution (100 μL) for the PCR consisted of 10 μL of 10 × PCR buffer, 8 μL of 2.5 mM dNTP mix, 100 ng of isolated DNA, 5 U of Ex Taq DNA polymerase (TaKaRa Bio Inc., Otsu, Japan) and 20 pmol of each primer. The PCR was performed under the following conditions: 94°C for 1 min followed by 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1.5 min. The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. The PCR products from the isolates were directly used for sequence determination. The PCR product for the 16S rRNA clone library (culture-independent analysis) was cloned in *Escherichia coli* DH5α with the pT7Blue-2 vector system (Novagen, Madison, WI, USA) according

Table 1. Different media used in this study to isolate bacteria from elevated-temperature compost.

Medium name	Medium composition
SMA*	5 g casein peptone, 2.5 g yeast extract, 1 g glucose, 15 g agar in 1 L distilled water (pH 7)
PYG	5 g casein peptone, 3 g yeast extract, 1 g K_2HPO_4, 30 g gellan gum in 1 L distilled water (pH 8)
A-1	5 g casein peptone, 3 g bonito meat extract, 20 g agar in 1 L compost extract (pH 8)
A-2	5 g casein peptone, 3 g bonito meat extract, 20 g agar in 1 L distilled water (pH 8)
B-1	5 g casein peptone, 3 g bonito meat extract, 20 g agar in 1 L distilled water (pH 7.5)
B-2	5 g casein peptone, 3 g bonito meat extract, 20 g agar in 1 L compost extract (pH 7.5)

*Standard method agar.

Table 2. Elevated-temperature compost samples used for isolation and clone analysis.

Sample name	Temperature of compost (°C)	Steady state or early phase	Method	Medium used	Culture temperature (°C) and pH
CS1	96	Steady state	Isolation	SMA	50, pH 7
CS2	100	Steady state	Isolation	SMA	50, pH 7
CS3	90	Steady state	Isolation, clone analysis	PYG	60, 70, pH 8
CS4	90	Steady state	Isolation	A-1, A-2	60, pH 8
ChS	90	Early phase	Isolation	B-1, B-2	60, pH 7.5

to the manufacturer's instructions. Approximately 30 randomly selected clones were examined for the correct insert size by vector-targeted PCR followed by gel electrophoresis. Approximately 400–600-bp partial sequences from the amplified approximately 1,500-bp full-length sequence were analyzed as described below.

DNA sequencing and sequence assignment

The DNA sequence was determined by the dideoxy chain termination method using a BigDye Terminator Cycle Sequence Kit (Applied Biosystem, Foster City, CA, USA) and an automated DNA sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems). The sequence assignments were determined by a BLAST search (Altschul et al., 1990). The sequences reported in this study were deposited in the DDBJ database under DDBJ/EMBL/GenBank accession numbers LC315695–LC315768 for the isolates and LC315983–LC316008 for the culture-independent clone libraries.

Phylogenetic analysis

Phylogenetic analysis was performed using the determined 16S rRNA gene sequence of the family Bacillaceae. The sequences were aligned, and the consensus sequence was determined using CLUSTAL W (Thompson et al., 1994). A phylogenetic tree was constructed using the neighbor-joining method, and the distances between sequences (K_{unc} value) were calculated using Kimura's two-parameter model (Kimura, 1980; Saitou and Nei, 1987) in MEGA 7 (Kumar et al., 2016). The confidence values for the branches of the phylogenetic tree were determined by bootstrap analysis (Felsenstein 1985) based on 1000 resamplings.

RESULTS

Isolation of bacteria

To understand the species of bacteria that can be isolated from high-temperature (≥90°C) compost, 80 strains were isolated from 5 samples that exhibited different temperatures (90–100°C). In addition, although four samples were obtained from the steady-state compost (including 2 composts at temperatures higher than 95°C), one sample was obtained from newly prepared small-scale compost without added substrate for microbial growth (Table 2). However, several types of media were used for bacterial isolation from each sample. Notably, these media used agar or gellan gum for solidification and included or lacked compost extract (Table 1). Although the *in situ* temperature was ≥90°C, no growth was observed at temperatures greater than 80°C for any of the samples used in this study in either solid or liquid medium. Although the medium composition and temperature for isolation were different for each sample, the differences in the isolated bacteria were dependent on the sample. This result suggests that the microbiota of high-temperature compost varies depending on the *in situ* temperature, sampling location or waste degradation phase.

The most diverse species of bacteria were isolated from the CS3 sample using PYG (peptone, yeast extract and gellan gum) medium (incubated at 60°C), which contained gellan gum (Table 1) because of the characteristics of the sample and the use of gellan gum. Only *Thermus thermophilus* or *T. thermophilus*-related strains were isolated when PYG medium was used for the CS3 sample when the incubation temperature of the culture was 70°C (Table 3). Based on a comparison of results for media A-1 with A-2 and B-1 with B-2, the combined use of the compost extract with water-solvent medium has led to the isolation of highly diverse species.

Table 3. Summary of the isolates recovered using the different media. Bacterial identification was based on ≥99% similarity in the 16S rRNA gene sequence.

Medium/Sample names Sample/Culture temp. (°C)	SMA/CS1 96/50	SMA/CS2 100/50	PYG*/CS3 90/60	PYG*/CS3 90/70	A-1**/CS4 90/60	A-2/CS4 90/60	B-1/ChS 90/60	B-2**/ChS 90/60	Total number of isolates[¶]
Bacillus hisashii	3								3 (1)
Bacillus thermoamylovorans	1								1 (1)
Bacillus subtilis	1								1 (1)
Bacillus thermoamylovorans	1								1 (1)
Bacillus thermocloaceae			1		4				5 (2)
Bacillus kokeshiformis			1						1 (1)
Bacillus termolactis			1						1 (1)
Bacillus borborid								1	1 (1)
Ureibacillus suwonensis		6						1	7 (2)
Ureibacillus thermosphaericus			3					1	4 (2)
Ureibacillus terrenus			11						11 (1)
Geobacillus thermodenitrificans			4			7	6		17 (3)
Aeribacillus pallidus						1	1	1	3 (2)
Noviobacillus thermophilus					2				2 (1)
Thermobifida fusca			2						2 (1)
Thermus thermophilus				5					5 (1)
No. of strains exhibiting ≤98% similarity to reported species	1	1[§]	1[§]	5	1[¶]				

*Only this medium used gellan gum for solidification. ** Compost extract-containing media. [¶]Numbers in the brackets indicate detected sample counts.
[§]This was due to the sequence quality. DNA extraction for 16S rRNA gene sequence analysis was performed by using the stain grown in the corresponding isolation medium (Table 2).
[¶]This strain was CS4-7 (LC315748) exhibited 91% similarity with *Melghiribacillus thermophilus* (NR_134761) (data not shown).

Ureibacillus spp. were isolated from medium that contained compost extract, while *Geobacillus thermodenitrificans* was not isolated from this medium (Table 3). Although most of these isolates were not very distantly taxonomically related to reported species, one strain, namely, CS4-7, was very different from a reported species (*Melghiribacillus thermohalophilus*, 91%). This strain was isolated from A-1 medium, which contained compost extract.

Although *Ureibacillus terrenus* and *T. thermophilus* were isolated from only one sample (CS3), based on the identification of isolates by 16S rRNA gene sequencing, strains that exhibited higher than 99% similarly with *G. thermodenitrificans*, *Ureibacillus suwonensis*, *Ureibacillus thermosphaericus*, *U. terrenus*, *Bacillus thermocloaceae*, *Aeribacillus pallidus* and *T. thermophilus* were frequently isolated when the incubation temperature of the medium was 50–70°C (Table 3). Although high-temperature compost was used in the present study as the source of these isolates, the isolated species are commonly observed in ordinary composts. In addition, the isolates did not exhibit growth at temperatures greater than 80°C (data not shown), and there have been no reports of the corresponding identified species growing at temperatures greater than 80°C (Table 4), which indicated a discrepancy between the isolated taxa and the *in situ* temperatures of the sources. However, this study indicates that the frequency of isolation of mesophilic microorganisms was lower in high-temperature compost (only two strains, identified as *Bacillus subtilis* (>99% similarity) and *Sphingomonas melonis* (98% similarity) isolated from CS1 sample) than in moderate-temperature compost (Dees and Ghiorse, 2001; Li et al., 2014; López-González et al., 2015).

Table 4. Growth temperature ranges and substrate utilization of the isolates from the CS3 sample.

Strain	Closely associated phylogenetic relative	Growth temperature range (°C)*	Cellulase	Xylanase	Protease (casein)	Amylase	Catalase
CS3-1	*Bacillus thermocloaceae* (NR_036986)	55-65	ND	ND	–	ND	ND
CS3-2	*Ureibacillus thermosphaericus* (NR_040961)	37-60	–	–	–	–	+
CS3-3	*Ureibacillus thermosphaericus* (NR_040961)	37-60	–	–	–	–	+
CS3-4	*Ureibacillus terrenus* (NR_025394)	42-65	–	–	–	–	+
CS3-5	*Geobacillus thermodenitrificans* (NR_0432021)	45-70 (60)	–	–	–	+	+
CS3-6	*Ureibacillus thermosphaericus* (NR_040961)	37-60	–	–	–	–	+
CS3-7	*Ureibacillus terrenus* (NR_025394)	42-65		–	+	–	+
CS3-8	*Geobacillus thermodenitrificans* (NR_0432021)	45-70 (60)	–	–	–	+	+
CS3-9	*Ureibacillus terrenus* (NR_025394)	42-65	–	–	–	–	+
CS3-10	*Thermobifida fusca* (NR_14411)	35-60	+	+	+	+	+
CS3-11	*Ureibacillus terrenus* (NR_025394)	42-65	–	–	–	–	+
CS3-12	*Bacillus kokeshiformis* (NR_133	35-61 (50)	–	–	ND	+	+
CS3-13	*Thermobifida fusca* (NR_14411)	35-60	+	+	+	+	+
CS3-14	*Ureibacillus terrenus* (NR_025394)	42-65	–	–	–	–	+
CS3-15	*Ureibacillus terrenus* (NR_025394)	42-65	–	–	–	–	+
CS3-16	*Ureibacillus terrenus* (NR_025394)	42-65	–	–	–	–	+
CS3-17	*Ureibacillus terrenus* (NR_025394)	42-65	–	–	–	–	+
CS3-18	*Ureibacillus terrenus* (NR_025394)	42-65	–	–	–	–	+
CS3-19	*Geobacillus thermodenitrificans* (NR_0432021)	45-70 (60)	–	–	–	+	+
CS3-20	*Geobacillus thermodenitrificans* (NR_0432021)	45-70 (60)	–	–	–	+	+
CS3-21	*Ureibacillus terrenus* (NR_025394)	42-65	–	–	–	–	+
CS3-22	*Ureibacillus terrenus* (NR_025394)	42-65	–	–	–	–	+
CS3-23	*Bacillus thermolactis* (NR_115226)	40-60	–	–	–	+	+
CS3-24	*Ureibacillus terrenus* (NR_025394)	42-65	–	–	ND	ND	ND

All isolates exhibited 99% similarity with closely associated phylogenetic relatives. *Growth temperature ranges were cited from Zarilla and Rerry (1987); Zhang et al. (1998); Fortina et al. (2001); Kim et al. (2006); Cihan et al. (2011); Coorevits et al. (2011); Wang et al. (2013); Poudel et al. (2014); and Yang et al. (2015). The numbers in brackets are the optimum growth temperatures.

It is expected that many strains can produce macromolecule degradation-related enzymes because various wastes were frequently added to this compost. Although eight strains exhibited amylase activity among the twenty-four strains, only three strains exhibited protease activity. *Thermobifida fusca* strains, belonging to the phylum Actinobacteria, were isolated from the CS3 sample. Only these two strains produced multiple enzymes that degraded various macromolecules (Table 4).

The temperature of the compost exceeded 100°C when the CS2 sample was obtained. Although the heat was apparently produced as a result of vigorous microbial activity, the diversity of the isolates decreased at this temperature (Table 3). The colony morphology on the plate inoculated with a sample from the compost with a temperature greater than 100°C was homogenous (data not shown). Seven colonies were picked from the plate, and the 16S rRNA gene sequences

of the isolates were determined. According to the results, although one strain exhibited 98% similarity to *U. suwonensis* due to the low quality of the sequence, 6 isolates were very similar (≥99% similarity) to *U. suwonensis* (Table 3).

There is a possibility that the generation of high temperatures (≥90°C) is dependent on the mass of the compost and the types of waste added. Therefore, we tried to prepare small-scale compost by adding only water without adding waste. In addition, to understand the types of bacteria that can be isolated from the early phase of high-temperature compost, bacterial isolation was performed with two kinds of medium one of which contained compost extract. As in the steady-state compost, *G. thermodenitrificans*-related strains (≥99% similarity) were most frequently isolated from this sample (6 strains; Table 3). In addition to these species, *A. pallidus*-related strains (≥99% similarity) were isolated from both types of medium (with and without compost extract; Table 3). Isolates sharing ≥99% similarity with *G. thermodenitrificans*, *Ureibacillus* spp. (*U. suwonensis*, *U. themosphaericus*) and *A. pallidus* were isolated from both steady-state and newly prepared small-scale composts. Therefore, these taxa were considered to be frequently observed isolates regardless of the composting process. The introduction of a mixture of these species at the start of composting may accelerate the initiation of high-temperature composts in the absence of materials such as compost seed, from which the expected microbiota originates.

To understand the phylogenetic relationships among the isolates, a neighbor-joining phylogenetic tree was constructed (Figure 1). According to the tree, the predominant isolates throughout the samples, namely, the *G. thermodenitrificans*-related strains, were quite similar phylogenetically and had slightly different phylogenetic positions from the type strain of *G. thermodenitrificans*. In addition, the *U. suwonensis* and *U. thermosphaericus*-related strains exhibited slightly different phylogenetic positions from the type strains of each species belonging to this genus. In the clade containing *Bacillus kokeshiiformis*, various species exhibited similar phylogenetic positions (Coorevits et al., 2011). Therefore, to assess the novelty of the strains CS3-12 and CS3-23, additional detailed experiments, such as DNA-DNA hybridization, are necessary. Other isolates located at almost the same phylogenetic positions corresponded to the most closely related taxa.

Culture-independent analysis

The most diverse species of bacteria were isolated from the CS3 sample using PYG, which contained gellan gum. The culture-independent approach (clone analysis) was also applied to the CS3 sample (Table 5). Although many diverse bacterial strains were isolated from the samples, there was a difference in the bacterial diversity of the isolates between the culture-dependent and culture-independent approaches. More than 42% of the isolated clones shared less than 95% similarity with reported gene sequences. The results described above suggest that most of the bacteria in the high-temperature compost could not be easily isolated by ordinary bacterial isolation methods. The predominant group was the family Bacillaceae. Based on classification at the genus level, the genera *Bacillus*, *Ureibacillus* and *Paenibacillus* were major constituents. Although strains sharing 95-96% similarity with *Paenibacillus sabinae* were frequently detected in the culture-independent analysis, this taxon was not isolated. Although strains sharing greater than 99% similarity with *T. fusca*, which exhibited multiple enzyme activities (Table 4), were isolated in the medium containing gellan gum, this taxon was not detected by the culture-independent approach. Although the compost was aerated, obligate anaerobic bacterial clones belonging to the family Thermoanaerobacteraceae were detected. This finding indicates that although the compost was aerated, there were niches where obligate anaerobes could thrive in this compost.

DISCUSSION

To understand the differences between the bacteria isolated from high-temperature compost and ordinary compost, both culture-dependent and culture-independent approaches were used to study high-temperature compost. We examined the probability of isolating bacteria from high-temperature compost using different samples and media. However, there was a discrepancy between the *in situ* compost temperature (≥90°C) and the maximum growth temperature (<80°C) ranges in the medium used to grow the isolates. This discrepancy could be a result of the temperature inside the compost being higher than out sites (that is, at the surface), with most of the microbial activity occurring in these low-temperature niches. Our isolates, which were buried inside the compost, were not able to propagate at temperatures higher than 80°C, in the laboratory experiment. These isolates are thought to have remained alive *in situ* because they were isolated from the environment. However, it is not known whether these isolates were metabolically active *in situ*. On the other hand, according to the culture-independent analysis, there may exist unculturable microorganisms that can grow at temperatures greater than 80°C under specific culture conditions that were not tested in this study, such as strictly anaerobic, microaerobic or low-nutrient conditions (Janssen and Yates, 2002).

Although isolates from high-temperature compost (≥90°C) were not able to grow at temperatures higher than 90°C, the frequency of isolation of mesophilic bacteria was lower than that from ordinary temperature compost (50 to 80°C) (Dees and Ghiorse, 2001; Li et al., 2014; López-González et al., 2015). However, no isolate

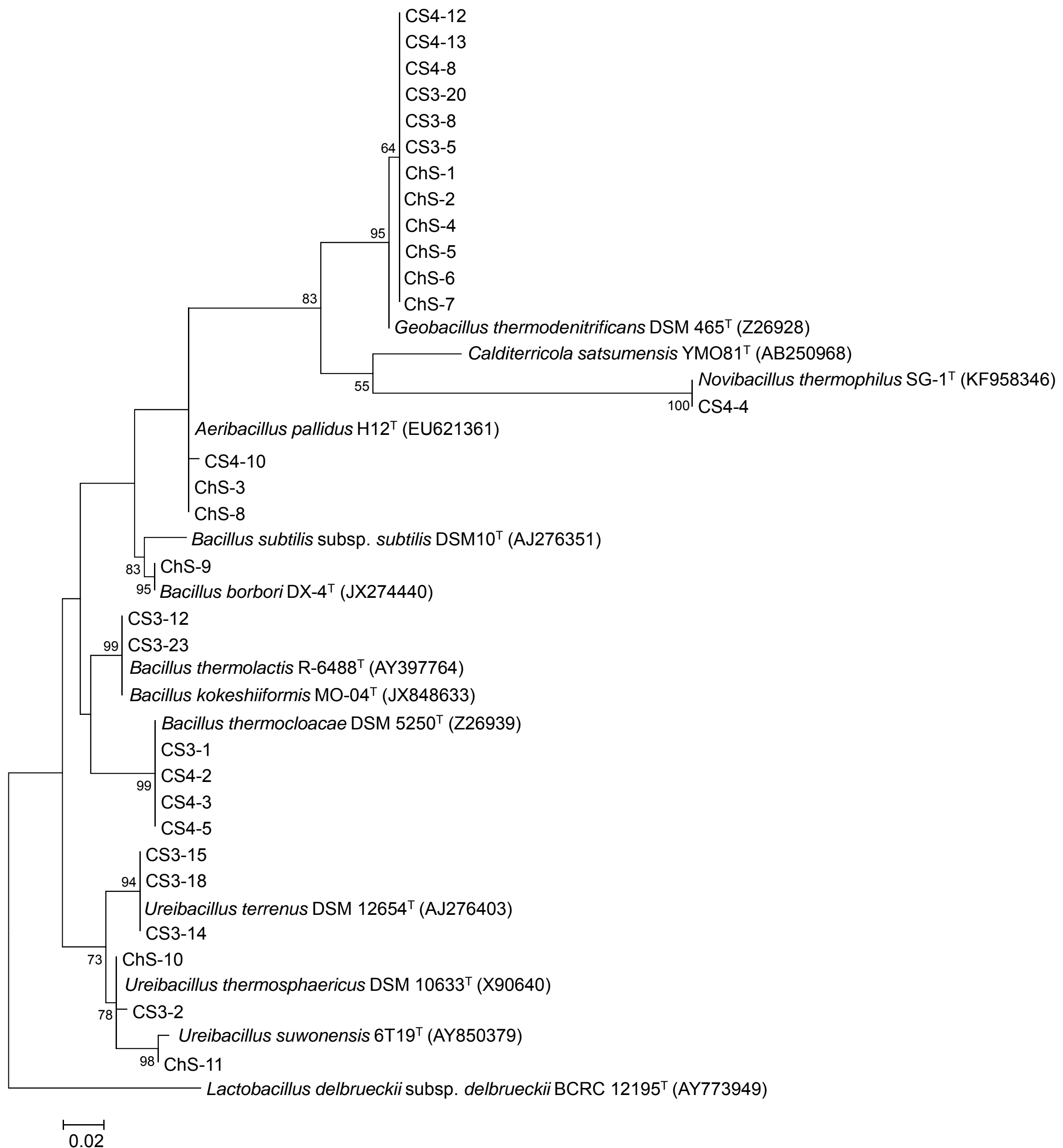

Figure 1. Phylogenetic tree showing the positions of isolates from three different samples of high-temperature compost. The strain name represents the sample name listed in Table 2. Bootstrap percentages (based on 1000 replicates) >50% are shown at the nodes. *Lactobacillus delbrueckii* subsp. *delbrueckii* BCRC 12195^T was used as an outgroup. Scale bar=0.01 substitution per nucleotide position.

could grow at temperatures higher than 90°C. This finding indicates that selective pressure against mesophilic bacteria in high-temperature compost was higher than that in ordinary compost. Although there are two previous examples of isolates of new species of bacteria that can grow at 80°C (Moriya et al., 2011), the isolation of bacteria that can grow at temperatures higher than 90°C from high-temperature compost has not been previously reported (Oshima and Moriya, 2008; Yoshii et al., 2013). We believe there was little possibility of isolating microorganisms that could grow at temperatures higher than 80°C from the compost used in the present study. Aliquots of the compost samples were used to inoculate broth medium, and microbial growth was never

Table 5. Sequence analysis of 16S rDNA obtained from clone library constracted from the samples from 90°C compost.

Clone	Length (bp)	Accessing no.	Closely identified phylogenetic relative	Similarity (%)	Clone	Length (bp)	Accessing no.	Closely identified phylogenetic relative	Similarity (%)
CC-1	438	LC315983	*Ureibacillus thermosphaericus* (NR_040961)	98	CC-14	590	LC315996	*Bacillus thermotolerans* NR_118456	99
CC-2	535	LC315984	*Ureibacillus thermosphaericus* (NR_040961)	97	CC-15	496	LC315997	*Anoxybacillus calidus* NR_125532	92
CC-3	547	LC315985	*Bacillus infernus* (NR_027227)	93	CC-16	593	LC315998	*Bacillus acidicola* NR_041942	94
CC-4	549	LC315986	*Bacillus massiliogorillae* (NR_133029)	93	CC-17	556	LC315999	*Bacillus thermocloacae* (NR_036986)	99
CC-5	466	LC315987	*Geobacillus icigianus* (NR_134736)	90	CC-18	427	LC316000	*Fervidicola ferrireducens* (NR_044504)	94
CC-6	556	LC315988	*Bacillus thermocloacae* (NR_036986)	98	CC-19	418	LC316001	*Paenibacillus sabinae* (NR_121732)	96
CC-7	553	LC315989	*Ureibacillus thermosphaericus* (NR_040961)	95	CC-20	430	LC316002	*Caldilinea aerophila* (NR_074397)	88
CC-8	561	LC315990	*Geobacillus thermodenitrificans* (NR_043021)	100	CC-21	403	LC316003	*Paenibacillus sabinae* (NR_121732)	96
CC-9	560	LC315991	*Paenibacillus sabinae* (NR_122066)	96	CC-22	528	LC316004	*Melghiribacillus thermohalophilus* (NR_134761)	87
CC-10	575	LC315992	*Bacillus thermocloacae* (NR_036986)	99	CC-23	459	LC316005	*Thermus thermophilus* (NR_037066)	98
CC-11	564	LC315993	*Bacillus thermocloacae* (NR_036986)	99	CC-24	565	LC316006	*Bacillus subterraneus* (NR_104749)	94
CC-12	570	LC315994	*Ureibacillus thermosphaericus* (NR_040961)	98	CC-25	580	LC316007	*Bacillus thermocloacae* (NR_036986)	90
CC-13	574	LC315995	*Thermacetogenium phaeum* (NR_074723)	92	CC-26	580	LC316008	*Paenibacillus sabinae* (NR_122066)	95

observed at 80°C (data not shown). This finding suggested that the *in situ* compost environment has not been adequately reproduced in the laboratory. Although we isolated as many as 80 colonies of bacteria from the compost using various conditions, none of the isolates could grow at 80°C. Therefore, the existence of many types of bacteria that can grow at temperatures higher than 80°C under laboratory culture conditions is highly unlikely. Bacteria that exhibited growth at sites with low localized temperatures (e.g., at the surface) were occasionally introduced into the interior of the compost and we isolated surviving microorganisms that were not able to grow at temperatures higher than 80°C.

Another possibility is that there is a difference in moisture content between the *in situ* compost and the recovery medium in the laboratory. The relatively higher moisture content in the media used in the laboratory may have hindered bacterial growth. Although some thermophiles exhibit metabolic activity at temperatures higher than 90°C, these organisms can be expected to exhibit growth at lower temperatures. Under such extreme conditions, there is a gap between the temperatures suitable for metabolism and those suitable for propagation. For example, it has been reported that *Amphibacillus iburiensis* can survive at pH values higher than 10.5 and exhibits indigo-reducing activity at those pH values (Hirota et al., 2013). However, bacterial propagation is observed at relatively low pH values under laboratory conditions. Although there was apparent metabolic activity in the form of acid production by the inoculated cells in broth adjusted to a pH value greater than pH 10, actual growth was initiated at pH 8.0–9.1, with optimum growth observed at pH 8.9–9.1 (30°C) (Hirota et al., 2013). Thus, we hypothesize that there are bacterial niches that exhibit temperatures lower than that inside the compost and that the temperature range in which bacteria are able to perform metabolic activity is different from the range in which bacterial propagation can occur.

We attempted to isolate as many colonies as possible from various compost samples that exhibited different temperatures (90-100°C). However, in many cases, no colony diversity was observed when an ordinary agar plate was used for isolation using composts with >90°C temperatures. When gellan gum was used and in sample CS3 (90°C), relatively diverse micro-organisms were isolated. The superiority of gellan gum over agar has been reported previously due to the prevention of H_2O_2 production by this material (Tanaka et al., 2014). Although most of the isolates produced catalase when grown in this medium under laboratory conditions, the physiological states of these isolates *in situ* may not be as good as those under laboratory conditions. Therefore, the presence of H_2O_2 in the medium may inhibit colony formation. In addition, the effect of gellan gum may be attributed to not

only the suitability of the medium but also the high microbial activity of part of the sample used (CS3). Although different media were used for different samples, we believe that the microbial diversity observed was due to changes in the microbiota and bacterial localization. This finding was consistent with the fluctuation in temperature, the uniformity of the substrates and the inhomogeneity of the compost.

Compost extracts or extracts from isolates have been previously used to isolate bacteria from compost (Rhee et al., 2002; Bae et al., 2005; Yabe et al., 2011). Compost extract was used to expand the known diversity of identified bacterial strains. Although growth stimulation was observed upon addition of the extract, an adverse effect was also observed. This adverse effect may also be important for isolation of undiscovered bacteria. There are several examples of the isolation of novel microorganisms by the addition of antibiotics to the isolation medium (Yabe et al., 2009, 2010, 2011a, b). Furthermore, bacteria have been successfully isolated by the addition of bacterial cell extracts (Rhee et al., 2002; Bae et al., 2005). In the present study, one isolated strain, namely, CS4-7, exhibited low similarity with a reported species (*Melghiribacillus thermohalophilus*, 91%). Various nutrient concentrations in the medium, especially low nutrient concentrations are predicted to lead to the isolation of increasingly wide ranges of bacterial species (Janssen and Yates, 2002).

Isolates sharing ≥99% similarity with *G. thermodenitrificans*, *Ureibacillus* spp. (*U. suwonensis*, *U. themosphaericus*) and *A. pallidus* were isolated from both steady-state and newly prepared small-scale composts. These genera are frequently isolated from ordinary compost at temperatures higher than 50°C (Baharuddin et al., 2010; Li et al., 2014). Among the above described taxa, a *G. thermodenitrificans*-like strain was most frequently observed, and a similar observation was reported previously in ordinary compost (Li et al., 2014). This suggests that the *G. thermodenitrificans*-like strain plays a central role in high-temperature compost as well as ordinary compost. In this study, *A. pallidus* was isolated mostly from early-phase compost. On the other hand, *Ureibacillus* spp. was isolated mostly from steady-state compost. The introduction of a mixture of the three genera at the start of composting may accelerate composting or elevate temperature in both the early-phase and steady state.

In the process of identifying bacteria that play key roles in the initiation of high-temperature compost, adjustment of moisture conditions and aeration from the bottom successfully led to the generation of high-temperature compost without the addition of substrates (that is, wastes). Therefore, the origin of the microbiota was considered to be the composted mixture of tree materials (mostly tree roots and bark). In the present study, *G. thermodenitrificans*, *A. pallidus* and *Bacillus borbori*-related strains were frequently isolated. These strains are candidate bacteria for increased heat production during initiation of composting. There are clear indications of the mechanisms underlying the production of heat during the composting process. One direct possibility is that heat-producing bacteria are present in the compost. Heat-producing bacteria, such as *Pseudomonas putida*, have been previously reported (Tabata et al., 2013). Ordinary compost that lacks aeration does not reach temperatures higher than 90°C. Therefore, it is possible that excessive heat production is uncoupled from metabolism via stimulation by complex conditions including aeration. Energy dispersal from continuous bacterial processes may be useful for continuous operation without the production of residual biomass (Lapara et al., 2000).

Conclusions

Although the frequency of isolation of mesophilic microorganisms was lower in high-temperature compost (only one strain identified as *B. subtilis*) than in moderate-temperature compost, the upper limit of the growth temperature (approximately 70°C) of the isolates in the present study was much lower than the *in situ* temperature (90–108°C). There are several possible explanations for this discrepancy: 1) the *in situ* compost environment is different from the culture conditions in the laboratory; 2) the temperature range in which the bacteria exhibit metabolic activity but not propagation is different from that in which bacterial metabolism is coupled with propagations; 3) the bacteria that exhibited propagation at localized low-temperature sites (for example, at the surface) were occasionally introduced into the interior of the compost, and we isolated surviving microorganisms that were not able to grow at temperatures higher than 80°C. Based on the above possibilities, further studies are needed to resolve the contradiction that none of the isolates from the high-temperature compost (≥90°C) could grow at temperatures ≥80°C. Isolates that shared ≥99% similarity with *G. thermodenitrificans*, *Ureibacillus* spp. (*U. suwonensis*, *U. themosphaericus*) and *A. pallidus* were isolated from both steady state and newly prepared small-scale composts. Introduction of a mixture of these species at the start of composting may accelerate the initiation of high-temperature composts in the absence of materials such as compost seed, from which the expected microbiota originates.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Microbial contamination of cell phones of nursing department students in Technical Institute of Baqubah, Iraq

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This study was aimed to investigate microbiological colonization of cell phones used by nursing students of Baqubah Technical Institute, from June to August 2018. In 150 randomly collected cell phones, 133 bacterial and 74 fungal species were isolated using sterile swabs from cell phones surface. The microbe were identified using conventional methods, bacterial species isolated were: *Staphylococcus aureus* (48%), *Staphylococcus epidermidis* (25.2), *Pseudomonas aeruginosa* (14.1), *Bacillus subtilis* (7%), *Escherichia coli* (2.3), *Streptococcus viridians* (2%) and Proteus spp. (1.2%) whereas, the fungal species isolated were Cladosporium spp. (36.1%), Alternaria spp. (17%), Penicillium (9%), *Aspergillus fumigates* (6.3%), and *Aspergillus niger* (32%). The bacterial isolates were tested by antibiotic disks diffusion method. High percentage of bacterial isolates was found resistance to erythromycin, cefoxitin, ciprofloxacin and clindamycin. Several *S. aureus* and *S. epidermides* isolates were resistant to erythromycin and cefoxitin. The Proteus spp. and *E. coli* were found highly sensitive to ampicilin, amikacin, cefepime, cefroxain and imipenem. However, the *P. aeruginoae* spp. showed two different antibiotics sensitivity profiles for the similar antibiotics. This study confirmed that the students cell phones were contaminated with several pathogenic bacterial and fungal species thus might act as an important source of cross-transmission of human and antibiotics resistant.

Key words: Cell phones, microbial contamination, nursing students, fungal species, *Staphylococcus aureus*.

INTRODUCTION

A cell phone is an important device for private telecommunication in daily life and is frequently kept in close contact with the human body. In most countries, mobile phones became more than landline telephones, since most adults and many teenagers currently own mobile phones. At present, Middle East geographic area has the fastest growth rate of cellular phone subscribers in the world (Ibrahim et al., 2014). Persistent handling of cell phones by different users exposes it to many species of microbes; thus, making phones perfect carrier for microorganisms. Particularly, those related to the skin resulting in the spread of different microorganisms from

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user to user (Al-Abdalall, 2010).

The problem of cell phones contamination with microbes is aggravated from the fact that several cell phone users neglects their personal hygiene (Roy et al., 2013). Continuous usage of cell phones in almost every place and occasions exposed it to a large number of microorganisms. It can be an important source for variety of zoonotic pathogens, which lead to infections and may be a potential health hazard for users and their family (Gurang et al., 2008). Handling of mobile phones by lectures and teachers makes it a good vehicle to spread many pathogenic microbe (Ibrahim et al., 2014; Brady et al., 2006). Various species and genera of bacteria, including *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* spp., *Enterococcus* spp. and *Proteus* spp. are known as the etiologic pathogenic agents.

In addition, the normal Microflora is harmless and may be useful when they found in the normal sites in host. However, it can also produce disease if replaced into another locations or a compromise host (Roy et al., 2013; Amadi et al., 2013). Fungal species like *Trichophyton mentagrophytes, Aspergillus niger*, *Pencillium* sp. have the ability to grow on cell phones when exposed to mobile waves for 20 min (Fawole and Ose, 2001).

Hand washing may not be usually performed enough thus, personal mobile phones may be used in work all of the day. Thus, mobile phones are considered as a potential source for transmission of microbe (Ibrahim et al., 2014; Suganya and Sumathy, 2012).

Over the last decade, the use of mobile has increased rapidly from being rare and priced items of device used primarily by the wealthy category, to a common cheap personal asset. According to many Microbiologists the warmth generated by continuous handling of phones make it a perfect ground for the normal flora of the skin which may be resistant to some antibiotics (Dave and Shende, 2015; Zakai et al., 2016).

This research investigates microbial contamination of cell phones used by the students at the Nursing department in Baqubah Technical institute. This research also identifies the microorganisms that are regularly associated with mobile phones. This research also studies the sensitivity of bacterial isolates to some antibiotics.

MATERIALS AND METHODS

Sampling

This study was performed during summer training from June to August 2018, at the laboratory of Medical Microbiology, Baqubah Institute, Middle Technical University (MTU), Diyala, Iraq. One hundred and fifty randomly collected cell phones of second year nursing department students were examined by taking swabs for isolation of bacteria and fungi.

The surface of cell phones were swabbed with sterile cotton swab immersed in sterile saline. Each cell phones were sampled and inoculated separately into tubes containing 3 ml Luria - Bertani broth (LB broth) and Sabouraud dextrose broth.

Bacterial isolation

The inoculated LB broth were incubated overnight and streaked into blood agar and MacConkey's agar. The culture plates were incubated aerobically at 37°C for 24 h. The identification of isolated bacteria were based on standard protocol beginning with morphology of colonies, gram stain, and observed for growth as well as colonial description of the isolates (Roy et al., 2013; Arora et al., 2009). Mobility tests, biochemical tests and Microorganisms plates were identified grown on with conventional techniques. A slide coagulase test (Microgen Staph, Microgen Bioproducts, Camberley, UK) was used to differentiate *S. aureus* from other coagulase-negative Staphylococci (Zakai et al., 2016; Brooks et al., 2013).

Biochemical analysis

Following purification, single colonies of bacteria were subjected to biochemical tests according to standard procedures, which include carbohydrate fermentation test, mannitol motility test, IMViC tests (Methyl Red test, Indole test, Voges Proskauer test and Citrate test), urease test, nitrate reduction test, growth in triple sugar iron agar (TSI) (Brooks et al., 2013; Kumar and Aswathy, 2014).

Antibiotic susceptibility test

The antibiotic susceptibility test was conducted on 0.5 McFarland (is a chemical solution of 1% barium chloride $BaCl_2$ and 1% sulfuric acid H_2SO_4 Solution in appropriate proportion), using the Kirby-Bauer disk diffusion method according to NCCLs recommendation M100-S25(2015). The bacterial suspension (0.5 MaFarland) was streaked over Muller-Hinton agar surface (Shahlol et al., 2015); then available suitable antibiotic disks were placed onto the surface of medium and incubated for 18 h at 35°C. The zones of inhibitions were measured and interpreted according to the Clinical and Laboratory Standards Institute (Wayne, 2011). The antibiotics disks used include: Tetracycline, erythromycin, cefoxitin, ciprofloxacin and clindamycin for Gram positive bacteria, ampicillin, amikacin, cefepime, ceftriaxone and imipenem for Gram negative bacteria were used and the results were indicated by sensitive or resistant test according to standard measure (Zakai et al., 2016; Julian et al., 2012).

Fungal isolation

After incubation for 24 h at room temperature, swabs were streaked on the Sabouraud dextrose agar and potato dextrose agar. The samples were cultured for the growth of isolated colonies on potato dextrose agar. Then the plates were incubated at 37°C for 24 h, the colonies grown on two media were examined for their morphology and staining. The isolated fungal species further identified and characterized by using standard microbiology method (Kampf and Kramer, 2004).

RESULTS AND DISCUSSION

An inanimate object as mobile phone, may pose as a potential for survival of microorganisms. Some viruses such as corona, coxakie and influenza can survive few days and herpes virus for a week, while bacteria can persist for months (Kampf and Kramer, 2004). Many studies conducted around the world show that there is a

Table 1. Grouping of microbial content of cell phones.

Number of isolated microorganisms	Bacteria		Fungal	
	Number	%	Number	%
0	17	11.3	76	28
1	33	22.0	24	17.3
2	47	31.3	33	30
3 or more	53	35.3	17	24.7

Table 2. Bacterial species isolates from cell phones.

Isolates of bacterial	Number	%
Staphylococcus aureus	264	48
Staphylococcus epidermidis	137	25.2
Pseudomonas aeruginosa	77	14.1
Bacillus subtilis	36	7
Escherichia .coli	13	2.3
streptococcus viridians	9	2
Proteus spp	7	1.2

high prevalence of microbial contamination in cell phones (Karabay et al., 2007).

The results in Table 1 refers to the highest rate which belongs to cell phone contaminated with 3 or more types of bacteria (35.5%), while the non-contaminated cell phone recorded as lowest rate (11,3%). These results approximate Chawla et al. (2009) with his findings, which included the total number of cell phones that showed no growth of bacteria, the contaminated phones with 2 types of bacteria reported as the highest rate. Cell phones, which show no fungal growth, recorded the highest rate 76%, while those that appear in the lowest rate 17% show growing of 3 or more fungal types. Many researches carried the entire world refers to high propagation of contaminated cell phones (Karabay et al., 2007).

The rate and number of isolated bacterial types (spp.) are summarized in Table 2. *S. aureus* and *S. epidermidis* were the predominant bacteria in rate of 48 and 25.5%. These results were parallel with Akinyemi et al. (2009) and with Datta et al. (2009) in their study reporting that coagulase-negative staphylococci were the most prevalent bacterial agents isolated from mobile phones, followed by *Staphylococcus aureus* (Chawla et al., 2009) in which *S. aureus* were the predominant bacterial spp. In rate of (48%), among other species including 7 types of bacteria were isolated from totally 150 cell phones which are in accordance with frequency as follows: *S. epidermidis* (25.2%), *P. aeruginosa* (14.1%), *B. subtilis* (7%), *E. coli* (2.3%), *S. viridians* (2%) *and Proteus* spp. (1.2%) sequently.

S. aureus is carried by healthy people on the skin and nose. It can cause mild to serious infections if it enters the body through cuts, wounds, etc. (Angadi et al., 2014). *S. aureus* mainly introduced from hands which is the main reservoir for this bacteria and introduced to food while preparation. (Suganya and Sumathy, 2012; Morubagal et al., 2017).Many pathogens like *S. epidermidis* can transfer by cell phones to the body by contacting with other plastic surface such as catheters or prostheses. The most prevalent cause of sepsis and the etiologic agent of most cases of urinary tract infection is *S. epidermidis* (Al-Abdalall, 2010; Akinyemi et al., 2009; Jalalmanesh et al., 2017). *P. aeruginosa*. was observed at the rate of 14.1%. This is close to Famurewa and David (2009) who observed that 22.6% of the investigated cell phones owned by volunteers in the university premises were contaminated with *P. aeruginosa*.

The contamination of hospital device and food products with species of bacteria is a major concern (Gurang et al., 2008; Julian et al., 2012) since the cell phones can play a role as a vector. The prevalence of other bacterial spp. isolated from student's cell phones were *B. subtilis* (7%), *E. coli* (2.3%), *S. viridance* (2%) and the lower percentage (1.2%) was Proteus spp. The prevalence of Bacillus species according to previous researches processed in Iran, were 60 and 26.3%, respectively (Karabay et al., 2007; Jalalmanesh et al., 2017). These results do not agree with another study performed by Sedihgi et al. isolates *Bacillus* spp. By about (0.8%) from the cell phone of Health Care Providers in a Teaching Hospital in Hamadan Province, Iran (Sedighi et al., 2015).

E. coli, *S. viridians* and *Proteus* spp. were isolated by a small percentage compared with other isolates mentioned.

Table 3. Fungal species isolates from phones.

Isolates of Fungal	Number	%
Cladosporium spp.	17	36.1
Alternaria sps.	15	32
Penicillium	8	17
Aspergillus fumigates	4	9
Aspergillus niger	3	6.3

Table 4. Pattern of antibiotic sensitivity for Gram-positive bacterial isolates.

Antibiotics	Bacteria							
	S. aureus		*S. epidermidis*		*Bacillus subtilis*		*S. viridians*	
	S	R	S	R	S	R	S	R
TE	232	32	95	42	26	10	7	2
E	193	71	83	54	21	15	5	4
CX	213	51	76	61	17	19	9	-
CIP	231	33	104	33	27	9	6	3
CD	217	47	97	40	29	7	8	1

TE, tetracycline; E, erythromycin; CX, cefoxitin; CIP, ciprofloxacin; CD, clindamycin.

Significance of fecal contamination of hands can be confirmed by presence of *E.coli* through bed pans or poor personal hygiene (Amadi et al., 2013). Ibrahim et al. (2014) observed that 9.77% of examined cell phones were contaminated with *E. coli* and *Proteus* spp. in a rate of (7.47%) with many other bacterial species in different rates. The results were also close the findings of Zakai et al. (2016) in regards to total isolation of bacteria which was about (20%).

Cell phones are likely to be a soruce of microbial transmission, inculding human pathogens and that can increase the incidence for bacterial and fungal infections. Recently many rersearches researched the contamination of cell phones surfaces with bacteria and fungi (Nowakowicz-Dębek et al., 2013).

Table 3 shows the pathogenic fungi isolated based on mycelia, colour and spores from swabs taken from the cell mobile device with different values started from *Cladosporium spp.* at a higher rate (36.1%) to *A. niger* which was in the lower percentage (6.3%). Many of recent studies Confirmed high contamination with mycotic agents, especially of Aspergillus and *Penicillium* (Nowakowicz-Dębek et al., 2013).

Present research also is in parallel with Coutinho et al. (2007) who analyzed the incidence of fungal contamination of mobiles in high level when he isolate 34 species of fungal from public telephones in Brazil.

These isolates influence food infectious and cause food spoilage by producing toxins. Filamentous fungi, have strong allergenic properties, and can induce dermal mycoses, which is considered as opportunistic human pathogens (Nowakowicz-Dębek et al., 2013). The results are consistent with isolation of *cladosporium spp*. In a rate of 20.9% and *Aspergillus fumigates* at a rate 2.3% among fungal isolates including *A. niger* 20.7%, and other pathogenic species from mobile phones in eastern Saudi Arabia (Al-Abdalall, 2010).

Dave and Shende (2015) pointed out to the isolation of a group of pathogenic fungi in similar proportion to the same rates obtained by us but differ with the isolation rate of *A. niger* (32.0%) which was reported as a high percentage.

The sensitivity tests for bacterial isolates were presented according to Gram positive and Gram negative in Tables 4 and 5, respectively. Generally antibiotic sensitivity test results revealed that all bacterial strains were sensitive to the studies antibiotics but at different rates.

Most of *S. aureus*, *S. epidermidis*, *B. subtilis* and *S. viridians* isolates were sensitive to tetracycline, erythromycin, cefoxitin, ciprofloxacin and clindamycin. *P. aeruginosa, E. coli* and *Proteus* spp. were moderately sensitive to the following antibiotics ampicillin, amikacin, cefepime, ceftriaxone and imipenem. *Proteus* spp. did not show any resistance to amikacin and imipenem same as *E. coli* to cefepime and was to imipenem .This agree with Roy et al. (2013) findings according to *E. coli* and *Proteus* spp. isolates that showed highly sensitivity to ciprofloxacin, erythromycin, amikacin.

There is increase in the use of mobile devices without awareness of the risks that it may cause; especially the contamination of these devices with microbes may lead

Table 5. Pattern of antibiotic sensitivity for Gram-negative bacterial isolates.

Antibiotics	Bacteria					
	P. aeruginosa		*E. coli*		*Proteus spp.*	
	S	R	S	R	S	R
A	49	28	10	3	6	1
AK	46	31	12	1	7	-
CPM	32	45	13	-	5	2
CTR	55	22	9	4	6	1
IPM	37	40	13	-	7	-

A, ampicillin; AK, amikacin; CPM, cefepime; CTR, ceftriaxone; IPM, imipenem.

to serious health problems especially when it is used without caring heygin precautions (Martínez-Gonzáles et al., 2017).

Recent research included contamination of 133 out of 150 mobile devices with bacterial and among of 150 total examined cell phones only 74 devices were contaminated with fungal spp. The ability of pathogens to grow on the surface of cell phones, survival time, and the risk of transmitting these pathogens to patients should be taken into account. This study aimed to isolate and identify microorganisms and create awareness that mobile could also serve as a vector for transfer pathogenic agents from one individual to another, and causes of infections. Therefore, it is important to take care of personal hygiene and mobile decontaminations by regular cleaning of mobile phones with methylated spirit or alcohol to eradicate and reduce growth of pathogenic micro-organisms.

Conclusion

This study reveals that there is colonization of pathogenic bacteria and fungal agents on the mobile phones, in which it may act as disease - producing and help in transforming microbes among the students of 2nd year Nursing department especially when they start training in health center during summer. These contaminated phones may be an important facility in the spreading of drug-resistant bacterial isolates. In order to reduce this potential risk, everyone should have an education about hygiene, comprehensive guidelines and strict hand wash, and regular decontamination of mobile phones by appropriate cleaning of the device.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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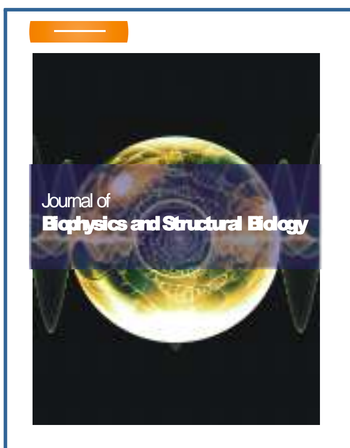

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