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Full Length Research Paper

Impact of land use and soil types on arbuscular mycorrhizal fungal diversity in tropical soil of India

Sanjeev Kumar[1]* and Alok Adholeya[2]

[1]Department of Genetics and Plant Breeding, School of Agriculture, Lovely Professional University, Jalandhar, Punjab, India.
[2]Centre for Mycorrhizal Research (CMR), Biotechnology and Bioresources Division, The Energy and Resources Institute, Darbari Seth Block, IHC Complex, Lodhi Road, New Delhi–110003, India.

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A study was undertaken along land use gradients with different soil types in subtropical ecosystem of Northern India to evaluate the accurance of arbuscular mycorrhizal fungi (AMF). The gradients was from natural land with forest tree, farmer's field under chemically managed, farmer's field under organically managed and Industrial wasteland with five different plant species. We investigate the total AMF species in land use system of different soil types and also in trap culture set from same land use system.Distribution of mycorrhizal species were calculated directly by quantification AMF spores and indirectly by sequencing the SSU-ITS and LSU regions of rDNA. A total 19 AMF morphotypes from direct field sampling and additional 35 morphotypes from trap culture were recovered, which represented seven genera and eighteen species. Result suggested that few *Rhizophagus* and *Funneliformis* species came from organically managed and natural land; most of the species came from sites representing chemically managed and industrial wasteland sites from which *Gigaspora* and *Scutellospora* species were absent. Organically managed land contributed the largest number of AMF species and diversity, even more than those found in natural sites, which suggests that factors contributing to the diversity of AMF are indeed complex: For example, chemically managed sites not only causes loss of fungal biodiversity but also selectively favors smaller spores of genera *Rhizophagus* and *Funneliformis*.

Key words: Tillage, diversity, ribosomal dna, raised bed plantation, arbuscular mycorrhizal (AM), morphotypes.

INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) form mutually beneficial associations with a large number of terrestrial plant species (Van der Heijden et al., 1998). These fungi promote phosphorous uptake and help plants in coping with different forms of stress. Communities of AMF are affected by many factors including plant genotype, agricultural practices, and pollution (Sander et al., 1995a). Farming practices such as intensive cultivation,

*Corresponding author. E-mail: sanjeev.19379@lpu.co.in.

Table 1. Major characteristics of different land use systems selected for AMF diversity study

S/N	Land use system	Major characterization
1	Chemically managed arable land (CML)	The site comprised three land use system (conventional tillage or ConT; zero tillage, or ZTL; and permanent raised beds, or RBP) supporting a rice–wheat production system under conventional management (120–150 kg/ha N, 40–60 kg/ha P2O5, 40–60 kg/ha K2O, and 25 kg/ha Zn).
2	Arable, zero tillage (ZTL)	Site comprised ZTL plot had been no tillage for the last two rotations
3	Arable, conventional tillage (ConT)	Site comprised ConT plot had been tilled using a tractor for the last 8-10 years
4	Arable, raised bed (RBP)	Site comprised RBP plot of wheat and rice had been grown on permanent raised beds with zero tillage for the last two years
5	Natural grassland land (NAL)	Site comprised 6 plots of wheat (PA1 to PA6) under chemically managed land (CML) and 14 plots (PA7-PA20) representing a natural land dominated by *Cyathea spp*, *Albizia procera*, *Shorea robusta* and *Phyllanthus emblica* plant
6	Organically managed land (ORG)	This site in eastern zone of Haryana, dominated by *Oryza* and *Triticum spp.* grown under organically managed soil. Sites comprised three plots, each with a different dose and mix of organic manure: PALF1 (poultry manure alone), PALF2 (poultry manure and 20 tonnes/ha of farmyard manure) and PALF3 (poultry manure and 40 tonnes/ha of FYM).

tillage and using sewage sludge as soil amendment may affect communities of AMF both qualitatively and quantitatively (Sieverding, 1990). More report by Jansa et al. (2003) observed that *Rhizophagus* species were dominant in highly tilled field, whereas *Scutellospora* species were prevalent in low tillage fields. Furthermore Treseder et al. (2004) observed that conventional agricultural practices such as application of chemical fertilizers and tillage intensity tend to decrease AMF spore abundance and alter community composition. Moreover Gaur and Adholeya (2002) suggested that application of low input fertilizers (Organic) promotes the growth of indigenous AMF in nutrient-limited soils. Only a few studies have explored the extent to which soils under conventional and organic farming systems in the temperate zone differ in terms of the composition and species richness of AMF (Oehl et al., 2003; Hijri et al., 2006). There are 102 AM fungi reported in tropical diverse habitats from India (Manoharachary et al., 2005).The occurrence of AM fungi in a forest and coastal regions of Andhra Pradesh reported by Manoharachary et al. (1991), distribution and identification of AM fungi in the rhizosphere soils of the tropical plains collected from Tamil Nadu, India by Ragupathy and Mahadevan (1993) and natural forest regions in the Old Delhi Ridge, Saraswati Range of Haryana by Thapar and Uniyal (1996). However, most of study surveyed AMF diversity in the subtropical region of Northern India based on morphological charcters of spores collected from single land use system (Karthikeyan and Selvaraj, 2009; Kumar and Grampalli, 2010). The comprehensive survey of land use intensity and different soil types strongly affect the AMF community composition in temperate soil earlier reported by Oehl et al., 2010; Stover et al., 2012; González et al., 2012; Bainard et al., 2015). Subseqent study by Dobo et al. (2016) were recorded 29 AMF morphospecies, belonging to nine genera originated from the rhizospheric soil of the three land uses system. However rich diversity of AMF found over a broad range of different land use (Cropping vs noncropping) in tropical soil, to our knowledge, has not been investigated so far using multiple methods.

Therefore aim of the present study was to investigate AMF diversity in different land use and soil types in tropical soil. It was hypothesized that AMF are more abundant and diverse in organically managed as well as natural soils than those in chemically managed soils and in soils affected by industrial pollution. Hypothesis was tested directly by quantifying the number of spores and species richness and indirectly by sequencing the SSU-ITS and LSU regions of rDNA. To determine AMF diversity, the following seven land-use systems from four agroclimatic zone with different soil types were selected: Agriculture land high-intensity farming (Chemically managed), Agriculture land under conventional tillage (ConT), Agriculture land under Zero tillage (ZTL), organic (low-input) farming, agricultural field highly contaminated tannery effluent, and natural land (NAL)..

MATERIALS AND METHODS

Sampling site

The investigation was carried out in seven different land use pattern (Table 1). The annual rainfall range 800 to 1200 mm and has ample irrigation resources. The first agroclimatic zone near Ghaziabad (28° 40' N, 77° 28' E) which is part of the western plains of Uttar Pradesh, India constitutes the sub-humid zone. The second agroclimatic zone near Palwal (28° 9' 0" N / 77° 20' 0" E) , in eastern zone of Haryana, India. The third sampling agroclimatic zone near Pachmari (22° 28' 0" N / 78° 26' 0" E) was part of the Malawa plateau zone of Madhya Pradesh which has medium black sandy soil. The fourth agroclimatic zone sampling was Kanpur

(80°21′ N / 20°38′ E) part of the central plains of Uttar Pradesh, India constituted sub-humid zone.

Sampling of AM fungi

All soils were sampled in 2008 in october from seven land use system of four agroclimatic zone. Each sample collection point was divided into four blocks. undisturbed core samples 20 soil cores/plot were collected (soil and roots) from the rhizosphere of wheat plants from a depth of 0 to 30 cm using core sampler. Thus, a total of 80 soil cores were collected from each collection sites. The samples were air dried in the shade to a point where there is no free moisture and were placed into zipped bags, and stored at 4°C in a cold room until processed. The samples were used for three different purposes: (i) Propagation of AM fungal isolate of each collection point for their identification; (ii) Analysis of AM fungal parameters; and (iii) Analysis of soil chemical parameters.

Physical and chemical analysis of soil

A soil suspension of 1:2.5 (soil-to-water mixture) was made. The pH of the soil suspension was measured by digital pH meter (Expandable Ion Analyser EA 940, Orion Research) and the electrical conductivity was measured by digital electrical conductivity meter (Controlled dynamics). A protocol by Datta et al. (1962) was followed for measuring % organic carbon. % Total nitrogen was calculated using Kjeldahl's method by Bremner (1960). Available phosphorus was determined using Olsen's method (Olsen et al., 1954) and the estimation of available potassium was done using flame photometer with filters (Wood and Deturk, 1940).

Trap cultures

Mycorrhizal fungi obtained from different land use system tropical soil are very difficult to identify even impossible to identify up to species level. This is due to tropical environments (high temperature, moisture) and those with high organic matter as well as high proportion of spores undergo so much structural changes or degradation. Therefore, pot culture established in year 2008 using soil samples to recover spores from AM fungal species present in field soil, including some of which may not have sporulated at time of sampling. The trap cultures were established using methodology described by Oehl et al. (2003). Plastic trays (460×290×240 mm^3) were used to establish AM fungal cultures in greenhouse using soil samples from all collection sites. For each collection site, four trays (comprising four blocks) were prepared. The plastic trays were provided with 50 mm hole at the bottom. A 20-mm thick drainage mat (Enkadrain ST, Schoellkopf AG, CH-8057 Zurich, Switzerland) was placed at the bottom of the tray and the tray was filled with 25 Kg of substrate (50% Terragreen: American aluminium oxide, oil dry US special, Type IIIR and 50% soil sediment: Nutrient deficient (Olsen P = 1.56 ppm; Organic C = 0.28%; Total N = 0.052 %; K = 52.66 ppm). The substrate was autoclaved at 120°C for one hour at 15 psi before filling. Substrate cores (50 g) were taken out from five different places in the tray and were replaced by five, undisturbed soil cores (50 g, containing collection site's AM fungi) to inoculate the hosts. Seeds of *Allium cepa*, *Tagetus* sp., *Daucus carotus*, *Medicago sativa* (alfalfa), and *Trifolium alexandrianum* (barseem) were pregerminated. Five pre-germinated seeds of each species were placed at the top of all five soil cores. The plants were watered to a moisture level of approximately 60% of the water holding capacity and were grown in greenhouse at 20 ± 5°C with 60% relative humidity. The pots were arranged on a greenhouse bench in a completely randomized design with 4 replications. Half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1938) was provided to the plants at fortnightly interval. After four months of growth cycle, the pots were left to drying undisturbed with a fairly stable temperature so that the drying period is not too rapid. After completion of the growth cycle the dried shoots were cut at the ground level without disturbing the substrate and seeds of different hosts *Gossypium*, *Vetiver*, *Vigna radiate*, *Sorghum* and *Tagetus* sp. were sown again. Three trap culture cycles were propagated. After each cycle, rhizosphere soil cores were taken from the vicinity of trap plants at a depth of 0 to 15 cm and species characterization was done.

AMF spore identification

Spores were collected separately and similar looking spores were grouped into different spores morphotypes according to colour, size and mycelial attachment with spores. Permanent slides was prepared in polyvinyl alcohol and polyvinyl alcohol plus Melzer's solution (1:1) as described by Walker and Trappe (1993). Spores from each morphotypes were observed under a Zeiss compound microscope equipped with a digital imaging system, and digital photographs was taken with a Zeiss Axiacam RTC (Germany). Spore diameter, wall thickness and hyphal thickness were measured using software Axio Vision (Version 4.7) attached to the microscope. The diagnostic slides of different species of AM fungal spores were prepared and features of the spores morphology were compared according to current taxonomic criteria (Schenck and Perez, 1990) and also using the internet information from the INVAM website (http://www.invam.caf.wvu.edu).

Data analysis

Spores were washed with distilled water and evenly over the entire grid. They were counted under a stereoscopic microscope (40×). The number of spores was expressed as the mean of four replicates. Diversity of AM fungi in four study sites was evaluated by observing the spores in four replicates each of 100 g of soil. Once the data were obtained, the following were calculated for AM fungal diversity analysis (1) Spore density (Total number of spores was expressed in mean of four replicate in 100 g of soil sample) (2) Relative Spores abundance = Number of given species spore/Total number of spores × 100 % (3) AM fungal species richness was measure known as $d = s/\sqrt{N}$ where s equals the number of different AMF species in site, and N equals the total number of individual organisms in site (4) Shannon–Weiner diversity index index (H′) of AMF spore morphotypes was calculated for each sites with equation $H' = -\sum (Pi) \ln (Pi)$. $Pi = ni/N$ (ni is the number of individuals of species i, and N is the total number individuals in all species). Species diversity (Shannon -weiner index) in each experimental trap culture were used in one-way ANOVA with soil treatment as a factor with seven levels an four replicates of the trap cultures for each treatment. All analyses were performed in JMP version 5.1.1 (SAS Institute Inc., Cary, North Carolina, 1989-2002), and differences between the means were analyzed using Tukey HSD multiple comparisons with $P < 0.05$. Similarity index (Legendre and Legendre, 1998) was calculated to compare the similarity of species among different sites as based on χ^2 distance using ward's minimum variance methods. The correspondence units (dimensionless) on the basis of spore number per 100 g of soil.

Molecular analysis

A single spore from each morphotype was transferred aseptically to eppendorf tube containing 10 µl of 10X PCR buffer (Invitrogen, USA) and used for DNA extraction. The spore was crushed, 10 µl of 20% Chelex resin added immediately, and the tube centrifuged

Table 2. Soil physico-chemical properties of different land use types chosen for diversity study.

Land use type	Soil texture	pH	Organic C (%)	P (ppm))	N (%)	K (ppm)
NAL	Medium black soil	7.18	1.142	4.95	0.02	60.8
CML,ConT,ZTL,RBP	Entosols of Alluvium Soil	7.54	1.02	7.52	0.02	52.8
ORG	Medium texture alluvial soils	7.00	0.62	5.12	0.03	71.9
IWL	Alluvial sandy loam soils	7.71	3.023	37.5	0.11	627

ConT, Conventional tillage; *RBP,* raised bed plantation; *CML,* chemically managed land; *ZTL,* zero tillage land; *ORG,* organically managed; *NAL,* Natural land; *IWL,* Industrial wasteland.

briefly for 2 s. The crude extracts were incubated at 95°C for 15 min, centrifuged at 8000 g for 3 min, and 5 µl of the supernatant was used as a template for PCR. The end region of an SSU with complete internal transcribed spacer (ITS) was amplified using parameters for the PCR described by Redecker (2000) with 56°C as the annealing temperature. The second step was carried out under identical conditions except that the annealing temperature was 60°C.

A fragment of a large subunit region of n-rDNA was amplified with a fungal-specific primer 28G1(F) and a *Glomeromycota* specific primer 28G2(R) as stipulated by Da Silva et al. (2006). The first PCR product of *Glomeraceae* morphotypes was used as a template for the nested PCR using the *Rhizophagus* and *Funneliformis* specific primer LSURK7r (Van Tuinen et al., 1998a) and 28G1 as the reverse primer whereas the Genera include *Gigaspora*, *Scutellospora* and *Acaulospora* morphotypes were amplified by using the primer pair LR1+ FLR2 (Van Tuinen et al., 1998b). The first PCR product of these spores morphotypes was diluted 10-fold and used as a template for the second PCR amplification using the primer pair FLR3(F) + FLR4(R) under the conditions specified by Gollotte et al. (2004). Nested PCR products were purified using Qiagen PCR purification system (Qiagen, USA). The purified PCR product of the SSU-ITS and LSU rDNA fragment was cloned using the pCR4-TOPO vector supplied with TOPO TA cloning kit for sequencing (Invitrogen, USA). 2 to 3 transformed colonies were picked and plasmid was extracted using Wizard Plus SV Mini Kit (Promega, USA). SSU-ITS and LSU rDNA insert was used for cycle sequencing reaction with PCR primers. The sequencing was performed on an automated multicapillary DNA sequencer, namely ABI Prism 3130×l Genetic analyzer (Applied Bio systems, Foster City, California, USA) using the Big Dye Terminator ver. 3.1 Ready Reaction Cycle Sequencing Kit (Applied Bio systems) at the sequencing laboratories of TERI, New Delhi. The sequences have been deposited with GenBank (NCBI, www.ncbi.nlm.nih.gov) under accession numbers shown in Figures 4 to 6. Sequence similarities were determined using BlAST similarity search algorithm (Atschul et al., 1990) as available on the NCBI home page. Sequences obtained from this study along with the reference SSU-ITS and LSU rDNA sequences retrieved from the NCBI database were aligned pair by pair using ClustalW (Higgins et al., 1994). The phylogenetic tree was constructed using Mega version 4.1 (Tamura et al., 2007) and evolutionary history was inferred using the neighbor-joining algorithm (Saitou and Nei, 1987).

RESULTS

The soil types varies range black medium texture alluvial soil to gangatic alluvial soils and pH was found to be slightly basic (7.18-7.71) in all the sites. The soil organic carbon was highest in industrial waste land (IWL) and lowest in the chemically managed land (CML). The available phosphorus ranges between 4.95 and 37.75 ppm and it was highest in the industrial wasteland sites than the other sites (Table. 2).

Based on the classification of *Glomeromycota* reported by Schüβler and Walker (2010), the ten species of AMF were recorded in the field soil and eight additional species of AMF observed in the trap soil. At the genus level, *Rhizophagus* was dominant AMF species. The most common species sporulating in trap cultures set up from soils from all the sites were *Rhizophagus irregularis* and *Rhizophagus intraradices* (Table 4). Myocrrhizal spores denesity differed significantly among trap cultures set up from both managed and natural ecosystem (Figure .2). Highest number of mycorrhizal spores was present in zero tillage land (ZTL) and lowest in natural land (NAL) showed in Figure 2. However species richness was decrease in order of ORG>NAL>ZTL>RBP >CML>ConT and the AMF species diversity as expressed by the H′ decrease in the order of OGR>RBP>NAL>ZTL> IWL>CML>ConT (Table 3). The H′ value was greater in trap culture originated from ORG field and lowest in trap culture originated from Conventional tillage soil (ConT) (Table 3). Moreover relative spores abundance of *Gigaspora* and *Scutellospora* species were found more among trap cultures set up from both organically managed and natural ecosystems and some were restricted in their accurrence (Table 4). Simlarity index based on cluster analysis showed that the highest similarity of AMF species composition between the sampling sites of ORG, RBP, NAL and ZTL on the other hand between sampling sites of IWL and CML (Figure 3).

Diversity in the Industrial wasteland soil

Spores of AMF collected from trap cultures originated from gangatic alluvial soils contaminated with tannery sludge were classified into 08 spores morphotypes all of these belonged to the genus *Rhizophagus*. Spores of these genera appeared yellow to reddish brown in reflected light and were globose to subglobose, 60 to 130 µM in diameter, although more than 75% of them were intermediate in size (80 to 100 µM).

Table 3. Species richness and Shannon-Weiner diversity index in different land use type.

Land use type	No. of species	Species richness (d)	[a]Diversity index (H)
Chemically managed arable land (CML)	3	0.38	0.63[c]
Arable, zero tillage (ZTL)	5	0.59	1.12[b]
Arable, conventional tillage (ConT)	4	0.32	0.51[c]
Arable, raised bed (RBP)	5	0.59	1.27 [b]
Natural grassland land (NAL)	7	0.84	1.06[a]
Organically managed land (ORG)	8	0.85	1.76[b]
Industrial waste land (IWL)	4	0.38	0.91[c]

[a]Non significiant difference between sites are shown by identical letter and determined using one way ANOVA of Tukey HSD of multiple comparisons with P <0.05.

Table 4. Relative abundance (%) of AM fungal species isolated from different land use systems.

Species	ConT	RBP	CML	ZTL	NAL	ORG	IWL
Rhizophagus intraradices	86.95	11.6	48.78	40.74	12.34	19.08	25.21
Rhizophagus irregularis	13.04	17.4	25.2	9.25	12.34	18.06	16.8
Rhizophagus proliferus	-	-	-	-	-	-	12.4
Septoglomus deserticola	-	-	-	-	-	-	45.57
Funneliformis mosseae	-	-	-	8.33	-	-	-
Funneliformis coronatum	-	-	-	41.66	-	29.08	-
Gigaspora margarita	-	45	-	-	75.3	19.09	-
Scutellospora gregaria	-	-	-	-	-	14.09	-
Scutellospora calospora	-	-	-	-	-	-	-
Acaulospora cavernata	-	-	-	-	-	-	-
Entrophospora infrequens	-	26	-	-	-	-	-
Scelerocystis spp.	-	–	26.01	-	-	-	-

ConT, Conventional tillage; *RBP,* raised bed plantation; *CML,* chemically managed land; *ZTL,* zero tillage land; *ORG,* organically managed; *NAL,* Natural land; *IWL,* Industrial wasteland.

Diversity in natural and organically managed land

Spores of AMF collected from trap cultures originated from medium black soil and medium texture alluvial soil under organically managed and natural land respectively were classified into 08 spore morphotypes, 5 of which represented the *Rhizophagus* species and 3 represented *Gigaspora, Scutellospora*, and *Aculospora* species (Figure 1). Spores abunance of *Scutellospora* species were more in trap culture originated from natural sites as compared with under organically managed sites whereas *Gigaspora* species were more predominant in trap culture established from organically managed land (Table 4).

Diversity in chemically managed land

AM spores morphotypes collected from field and trap cultures consisted of entosols of alluvium soil under intensive cultivation, which had received chemical fertilizers; fell into six spore morphotypes, representing four taxa of the Glomeraceae on each from Gigasporaceae and Acaulosporaceae and one from the Sclerocystis. Glomeraceae spores significantly more dominant in trap culture set up from ConT field than in trap culture originated from RBP and ZTL field (Table 4).

Molecular analysis

All 35 morphotypes of AMF collected from the trap cultures were used for molecular analysis; of these, 11 Glomeraceae morphotypes were used for SSU-ITSrDNA analysis and 17 Glomereaceae, 6 Gigasporaceae and 1 Acaulosporaceae morphotypes for LSU rDNA analysis. The sequencing reaction was performed on PCR/nestedPCR/plasmid of 35 morphotypes of AMF; 39 sequences of n-rDNA consisting of the SSU-ITS and LSU regions were isolated from the sequence analysis. Out of the 39 sequences, 36 appeared homologous with known Glomeromycota whereas the remaining three did not show any degree of homology with Glomeromycota. Neighbor joining (NJ) analysis using 11 SSU-ITS rDNA sequences obtained from 11 AMF morphotypes including

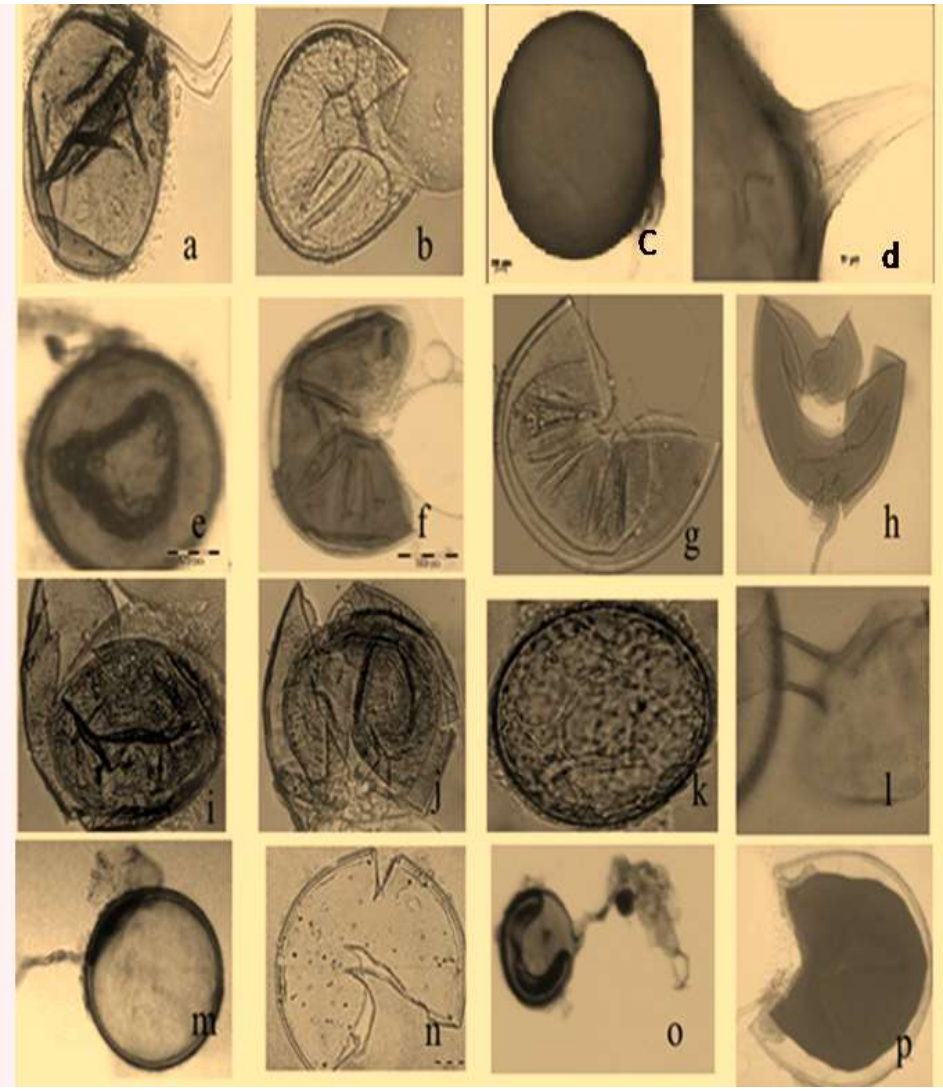

Figure 1. Spores of arbuscular mycorrhizal fungi isolated from trap culture originated from organically managed soil. (a) *Rhizophagus* sp. morphotype PALF1 in PVLG + Melzer reagent, (b) *Rhizophagus* sp. morphotype PALF2, (c-e) *Funneliformis coranatum* morphotypes PALF2AM1 in PVLG + Melzer reagent, (f) *Gigaspora margarita* of morphotypes PA16AM2 in PVLG + Melzer reagent, (g-h) *Gigaspora margarita* of morphotypes PA13AM1 in PVLG + Melzer reagent, (i-l) *Acaulospora species* M38AM1 in PVLG + Melzer reagent, (m-p) *Scutellospra* sp. of morphotypes PA13 in PVLG + Melzer reagent: Bars (a-p) 50 µM.

those retrieved from GenBank grouped all of them into a single major cluster of Glomeraceae (Figure 4). Sequences obtained from 09 AMF morphotypes formed a subcluster (*Rhizophagus*) along with reference *R. irregularis* n-rDNA sequences obtained from GenBank. On the other hand, sequences obtained from 2 morphotypes fell in a clade of known *Funneliformis mosseae*/*Funneliformis coronatum* with 94% bootstrap support (Figure 4). Out of the 24 morphotypes subjected to LSU n-rDNA analysis, 13 Glomeraceae isolates were identified by the nested *Rhizophagus* primer pair 28G1(F) + LSURK7r (R) and 11 by the *Glomeromycota* primer pair FLR3 + FLR4 (R). Two phylogenetic trees were generated using the sequences obtained from two different sets of primer pairs (Figures 5 and 6). The NJ tree obtained from the 13 sequences obtained from *Rhizophagus* and *Funneliformis* morphotpes with known Glomeraceae sequences retrieved from GenBank showed a single major cluster of Glomeraceae (Figure 5). Out of the 13 sequences, 05 were grouped with a known *R. intraradices* and 06 with *R. irregularis* clade and 02 with the reference *Septoglomus deserticola* sequence with 100% bootstrap support (Figure 5). The NJ tree obtained from the 12 sequences generated by the

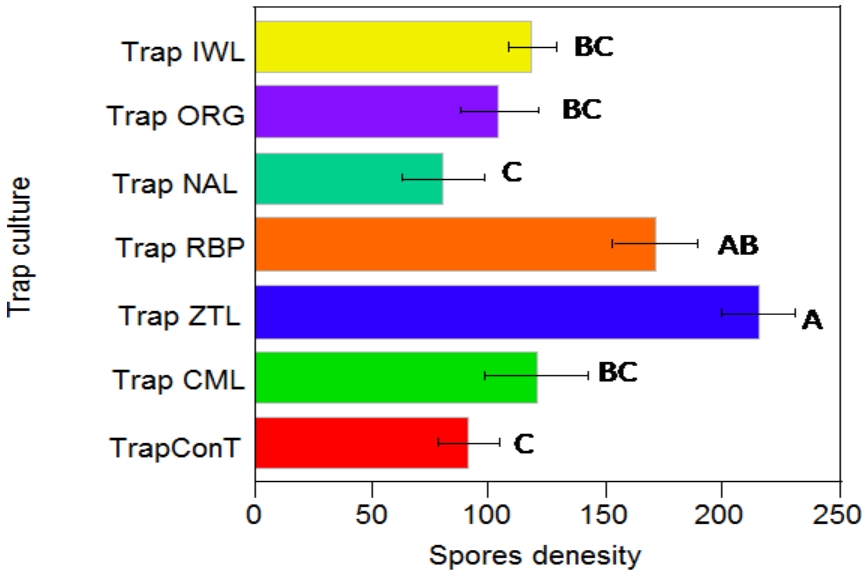

Figure 2. Spores density of mycorrhizal fungi in trap culture originated from different land use system.

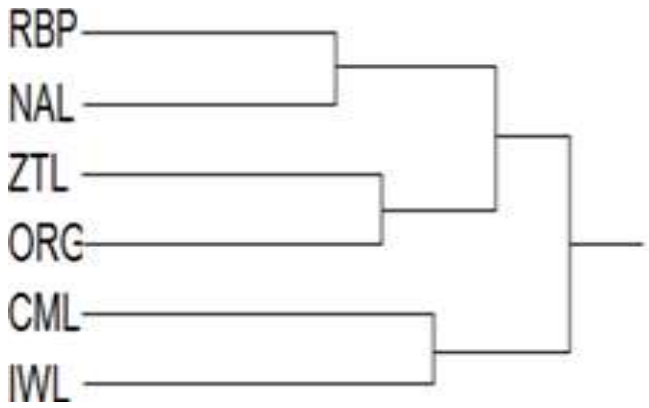

Figure 3. Simlarity index based on hierarchical cluster analysis of arbuscular mycorrhizal fungi in different land use system.

Glomeromycota primer along with reference sequences retrieved from GenBank showed three major clusters, one each of Glomeraceae, Gigasporaceae, and Acaulosporaceae (Figure 6). Out of these 12 sequences, 2 were grouped with *Rhizophagus proliferus*, 1 with *Funneliformis mosseae*, and 2 with known *Funneliformis coronatum* retrieved from GenBank. Sequences obtained from 7 *Gigasporaceae* morphotypes clustered with known *Gigasporaceae* retrieved from GenBank (Figure 6).

DISCUSSION

Previous report on distribution of AM fungi across a gradient of land use system in India mainly based on the morphological charcters of spores collected from field soil (Lakshmipathy et al., 2012; Bordoloi et al., 2015). The study present intervention using AMF morphotype collected from rhizospheric soil of field as well as trap culture to detect wide AMF diversity. The report by Bordoloi et al. (2015) suggested affect of mycorrhizal fungi in different landuse systems (Seven land use ecosystems of Arunachal Pradesh in Eastern Himalayan, India). However current investigation explore more under different land use systems in the tropical soil differ in terms of the composition and species richness of AMF. Moreover earlier surveys of populations of AMF, using either molecular or morphological approach, have focused on mycorrhizal roots (Helgason et al., 1999; Daniell et al., 2001) collected from field sites, and most

Figure 4. Neighbor joining tree obtained from alignment of partial region of the 26 SSU–ITS1 region of rDNA of *Glomeraceae* isolates with (*Acaulospora* sp.) as an out-group. Percentage bootstrap support (out of 1000 trials) is indicated. Names followed by accession no. represent sequences retrieved from GenBank. Names preceded by a different shape represent the sequence obtained in this work. There were a total of 367 positions in the final dataset.

studies of the diversity of AMF have used only rDNA as a marker. However present study using single spore DNA extracts, followed by nested PCR approach based on sequencing of LSU and SSU-ITS region of rDNA was further complemented method towards comprehensive detection and characterization of AM fungi in environmental soil (Kumar et al., 2013).

In the study, 18 mycorrhizal species were identified from field as well as trap culture in different land use system. Diversity of AM fungal species in present study is lower (35 species) than reported by Muthukumar and Udaiyan (2000) from seven different ecosystem of Western Ghat, India. Present study showed many additional species of AMF, for example group of sporocarpic fungi recorded in sorghum trap culture. However sporocarpic fungi were not detected in field soil because its induced sporulation during intensive cultivation in trap cultures. Similarly by Oehl et al. (2003), group of sporocarpic fungi were recorded in trap culture and suggested that the species thus undetected in field samples had initiated sporulation in trap cultures. Hence present study identified different species of mycorrhiza not only using direct field sampling but also through trap culturing so that cover all missing taxa of AM fungi. In addition, our study recorded S*. deserticola*, *R. proliferus*, *R. irregularis* and *R. intraradices* of Glomeraceae from industrial wasteland site. Four unidentified species of *Rhizophagus* were recorded from the field soil polluted with tannery sludge by Khade and Adholeya (2009). Our study suggested low specis diversity mainly *Rhizophagus* species in industrial wasteland soils due to higher pH level (7.71) and high precipitation. Moreover availability of more amount of organic carbon and phosphorus in the contaminated soils may be another factor which might have affected the growth of mycorrhizal mycelium and hence reduced the species diversity. However in contrast

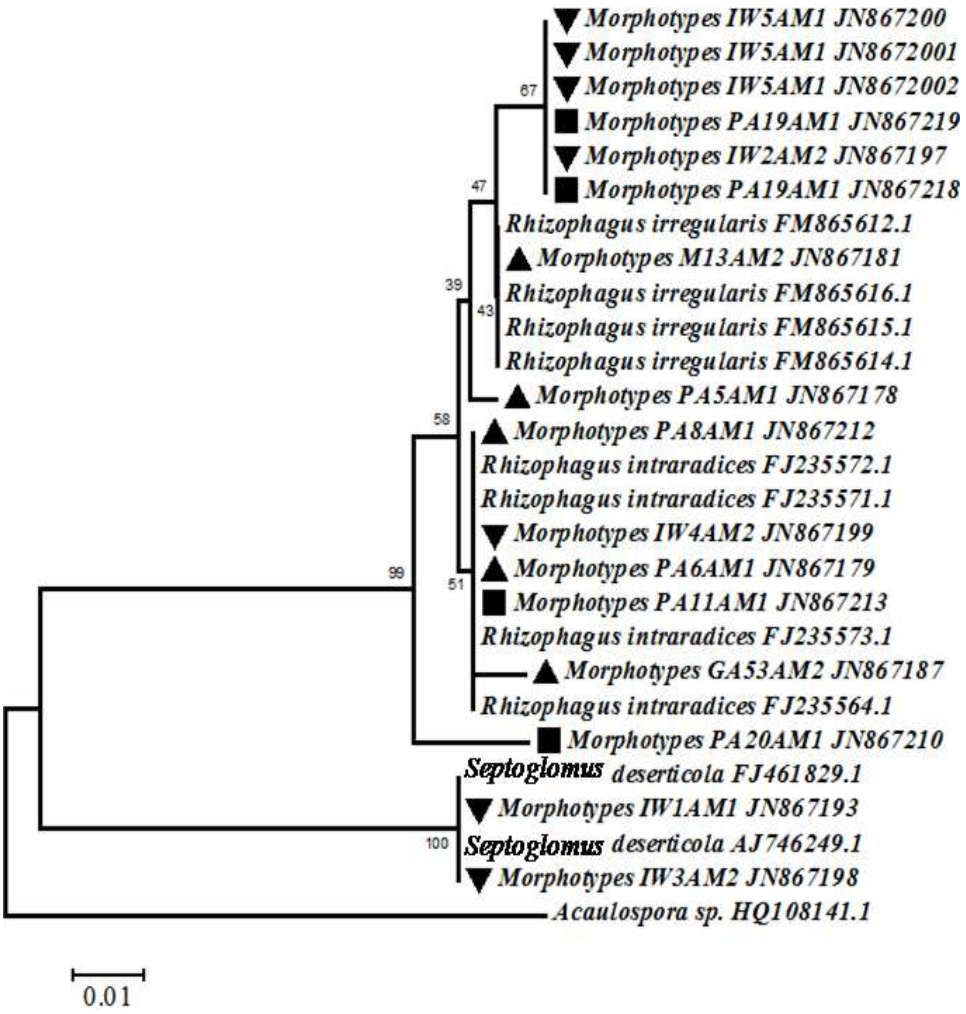

Figure 5. Sequences generated from amplicon of primer set 28G1+ LSURK7r used for phylogenetic analysis. Neighbor joining tree obtained from alignment of 13 LSU rDNA (400 bp from 5' end) used in this study including 21 LSU rDNA sequences retrieved from GenBank with (*Acaulospora*) as an out-group. Percentage bootstrap support (out of 1000 trials) is indicated. Names followed by accession no. represent sequences retrieved from GenBank. Names preceded by a shape represent the sequence obtained in this work.

study by Raman and Sambandan (1998), *Gigaspora* and *Scutellospora* were also observed in soil contaminated from Tannery effluents. In present investigation *S. deserticola* was recorded from this site may also be metal tolerant tolerant since it survived under given condition naturally. Recent studies by Arias et al. (2010) showed effect of metal observation when *Prosopis juliflora* was inoculated with *S. deserticola*.

In the study, Shannon Wieners diversity index in chemically managed land shows significantly low (0.63) than organically managed and natural forest land (Table 3). A similar result was suggested by Sharmah and Jha (2011), who reported that mean spore density of AMF was significantly lower in disturbed forests land as compared to the slash-and-burn fields of Karbi Anglong Hill district of Assam. Spore density and species richness were significantly more in natural savannas than cultivated soil and lowest in intensively managed cotton soil of West Africa (Tchabi et al., 2008). Moreover most of ribosomal rDNA sequences obtained from this site clustered with known *R. irregularis* (Figures 4 to 6). Similar study using molecular methods by Mathimaran et al. (2005) found that *R. intraradices* was the dominant species of AMF in soils under conventional farming practices and suggested that, as with temperate ecosystems, addition of chemical fertilizers may dramatically decrease the availability of propagules of AMF in tropical soils. In contrast, study by Gai et al. (2006) recorded higher diversity index in the agricultural field. Present study relative spores abundance of genera Gigasporaceae were recorded low in chemically managed soil under intensive cultivation (Table 4).

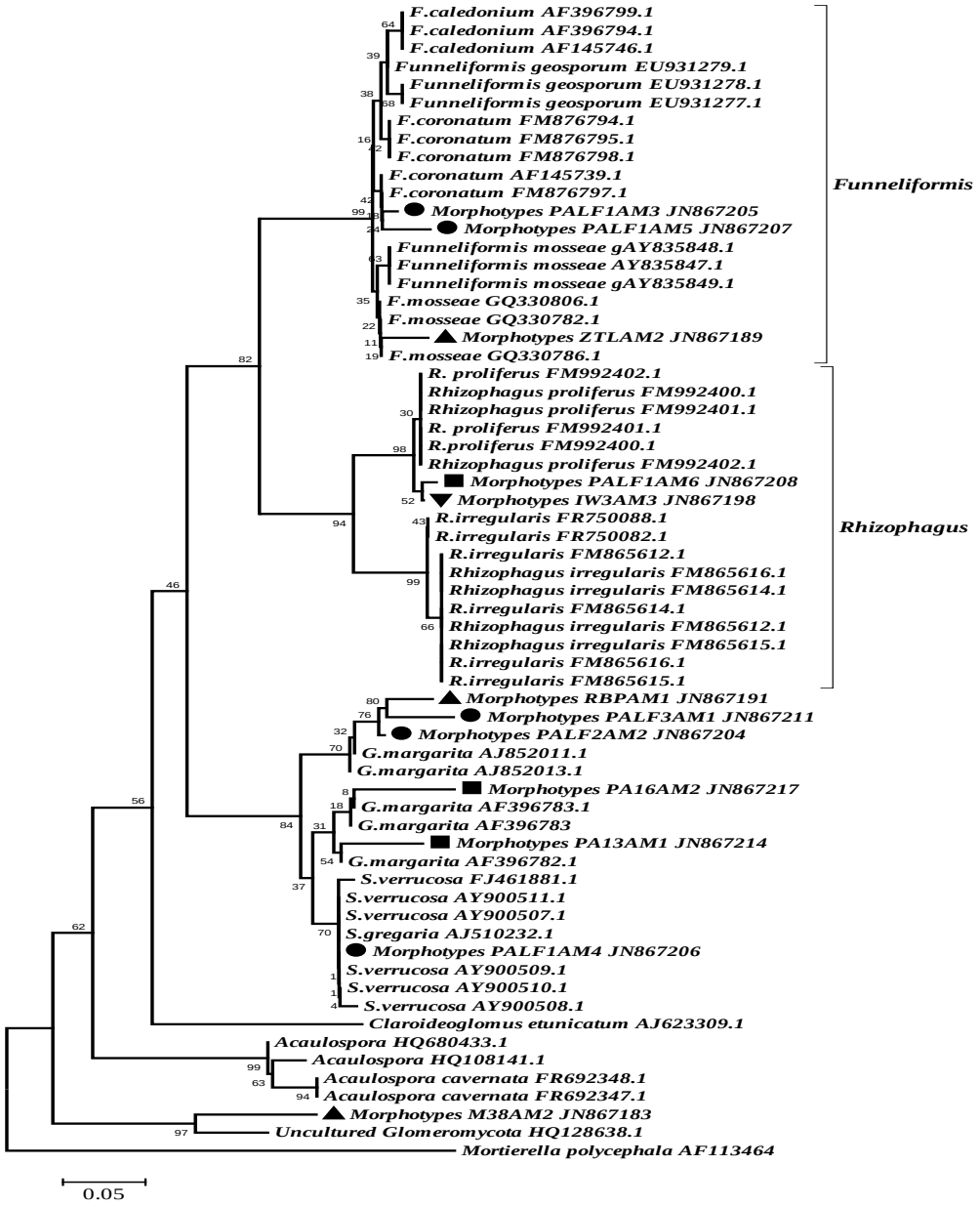

Figure 6. Sequences generated from amplicon of primer set (FLR3+FLR4) used for phylogenetic analysis. Neighbor joining tree obtained from alignment of 12 LSU rDNA (400 bp from 5' end) used in this study including 50LSU n-rDNA sequences retrieved from GenBank with (*Mortierella polycephala*) as an out-group. Percentage bootstrap support (out of 1000 replicates) is indicated. Names followed by accession no. represent sequences retrieved from GenBank. Names preceded by a shape represent the Sequence obtained in this work.

Futhermore, none of AMF morphotypes originated from chemically managed soil was clusterd with genera Gigasporaceae (Figures 4 to 6). The present study supports earlier hypothesis by Jansa et al. (2003), suggested significantly lower Gigasporaceae species in chemically managed soils. It was also reported by Johnson (1993) that application of inorganic fertilizers (High input) increased the abundance of *R. intraradices*, whereas other species like *Gigaspora gigantea, Gigaspora margarita, Scutellospora calospora* or *Paraglomus occultum* disappeared. Recent investigation by Mirás-Avalos et al. (2011) based on denaturing gradient gel electrophoresis (DGGE) sequencing found that increased presence of *Glomus* fungi in agricultural soil under conventional tillage practices.

Shannon-Weiner diversity index were significantly more

in trap culture set up field soil of raised bed plantation (RBP) and zero tillage (ZTL) soil (Table 3). Subsequently, sequencing of LSU rDNA also revealed that *Gigaspora* and *Acaulospora* species were present in RBP and ZTL sites respectively (Figures 5 to 6). Most of the AM fungal species that occurred in the RBP and ZTL do not occur in the other sites as the species cannot endure high degree of disturbances. Diversity index and species richness was more in organic and natural sites, a finding in line with the report by Gosling et al. (2010), who found that long-term application of organic manures results in rapid build-up of diverse range of AMF texa. Higher species richness in organic and natural land than the other sites due to higher diversity of host plant and sites have higher soil organic carbon that is more suitable for AM fungal growth (Bordoloi et al., 2015). Higher similarity index of species composition between chemically managed site with Industrial wasteland site may be due to lower tree diversity exists in both the site (Figure 3). Disturbance produce in the agriculture land is well known to all which not only suppress the plant diversity but also the microbial community that exists in association with them. Studies by Brokaw (1985) suggested that disturbance inhibit competitive interactions and minimize dominance of species, maintaining species diversity and richness.

Conclusion

Study revealed diversity of myocrrhizal fungi significantly affected by the different farming practices. The study provide lists of AMF species present in different soil type and land use system of subtropical soil and also provide data with which further studies can be compared. The result of our finding indicate that AMF diversity in organically managed soils was higher because of organic sources of nutrients such as farmyard manure and compost do not suppress sporulation of Gigasporaceae. Now recent advancement of next generation sequencing may provides more complete picture of distribution of arbuscular mycorrhizal fungal communities in different land use system especially to understand association of AM fungi with rare and endangered plant species as well as the medicinal plant species widespread in the tropical forest soil.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Development and evaluation of sundae-type "Coalhada" containing *Lactobacillus paracasei* and blueberry (*Vaccinium ashei*) preparation

Maria Eduarda Daussen Dutra[1]*, Daiane Aparecida Paggi[1], Luiza Martins Reguse[1], Ana Carolina Sampaio Doria Chaves[2] and Deise Helena Baggio Ribeiro[1]

[1]Centro de Ciências Agrárias, Departamento de Ciência e Tecnologia de Alimentos, Universidade Federal de Santa Catarina, Rod. Admar Gonzaga, 1346, Itacorubi, Florianópolis, SC, Brazil.
[2]Embrapa Agroindústria de Alimentos, Av. das Américas, 29501, Guaratiba, Rio de Janeiro, RJ, Brazil.

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"Coalhada" is a type of fermented milk (made with *Lactococcus lactis* spp. *lactis* and *Lactococcus lactis* spp. *cremoris*) very popular in Brazil, which is widely consumed due to its pleasant texture and mild taste and aroma. Its acceptance is possible to increase by adding a fruit preparation. Fermented milk is the most common food matrix used for delivery probiotic bacteria, which are able to survive to the passage through the gastrointestinal tract, reaching the colon in high concentration where they can effectively bind and subsequently, improve the body functions as a whole. In this study, two formulations of "Coalhada" were developed which resemble a sundae (in which, the fruit preparation stays on the botton of the package and the fermented milk on the upper part) used for a blueberry preparation. The formulation F1 was the control, with no addition of probiotic bacteria and to the formulation F2 the probiotic *Lactobacillus paracasei* was added. The formulations were evaluated over 14 days of storage under refrigeration. The characteristics evaluated in the product were: moisture, pH, acidity, lipids and ash content and all of them were not significantly affected ($p > 0.05$) by the addition of the probiotic bacteria. However, the addition of *Lactobacillus paracasei* significantly decreased ($p < 0.05$) the syneresis of the product during the refrigerated storage. Blueberry preparation had a high antioxidant activity which was 330.8 µmol TEAC 100 g^{-1} and the total phenolic compounds content was 36.65 mg GAE 100 g^{-1}, showing that the addition of blueberry can also improve the nutritional characteristics of the final product. The count of the lactic acid bacteria (including the probiotic one) remained high (> 10^9 CFU g^{-1}) during the 14 days of storage, but the shelf life of the two formulations was around seven days, since in the second week of storage the counts of yeasts and molds reached 10^2 CFU g^{-1} (which is the maximum allowed by Brazilian legislation). It is important to note that there was no addition of preservatives to the blueberry preparation, although the Brazilian legislation allows this and it could extend the shelf life of the final product, because yeasts and molds found in the product, probably comes from the fruits. Concluding that it was feasible to use blueberries in functional fermented milk such as "sundae-type Coalhada" and that it was a good way to innovate and to add value to the final product.

Key words: Fermented milk ("coalhada"), *Lactococcus lactis*, *Lactobacillus paracasei*, blueberry (*Vaccinium ashei*), shelf-life, lactic acid bacteria.

INTRODUCTION

In Brazil, “Coalhada” is a dairy product obtained through the fermentation of pasteurized or sterilized milk, being used with one or more species of mesophilic lactic acid bacteria (*Lactococcus lactis*). The microorganisms used should be viable, active and until the end of the shelf life of the product, the minimum concentration required in the final product should be 10^6 CFU g^{-1}. Its acidity must be between 0.5 and 1.5% of lactic acid according to the Brazilian Legislation (BRASIL, 2007).

The processing procedure of “Coalhada” is very similar to that used for yogurt production, but it differs in terms of the fermentation temperature. The ideal temperature is around 30°C and the microorganisms used for fermentation can be: *Lactococcus lactis, Lactococcus cremoris* and/or *Lactococcus diacetylactis* (Lerayer et al., 2009).

According to the Brazilian Legislation (BRASIL, 2007), fermented milks comprised standardized milk and/or reconstituted milk and the specific lactic acid bacteria for which type of fermented milk. Other ingredients, such as milk powder or milk protein concentrated, whey protein, caseinate, among others can be added and the addition of fruit pulp or fruit preparation is also permitted up to a maximum of 30% (w/w).

According to Lerayer et al. (2009), it is possible to divide the lactic acid bacteria (LAB) used to ferment milk into two main categories. The first one are the technological cultures, which are the acidifiers, responsible for the milk fermentation (to obtain the nutrients to multiply themselves) and they also give to the product a palatable characteristic; and the second group are the probiotic cultures, which are beneficial to the health of the host.

Probiotic are living microorganisms, which when ingested in sufficient amounts, will beneficially influence the health of the host by improving the composition of intestinal microbiota. They should be able to survive to the passage through the gastrointestinal tract and to bind to the colon (FAO/WHO, 2006).

A healthy and balanced enteric microbiota results in the normal performance of the physiological functions. With a high amount of probiotics in the colon, it is possible to maintain an environment hostile to the pathogens microorganisms (which could cause infections, besides the fact that they can produce toxins), by inhibiting their multiplication. In addition to the antimicrobial action, due to the production of compounds such as nisin, the probiotics also have hydrolytic activity on bile salts, contribute nutritionally and modulate immune activity. Probiotic bacteria should remain stable and viable in high amount during all the shelf life of the product (Saad et al., 2013). Lactobacilli are non-spore-forming, rod-shaped, anaerobic Gram-positive bacteria used for fermentation, especially, in the dairy industry. *Lactobacillus paracasei* is a probiotic bacteria used for cheese, yoghurt and other fermented milk production (Schmid et al., 2006). There are several scientific studies on the beneficial effects of *L. paracasei* ssp. *paracasei* in fermented products, which had shown that these bacteria helps in the reduction of hypercholesterolemia, hypertension, allergies, gastric damage, osteoporosis and obesity (Saad et al., 2013).

Many fruits are highly protective for human health, especially against ageing and oxidative-stress related to many diseases, due to their content of healthy phytochemicals. The berries are generally a small fruit that lacks big seeds and often are the richest source of natural antioxidants phytochemicals. Anthocyanin is a pigment that can be red, purple, violet or blue, it is a water soluble polyphenolic pigment widely found in the “berries fruits” which can prevent oxidative stress (Pojer et al., 2013).

Blueberry is a small fruit native from the United States; it is a flowering plant which belongs to the genus *Vaccinium* and to the family Ericaceae. In Brazil the cultivation of the species of *Vaccinium ashei* is still small but growing and it is concentrated in the southern states, where there are several days of cold during the winter, which allows the production of this berry (FACHINELLO, 2008).

Blueberry is one of the “super fruits” due to their potential bioactive compounds and with this fruit is possible to produce many functional foods. Blueberries have a flattened shape with a diameter between 1 and 2.5 cm and weight from 1.5 to 4 g. Its taste varies from sweet acid to acid and it has many seeds inside. It is known that anthocyanins are the nature’s most potent antioxidants and have demonstrated properties that extend well beyond suppressing free radicals (Srivastava et al., 2007). The blueberries may alleviate the cognitive decline in Alzheimer’s disease and other conditions of aging (Krikorian et al., 2010).

The high content of functional compounds makes the blueberry one of the richest fruits in terms of antioxidant compounds, since it contains significant levels of phenolic compounds, including anthocyanins, flavonoids and procyanidins, with a high level of biological activity, providing different health benefits (Koca and Karadeniz, 2009). Continued intake of phenolic compounds is associated with prevention of some degenerative diseases (Silva et al., 2010).

Blueberries are widely consumed *in natura* and often

*Corresponding author. E-mail: dudsdutra@gmail.com. Tel: +554837212897.

they are processed into a fruit preparation that can be used in production of fermented milk, in yoghurt, in ice cream, or in bakery products (Stewart, 2004). An attractive red color dependent on the pigment concentration is one of the most important quality characteristics of berry yoghurts.

The consumption of fermented milks is increasing worldwide and consumers are demanding new products, different formulations, organic and minimally processed fruit. In this research, fermented milk with a fruit preparation, the “Blueberry Coalhada”, which resembles a sundae, with and without the addition of the probiotic *Lactobacillus paracasei* was developed. The “Blueberry Coalhada” sundae type was characterized to evaluate the influence of the probiotic bacteria on the technological characteristics of the final product during the refrigerated storage.

MATERIALS AND METHODS

Ingredients (UHT milk, skimmed powdered milk and sugar) were purchased from a local store in Florianopolis, Santa Catarina State (SC), in Brazil for the preparation of two formulations of “Blueberry Coalhada”. Blueberries (cultivar of highbush blueberry - *Vaccinium corymbosum* L.) were purchased from a local market, in São José, SC, Brazil. Lyophilized bacteria used for the fermentation were the commercial lactic acid bacteria (DVS), a mixture of *Lactococcus lactis* and *Lactococcus cremoris* (R-704, from Chr Hansen®) and the probiotic *Lactobacillus paracasei* (BGP 93, from Sacco®).

“Coalhada” were produced on a laboratory scale using UHT milk (fat content 32 g L^{-1}) and was added 5% of sugar (w/w) and the mixture was heat-treated at 95°C for 5 min. The protein content of the “coalhada” was increased by addition of 5% of non-fat milk powder. This mixture was cooled to 30°C for the inoculation with the lactic acid bacteria. Both formulations (F1 and F2) were inoculated with 1% of a mixture of *Lactococcus lactis* and *Lactococcus cremoris* starter and afterwards, at formulation F2, 1% of the probiotic *Lactobacillus paracasei* was added. F1 was the control formulation, without the addition of *L. paracasei*. The fermentation was carried out at 30°C until a pH decrease at 4.6, when the products were cooled to 4°C to dramatically reduce the fermentation.

The fresh blueberries were washed and sanitized with sodium hypochlorite solution (100 mg L^{-1} during 15 min), then they were rinsed in potable water. To the blueberry preparation, it was added 10% of water and 40% of sugar in relation to fruit weight. This mixture was concentrated and also pasteurized by heat treatment (95°C during 10 min), then 1% (w/w) of pectin was added to the mixture and boiled for 5 min. The addition of pectin was in the end of the heating process to avoid its degradation during the heat treatment. The preparation was hot fill packaged into a previously sanitized glass container and it maintained under refrigeration at 4°C until usage. The pectin was added to increase the viscosity of the blueberry preparation, a slight jellification is important to avoid the mixture with the inoculated milk which was added in the upper part of the product.

Blueberry *in nature* and the blueberry preparation were analyzed by the following determined parameters: content of total soluble solids (°Brix) using a digital refractometer (Mark Mettler, Toledo) and pH using a digital potentiometer (from Digimed Brand), according to the recommendation of the manufacturers. Determination of the phenolic compounds content was performed by the Folin-Ciocalteu spectrophotometric method (Singleton and Rossi, 1965) and the antioxidant capacity according to Brand-Williams et al. (1995).

Physicochemical characterization of final product: content of moisture, protein, lipids, carbohydrates, ash and total acidity was carried out according to the methods described in AOAC (2010). To evaluate the spontaneous syneresis was applying the method described by Amatayakul et al. (2006).

The microbiological analyses of the two formulations were performed every seven days, by means of the counts for the yeasts and molds, lactic acid bacteria and total and thermotolerant coliforms according to APHA (2001, 2004).

The analyses were performed in triplicate and the results were subjected to variance analysis (ANOVA) and Tukey test to identify the significant differences ($p < 0.05$) between the means, using the software Statistica 7.0®.

RESULTS AND DISCUSSION

After 5 h and 30 min of fermentation at 30°C and the pH reached 4.6, leading to the coagulation of the milk and resulting in the “Coalhada”. There was no difference regarding the final pH of the two formulations (F1 and F2), so the addition of the *L. paracasei* did not affect the fermentation time. According to Arruda (2013), the fermentation of five different formulations of “coalhada” with the addition of passion fruit required 8 to 12 h, much longer than it takes for yoghurt fermentation after probiotic addition, as reported by Gallina et al. (2011).

The pH reduction is attributed to the continuous production of lactic acid by the inoculated lactic acid bacteria that destabilizes the casein micelles and it aggregates therefore, leading to the formation of the casein acid coagulum. It occurs when the pH reaches the isoelectric point of the casein (pH of 4.6), when its solubility is minimal (Tamine and Robinson, 2007).

The pH evaluation during the fermentation is important, since the low acidity (pH > 4.6) can cause separation of the serum from the coagulum once it is not completely formed and on the other hand, a sharp fermentation with a pH < 4.0 promotes the coagulum contraction due to poor proteins hydration, causing a severe syneresis (Tamime and Robison, 2007).

During the 14 days of storage under refrigeration, a slight post acidification in both formulations, with a decrease in the pH from 4.83 to 4.53 was observed; although this pH variation was not significant ($p > 0.05$).

However, since the first week of evaluation, the syneresis was higher in F1 than in F2 (Table 1), and after 14 days of storage, the serum separation was 17.68 and 6.08% for F1 and F2, respectively. These results suggest that the addition of *L. paracasei* reduced substantially the syneresis without affecting significantly the post acidification, thus improving the technological characteristics of the developed product.

Although the blueberries have a low pH, it is still possible yeasts and molds growth, especially the acid-tolerant molds, due to that the blueberry preparation was pasteurized. The heat treatment also has the function to contribute in the dissolution of sugar (Moura et al., 2009). A partial evaporation of water during the processing

Table 1. The pH-values and percentage of syneresis of Sundae-type "coalhada" with blueberry (F1) and Sundae-type "coalhada" with blueberry containing *Lactobacillus paracasei* (F2) during storage.

Storage (days)	pH		Syneresis (%)	
	F1	F2	F1	F2
1	4.83^{aA}	4.85^{aA}	0^{xX}	0^{xX}
7	4.64^{aB}	4.54^{bB}	8.56^{xY}	3.16^{yY}
14	4.54^{aC}	4.53^{aC}	17.68^{xZ}	6.08^{yZ}

Values in the same line with different superscript small letters are significantly different ($p<0.05$). Values in the same column with different superscript capital letters are significantly different ($p<0.05$) according to the Tukey test.

Table 2. Centesimal composition of Sundae-type "coalhada" with blueberry (F1) and Sundae-type "coalhada" with blueberry containing *Lactobacillus paracasei* (F2).

Composition	Mean (± standard deviation)	
	F1	F2
Moisture	73.40 ± 0.02^{a}	73.35 ± 0.15^{a}
Protein	4.35 ± 0.08^{a}	3.91 ± 0.06^{b}
Lipids	2.70 ± 0.12^{a}	2.54 ± 0.15^{a}
Ashes	1.08 ± 0.01^{a}	1.09 ± 0.01^{a}
Carbohydrates*	18.49 ± 0.02^{a}	19.05 ± 0.12^{b}
Acidity**	0.95 ± 0.01^{a}	0.91 ± 0.02^{a}

*Carbohydrates calculated by difference. **Acid content in % of lactic acid. Values in the same line with different superscript letters are significantly different ($p < 0.05$) according to the Tukey test.

occurs and the combination of this effect with the sugar addition made the soluble solids increased from 17.4°Brix (in the fresh fruit) to 50°Brix at the blueberry preparation. The blueberry preparation showed a 366.59 mg of gallic acid equivalents (GAE) per 100 g of total phenolic compounds, which is consistent with the results found by other researchers. Gesser et al. (2012) observed that, in a blueberry preparation with 30°Brix (which was used for the production of a probiotic fermented milk), the content of phenolic compounds was 336.41. Rocha (2009), Silveira et al. (2007) and Rodrigues et al. (2011) analyzing the blueberry pulp, the blueberry extract and the fresh fruits found 317.6, 277.4 and 436.6 mg GAE 100 g^{-1}, respectively.

The antioxidant activity determined in this study was 3308.1 µmol of TEAC in 100 g of sample and it was in the range that Sellappan et al. (2002) reported that using the same method, they found values between 811 and 3829 µmol of TEAC 100 g^{-1} of sample for several varieties of blueberry.

Table 2 shows the chemical composition of the two formulations (F1 and F2), with the mean and the standard calculated. Regarding to the chemical composition of the two formulations, there was no significant difference ($p > 0.05$) in relation to the moisture, lipids and ash content. Taking into consideration the lipid content on "coalhada", the final product was partially defatted, since the fat contents ranging from 0.6 to 2.9%. Protein and carbohydrate content were statistically different (Table 2). Protein was lower in F2, and due to that, carbohydrate content was also lower because it was calculated by the difference.

According to Tamime and Robinson (2007), it is better to add skimmed milk powder than whey protein or caseinate to increase the protein content in order to have a firm gel with the minimum syneresis. The addition of 5% of skimmed milk powder to both formulations in this study aimed to raise the protein content of the final product, improving its texture without increasing the fat content.

It was observed that a normal acidity for both formulations, is within the values expected for "coalhada" (0.5 to 1.5), and the difference between the two formulations was not statistically significant. According to Tamime and Robison (2007), acidity is one of the factors limiting the acceptance of fermented milk.

The counts for the total and thermotolerant coliforms for both formulations were less than 3 MPN g^{-1}, indicating that good manufacturing practices (GMP) were applied during the production, once these microorganisms are easily eliminated by washing and sanitizing all the surfaces or by heating the ingredients (Franco and Landgraf, 2008).

The total count for the lactic acid bacteria remained constant at 10^9 CFU g^{-1} for both formulations during the 14 days of analysis. Identity and quality standards of "coalhada" provide that the starters used must be viable, active and present in a concentration higher than 10^6 CFU g^{-1} throughout the shelf life of the product (Brasil, 2007). And if the product has at least 10^8 CFU g^{-1} of *Lactobacillus paracasei*, it can have a probiotic claim.In the first day of the analysis, the counts of the yeasts and molds were less than 10 CFU g^{-1} for both formulations, but after seven days of storage, the F1 formulation had reached 5×10 CFU g^{-1} and after 14 days, both formulations had values above the upper limit established by the Brazilian legislation (10^2 CFU g^{-1}). Arruda (2013) observed a shelf life of 28 days for "coalhada" mixed with passion fruit and Gesser (2012) reported a shelf life of 14 days for a probiotic fermented milk containing a blueberry preparation.

The presence of molds and yeasts can also be an indicator of poor sanitary practices in the manufacture and packaging of the final product. However, to these products was added sugar and fruit that are especially susceptible to the development of spoilage yeasts and typically, the shelf life of these products is limited by the multiplication of such microorganisms.

Conclusion

Based on the results of this study, it was concluded that the sundae-type “coalhada” with a blueberry preparation had a shelf life of seven days, which was limited by the yeasts and molds count, although the total lactic acid bacteria remained high throughout the analysis period. It is important to note that there was no addition of conservatives to the blueberry preparation, which is allowed by Brazilian legislation and could extend the shelf life of the product up to three or four weeks. The addition of *L. paracasei* did not interfere with the fermentation, but it had a positive effect, since it significantly decreased the syneresis of the product.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Modification of pathogenic microbiota and histology of gastrointestinal tract of *Archachatina marginata* (Swainson, 1821) by *Carica papaya* seed meal

Odo G. E*, Ekeh F. N, Ekechukwu, N. E, Agwu, E. J, Aguzie I. O. E. and Ugwuezumba B.

Department of Zoology and Environmental Biology, University of Nigeria, Nsukka, Nigeria.

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This study investigated the bactericidal efficacy of the ethanolic extract of *Carica papaya* seed on snail gastrointestinal tract (GIT) microbiota and associated histological changes. The bacterial isolates were characterized based on colony morphology, culture characteristics and biochemical tests. Each portion of the gut was further subjected to histological examination to ascertain the effect of this extract on the various regions of the snail gut. Three bacterial species (*Salmonella*, *Klebsiella* and *Escherichia coli*) were isolated from the GIT. *Salmonella* was the major isolate from all the sections of the GIT in the control. *Klebsiella* was the major isolate from all the GIT sections after administering 50 mg/kg body weight (b. wt) of the extract while *Salmonella* was absent. *Klebsiella* was the main isolate after administering 50 mg/kg, 100 mg/kg and 150 mg/kg of extract. *E. coli* and *Salmonella* and *Klebsiella* were isolated after administration of 150 mg/kg b. wt of extract. The histological changes included vacuolation of the crop, and reduction in sub-mucosal fat in the intestinal wall. The extract altered the microbiota of *A. marginata* GIT in a concentration dependent manner.

Key words: *Archachatina marginata, Salmonella, Klebsiella, Escherichia coli,* antibacterial.

INTRODUCTION

Carica papaya (pawpaw) is a commonly consumed fruit in Nigeria. The leaf has been widely used in Nigeria and several parts of the world in traditional medicines (Awais, 2008; Nirosha and Mangalanayaki, 2013; Orhue and Momoh, 2013). In recent times attention has been drawn to ripe and unripe *C. papaya* seed as being medicinal. Recently, reports of their antimicrobial and antifungal properties against common human microbes and plant fungi were made (Dawkins et al., 2003; Akujobi et al., 2010; Chávez-Quintal et al., 2011; Singh and Ali, 2011; Eke et al., 2014; Peters, 2014). In an entirely different contest, deleterious effects of extracts of seed of *C. papaya* from ripe and unripe fruits were made. Azoospermia, degeneration of epididymis and reduced gonad development (reduced fecundity and gonadosomatic index) were among *C. papaya* extact associated negative consequences (Lohiya et al., 2002; Abdelhak et al., 2013; Madan, 2013).

*Corresponding author. E-mail: gregory.odo@unn.edu.ng.

In another contest not related to fertility, Adeneye et al. (2009) and Naggayi et al. (2015) reported hepato-protective and nephroprotective activities respectively of aqueous *C. papaya* seed extracts. These observations were contradicted by studies made by Dikibo et al. (2012) and Paul and Ligha (2015) where hepatotoxicity was associated with ethanolic and hydromethanolic *C. papaya* seed extracts respectively. Thus, further *in vitro* and *in vivo* studies to harmonize, where possible, these differences in observations are needed.

In Nigeria *C. papaya* trees are common sites. They flourish well adorned with fruits as mark of fertile soil in Nigeria. The full potential of these fruits are not fully utilized as several are allowed to rotten on the tree. Large quantities of the fruit are seen in waste dumps near fruit market because of lack of appropriate storage facilities in Nigeria. This creates the need for innovative strategies to adequately curtail such enormous waste in a nation where food sufficiency seems an intractable challenge, per capita income deplorable and need for diversification of national revenue sources overwhelming. These problems are further complicated by the dwindling oil revenue of the nation (Uzonwanne, 2015). Building storage facilities may not be feasible due to multiple financial commitments of the Federal Government of Nigeria. The use of the seeds of *C. papaya* fruits in traditional medicine and the incorporation of the whole fruit into snail feed may be a useful alternative.

Archachatina marginata (Swainson, 1821), a giant African land snail is widely consumed in Nigeria. Advocacies for snail farming as an alternative source of protein to help alleviate the problems of nutritional deficiencies in Africa has helped enhance snail farming in recent times (NAERLS, 1995; Agbogidi and Okonta, 2011; Afolabi, 2013). Thus, this study had two core objectives which were to (i) investigate the effect of the ethanolic extract of ripe *C. papaya* seed on the gastrointestinal microbiota of *A. marginata*; (ii) and ascertain histological changes of the gastrointestinal tract associated with the extract.

MATERIALS AND METHODS

Collection of samples

The snails used for the study were purchased from Ibagwa and Orba, Nsukka Local Government Area, Enugu State, Nigeria, and transported to the Animal and Breeding Unit, Department of Zoology and Environmental Biology, University of Nigeria, where they were allowed to acclimatize for 2 weeks.The *C. papaya* seeds used for this investigation were collected from ripe pawpaw fruits purchased within the Nsukka metropolis. The seeds collected were dried at room temperature until constant weight was attained.

Preparation of ethanolic extract

After drying, the seeds were ground and weighed. The powdered product was extracted using absolute ethanol in a ratio of 1: 4 (that is, 100 g of crude seed extract in 400 ml of ethanol). Extraction lasted for 24 h and Soxhlet apparatus was used. The extract obtained was filtered off with a muslin cloth; and the filtrate collected and evaporated to dryness. LD_{50} test was carried out, and the sub-lethal ranges that could be used for the experiment determined.

Experimental design

A completely randomized design comprising four treatment groups (A - D), each in triplicates. Each triplicate contained 8 snails placed in one-third sand filled lidded experimental basket. Group A served as control, feed only normal snail feed. The snail in groups B, C and D were, in addition to normal feed, administered 50, 100 and 150 mg/kg body weight respectively of ethanolic *C. papaya* seed extract for 21 days. Extract administration was by oral injection using a syringe aided by an improvised tube used to gently keep the mouth open and extract injected through it. Three snails were randomly selected from each triplicate, sacrificed and the gastrointestinal tract used.

Preparation for and dissection of snail specimen

Prior to examination, the shells were carefully removed and the snail body washed with Elizabeth leaf (*Chromolaena odorata*) collected from Department of Plant Science and Biotechnology, University of Nigeria, Nsukka. This leaf was used to remove slime from the snail before dissection.

The snails were dissected using the methods of Segun (1975). The alimentary canal was sectioned into buccal cavity-oesophageal zone, crop, stomach and intestine. Each region was homogenized separately in normal saline for the bacterial work and formal-saline for the histopathology examination.

Isolation and identification of microorganisms from *Archachatina marginata* gastrointestinal tracts

Each portion of the gut were streaked onto MacConkay's agar plates, using inoculating loop and incubated for 18 - 24 hours at 37°C for bacterial growth. The bacterial isolates were characterized based on colonial morphology, cultural characteristics and biochemical tests as described by Oyeleke et al. (2012). The isolates were identified by comparing their characteristics with those of known taxa using the Bergey's manual of determinative bacteriology (Holt et al., 1994). Four different agar media were used viz: MacConkay's, EMB, Simmon citrate and urease. The MacConkay's agar medium was used to test for lactose and non-lactose fermenters, in each region of the gut. The EMB agar medium was used to test for the presence of *Klebsiella* and as a confirmatory test for *E. coli*. A positive *E. coli* result gives a greenish metallic sheen on the agar plate. Simmon citrate agar medium was used to test for the presence of *Klebsiella* and as a confirmatory test for *Salmonella*. Urease agar medium was used to test for *Proteus enterobacter* and as a confirmatory test for *Klebsiella*. Gram staining was used to further categorize isolates.

Histopathology examination

Each portion of the gut was subjected to histopathological study to ascertain the effect of this extract on the various regions of the snail gut wall. Ten percent (10%) neutral formalin and Carnoy's solution were used as fixatives. Paraplast embedded tissues were sectioned at 5, 8, and 10 microns and stained with Harris' hematoxylin and

Table 1. Bacterial isolates from the gastrointestinal tract of *Acrchachatina marginata.*

GI Sections	MacConkay's	Gram staining	EMB	Simon citrate	Urease	Organism*
Control						
B-Oesophageal	LF	-ve rods	-	+	-	*Salmonella*
	NLF	-ve rods	-	+	-	*Salmonella*
Crop	LF	-ve rods	-	+	-	*Salmonella*
	NLF	-ve rods	-	+	-	*Salmonella*
Stomach	LF	-ve rods	-	+	-	*Salmonella*
	NLF	-ve rods	-	+	-	*Salmonella*
Intestinal	LF	-ve rods	-	+	-	*Salmonella*
50 mg/kg body weight						
B-Oesophageal	LF	-ve rods	-	+	+	*Klebsiella*
	NLF	-ve rods	-	+	+	*Klebsiella*
Crop	LF	-ve rods	-	+	+	*Klebsiella*
	NLF	-ve rods	-	+	+	*Klebsiella*
Stomach	LF	-ve rods	-	+	+	*Klebsiella*
Intestinal	LF	-ve rods	-	+	+	*Klebsiella*
100 mg/kg body weight						
B-Oesophageal	LF	-ve rods	-	+	+	*Klebsiella*
Crop	LF	-ve rods	-	+	+	*Klebsiella*
	NLF	-ve rods	-	+	+	*Klebsiella*
Stomach	LF	-ve rods	-	+	+	*Klebsiella*
Intestinal	LF	-ve rods	-	+	-	*Salmonella*
150 mg/kg body weight						
B-Oesophageal	LF	-ve rods	-	+	+	*Klebsiella*
Crop	LF	-ve rods	+	-	-	*E. coli*
	NLF	-ve rods	-	+	+	*Klebsiella*
Stomach	LF	-ve rods	-	+	-	*Klebsiella*
Intestinal	LF	-ve rods	-	+	-	*Salmonella*
	NLF	-ve rods	+	+	+	*E. coli*

-ve = negative, LF = Lactose fermenter, NLF = Non-lactose fermenter, B-oesophageal = buccal cavity-oesophageal section GI = gastrointestinal. + = present, - = absent. *main prevalent bacteria

eosin or Masson's trichrome. Fresh frozen, calcium-formol post-fixed cryostat sections were also stained with Harris' hematoxylin for histological reference.

RESULTS

Microbial Isolates

Salmonella was the main bacterial isolate from the gastrointestinal tract of the snails fed only normal feed (control group) (Table 1). Lactose and non-lactose fermenters were isolated from the buccal-oesophageal region, crop and stomach of the control, while only lactose fermenters was isolated from the intestine. *Klebsiella* was the major isolated from the gastrointestinal tract of the snails administered 50 mg/kg b.wt of *C. papaya* seed extract in addition to normal snail feed. Non-lactose fermenters were only isolated from the buccal-oesophageal region and crop in the 50 mg/kg b.wt treatment group.

Klebsiella and *Salmonella* were two major bacteria isolated from snails administered 100 mg/kg body weight of *C. papaya* ethanolic seed extract (Table 1). *Klepsiella* was the major isolate from the bucal-esophageal region to the stomach while lactose fermenting *Salmonella* were isolated from the intestine. Non-lactose fermenters were absent in all section in this group except in the crop. The diversity of major bacterial species isolates was highest in the snails administered 150mg/kg body weight of the extracts. *Klebsiella* was the major species isolated from

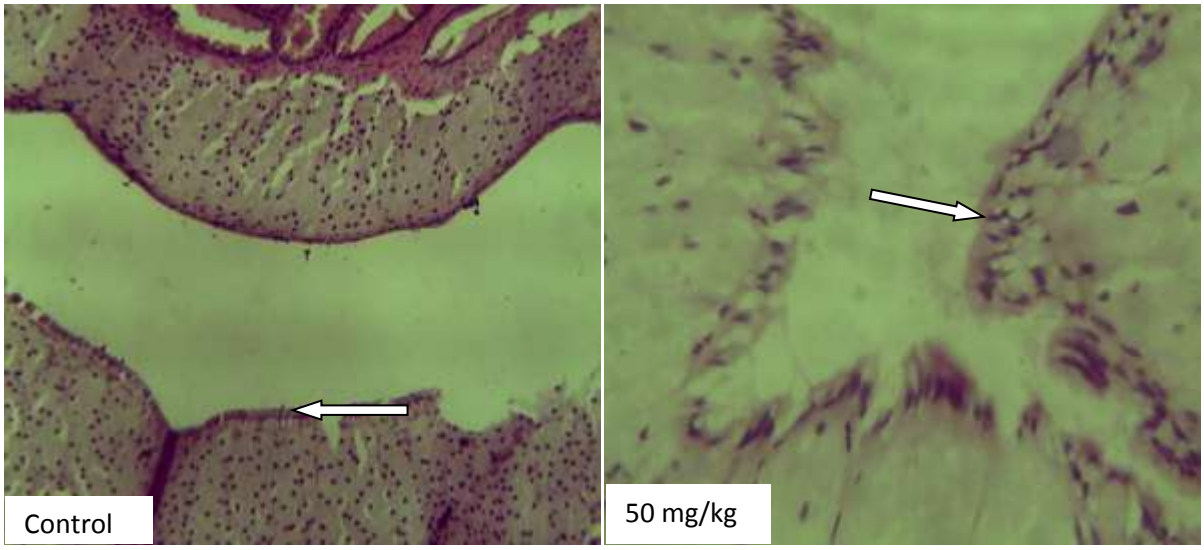

Figure 1. Histological section of the crop of the control and snails administered 50 mg/kg body weight of *C. papaya* seed extract (Magnification x 400). The epithelium of control was normal (white arrow) while that of snails administered 50 mg/kg b.wt of extract showed little vacuolation (white arrow).

the buccal cavity-oesophageal region, crop and stomach; lactose fermenting and non-lactose fermenting *E. coli* were the major in the crop and intestine respectively.

Histological sections

Notable changes in the histology of the gastrointestinal tract were observed in the crop and the intestinal sections (Figures 1 and 2). Slight vacuolations were observed in the cells lining the crop for snails administered 50, 100 and 150 mg/kg body weight of the extract. The layer of sub-mucosal fat thickness decreased in the intestinal wall of the snails administered 150 mg/kg body weight of *C. papaya* seed extract.

DISCUSSION

C. papaya is among the over 10,000 plants known to possess medicinal properties. Extracts of the leaf, seed, stem bark and fruit peels of *C. papaya* have been reported as possessing antimicrobial, antifungal and hepatoprotective potencies. Methanolic, ethanolic and aqueous seed extracts of *C. papaya* exhibited antifungal (Chávez-Quintal et al., 2011; Singh and Ali, 2011) and antimicrobial activities (Dawkins et al., 2003; Akujobi et al., 2010; Eke et al., 2014; Peters, 2014). Chávez-Quintal et al. (2011) reported antifungal activities of ethanolic seed extracts against *Fusarium* sp. and *Colletotrichum gloeosporioides*, even though ethanolic leaf of *C. papaya* was more potent. *Staphylococcus aureus, Pseudomonas aeruginosa, E. coli, Enterococcus faecalis* and *Salmonella typhi* were among the susceptible microbes. The potency of *C. papaya* seed as antimicrobial as shown from this study in *A. marginata* may be ascribed to its phytochemical constituents. Phytochemicals analysis by Chávez-Quintal et al. (2011) detected high concentrations of saponins and minute concentration of alkaloid and tripertenes in ripe *C. papaya* seed. Similarly, Eke et al. (2010) isolated saponins, flavonoids and alkaloids from unripe *C. papaya* seed. The types and percentage composition of phytochemicals in vegetal determines its efficacies as antimicrobial. Possibly, the low concentrations of alkaloids contributed to the reduced efficacy of ethanolic extract of ripe *C. papaya* seed as antimicrobial against gastrointestinal microbiota of *A. marginata*. This is as alkaloids are known to be effective as antimicrobial. A similarly opinion was shared by Chávez-Quintal et al. (2011), who investigated *in vitro* the efficacy of ethanolic extract of ripe *C. papaya* against fungi.

Susceptibility to antibiotics and antimicrobial extracts is relative to species and strains of microbes. While some strains and species are completely susceptible some are equally resistant. Structural architecture of microbes, biochemical constituents, genetic modifications and reproductive efficiency are among factors that determines an organism's response to unsuitable environments such as that presented by antimicrobial. The susceptibility of *Salmonella* in *A. marginata* administered 50mg/kg b. wt of the extract compared *Klebsiella* was probably due to resistant of *Klebsiella* to the extract. In human, *Klebsiella pneumoniae* is presently one of the microbes of global concern as a result of acquired antibiotic resistant (Barker, 1999; Fair and Tor, 2014; Calballero et al., 2015). The

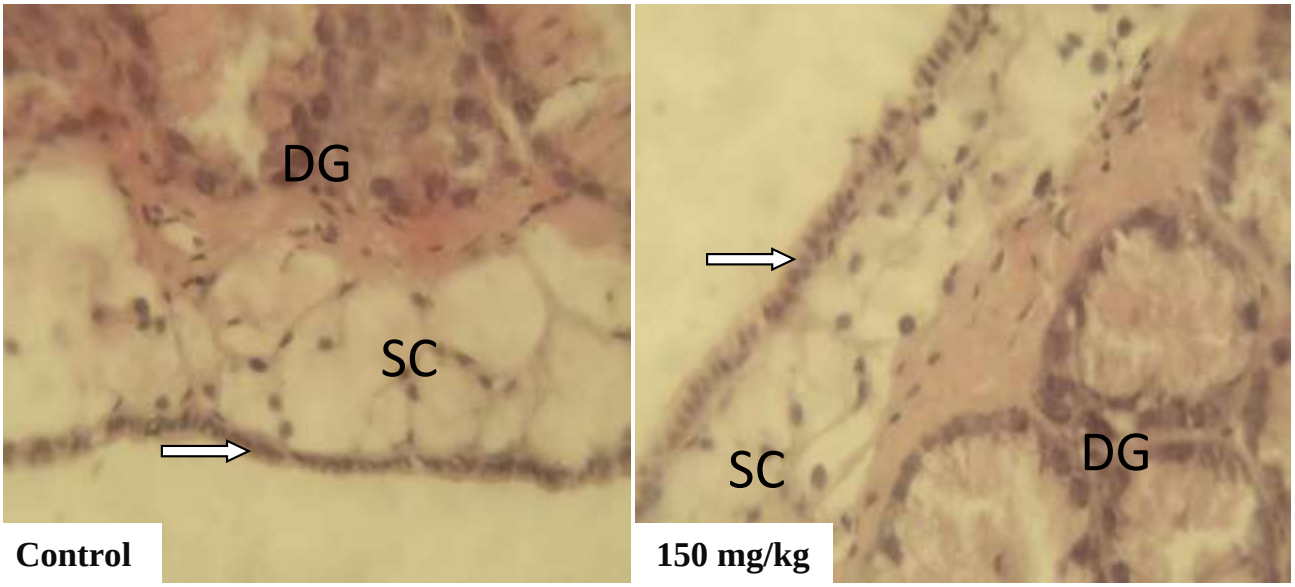

Figure 2. Photomicrograph of a longitudinal section of the intestine of the control and 150mg/kg body weight groups showing the intestinal wall (arrow) lying on typical digestive gland (DG). (Magnification x 400). The layer of sub-mucosal (SC) fat thickness decreased in snail administered 150 mg/kg b. wt of C. papaya compared to control.

inconsistencies in response of *Salmonella* as was observed by its survival at higher concentrations of the extract (100 and 150mg/kg b. wt), however, negates the notion of susceptibility. A susceptible species to a low concentration of an extract ought to remain susceptible at higher concentration even with tendency for increased susceptibility as extract concentration increased (Adetunji and Salawu, 2010; Aruljothi et al., 2014; Peters, 2014). *Salmonella* was susceptible to 100 and 150 mg/kg b. wt concentrations of *C. papaya* seed but not as much as was noticed for 50 mg/kg b. wt. Possibly, the absence or low concentration of antimicrobial phytochemicals such tannins and alkaloid as has been reported by previous studies (Chávez-Quintal et al*.,* 2011; Eke et al., 2014) may have contributed to this, such that response of microbe was irrespective of concentration of extract used. Differences in strains of *Salmonella* may have also contributed to resistance.

Histological modifications associated with *C. papaya* seed extracts are contradictory. Adeneye et al*.* (2009) reported hepatoprotective activities of aqueous extract of *C. papaya* seed in Wistar rats orally fed. Compared to the negative control that received carbon tetrachloride (CCl_4) without *C. papaya* seed extract that had severely congested hepatic central vein, improvement were noticed in terms of moderate to mild decongesting of this vein in those treated with concentrations of *C. papaya* in duration dependent manner (Adeneye et al*.,* 2009). Also, aqueous extract of *C. papaya* was observed to ameliorate the effect of paracetamol induced neprotoxicity in rats (Naggayi et al., 2015). Contrasting observations were made by Dikibo et al. (2012) and Paul and Ligha (2015), who observed hepatotoxic histological changes in Wister rats administered ethanolic and hydromethanolic extracts of *C. papaya* seed respectively; microvascular steatosis, ballooning necrosis of hepato-cytes, pyknosis, parachymal erosion, hemorrhages, mild vacuolation and embolism were some histological pathological changes noted from their study. We also noticed vacuolation and sub-mucosal fat reduction in the intestinal wall of *A. marginata* administered 50, 100 and 150mg/kg b. wt of *C. papaya* ethanolic seed extract. These pathologic histological changes probably resulted from the high saponins content of the seed extract. However, further studies may be needed to conclusively ascertain histological changes associated with *C. papaya* seed extract consumption as antimicrobial. Possible, the extraction solvent which determines the extraction products determine toxic or non-toxic potentials of *C. papaya* seed extracts. More so, anti-fertility activity of the powdered *C. papaya* seed meal in *Oreochromis niloticus* has been reported (Abdelhak et al., 2013). Chloroform and ethanolic extracts of *C. papaya* seed induced reversible azoospermia in langur monkey (Lohiya et al*.*, 2002) and albino rats (Madan, 2013). Toxic potencies of ethanolic extract of *C. papaya* seed was observed when it was used as molluscicide against schistosome intermediate hosts, *Biomphalaria pfeifferi* and *Bulinus globosus* (Adetunji and Salawu, 2010).

C. papaya ethanolic seed extracts possess antimicrobial activities against some gastrointestinal microbes of *A. marginata*. The *in vivo* use of this extract as antimicrobial and their consumption as food supplement require further studies, however, as it may possess some toxic

properties.

Conflict of interests

The authors have not declared any conflict of interests.

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Assessment of the microbial quality of some oral liquid herbal medicines marketed in Ile-Ife, South-western Nigeria

Oluwatoyin Abimbola Igbeneghu* and Adebayo Lamikanra

Department of Pharmaceutics, Obafemi Awolowo University, Ile-Ife, Nigeria.

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The extensive use of herbal medicinal products in the treatment and management of disease states within communities in Ile-Ife, south western Nigeria has made it imperative to investigate the microbial quality of a sample of these products in the light of the standards prescribed by regulatory bodies. This study was therefore carried out to assess the microbial quality of locally prepared and unregistered herbal oral liquid medicines available in Ile-Ife. A total of 50 herbal medicine samples were procured from various randomly selected markets in Ile-Ife. The microbial load of each sample was determined and the contaminants associated with each sample were identified. Samples that did not yield either bacterial or fungal growth were tested for their ability to elicit antimicrobial activity using the agar cup diffusion method. Results obtained in the course of the study showed that 90% of the samples carried microbial loads beyond officially permissible limits with *Escherichia coli* and *Salmonella* species being found in 2 and 6% of the samples, respectively. Since it was suspected that antibiotics were sometimes added to herbal products by some herb sellers to prolong the shelf life of their products, the screening exercise showed that the sample that did not yield bacterial growth exercised marked antimicrobial activity against both Gram positive and Gram negative organisms. The results of this study suggest that the herbal oral liquid products available to consumers in Ile-Ife are of unacceptable quality.

Key words: Microbial quality; herbal preparations; *Escherichia coli*; *Salmonella* species; antibiotics.

INTRODUCTION

Although oral pharmaceutical preparations are not required to be sterile, they are not supposed to be heavily contaminated by microorganisms or potentially pathogenic organisms including *E. coli*, *S. aureus* and *Pseudomonas aeruginosa* (USP, 2013). This is because apart from the safety of consumers, the presence of high microbial count in any preparation may lead to the proliferation of such organisms within the preparation leading to

*Corresponding author. E-mail: igbeneghuoluwatoyin@gmail.com.

spoilage (Bloomfield, 2007). This is why pharmaceutical companies are required to adhere to the principles of Current Good Manufacturing Practice (FDA, 2015) and their products must be subjected to total quality control measures, with the overall drug manufac-turing process being made to undergo quality assurance tests at every level. It has to be said however that, only the big pharmaceutical companies have the capacity to adhere to the principles of Current Good Manufacturing Practice. Okeke and Lamikanra (2001) noted that small pharmaceutical companies involved in the production of orthodox drugs, many of which are found in countries with challenged economies, are not able to invest in machinery, controls for production environments and the employment of qualified staff to see that their products are of consistently high quality. The situation is worsened in Nigeria by the existence of a large informal sector which is responsible for small scale production of a large number of unregistered and usually unstandardised medicines using rudimentary equipment and raw materials of plant derivation which are highly susceptible to extensive microbial contamination. In addition, the packaging of these products is often rudimentary with final products being packed in recycled plastic bottles which are frequently unlabelled. These unregistered herbal medicines are outside the control of the relevant regulatory bodies. However, they cannot be ignored as they are available virtually everywhere including and especially in rural areas, which are short of modern pharmaceutical cover.

The widespread use and availability of herbal medicines has been reported to be due to perceived efficacy, safety and absence of side effects from herbal products when compared to orthodox medicines (Kennedy, 2005; Clement et al., 2007). The use of herbal medicines is also likely to have increased because of emerging infections such as AIDS and drug resistant malaria (Gyasi et al., 2013; Lorenc and Robinson, 2013). The high cost of hospital consultation and orthodox drugs is an additional reason why herbal therapy may be attracting greater patronage (Gyasi et al., 2011). The use of improved packaging materials and increased public awareness through the organization of trade fairs on traditional medicine and the presence of NAFDAC registration numbers on registered herbal products are also likely factors that have increased the use of herbal products in Nigeria.

The issue of the safety of herbal products is however of great concern to many regulatory bodies who have therefore set out specifications on the quality of herbal products. Some regulatory bodies have given specifications on the microbial load and presence of specific organisms in herbal products (WHO, 2007a; European Pharmacopoeia, 2007). Many studies carried out over the years and in some parts of Nigeria have documented the different types of contaminants present in herbal products (Onawunmi and Lamikanra, 1987; Arias et al., 1999; Erich et al., 2001; Wolfgang et al., 2002; Adeleye et al., 2005; Okunlola et al., 2007; Abba et al., 2009). This study was carried out to determine the levels and identity of microbial contaminants of unregistered oral herbal liquid preparations available to consumers in Ile-Ife, south western Nigeria.

MATERIALS AND METHODS

Collection of herbal samples

Fifty (50) unregistered herbal oral liquid preparations produced and hawked by herb sellers were procured from major markets located in Ile-Ife. They were mostly aqueous decoctions produced from mixtures of several plant parts such as leaves, stems, roots and barks. The producers were found to be men and women, usually with no formal education. The markets included Ife New Market, Mayfair market, Sabo Market, Ede Road Market and Obafemi Awolowo University (O.A.U.) Central Market. Information concerning the uses and dosage of each preparation was obtained from the peddlers and documented. The samples were purchased as packaged by the herb-sellers and transported to the laboratory. Ile-Ife is a semi-urban city in south western Nigeria which lies on latitude 7°28' 0" N and longitude 4° 34' 0" E. It has a total area of 1,791 km^2 (692 sq mi) and as at 2006, the population was 509, 035. It is home to two universities; the Obafemi Awolowo University and the Oduduwa University as well as a teaching hospital, the Obafemi Awolowo University Teaching Hospitals Complex and several Public Health Centers and private clinics.

Determination of bacterial and fungal counts

In the laboratory, each of the samples was shaken properly to ensure a uniform distribution of the contents. Serial 10 fold dilutions in sterile water were then carried out and duplicate, 1ml portions of each dilution was aseptically placed into sterile petri-dishes. Twenty ml of molten nutrient agar (Oxoid, England) sterilized at 121°C for 15 min and cooled to 45°C for bacterial count or Sabouraud dextrose agar (Oxoid, England) for fungi was later added to each of the plates and gently mixed. The mixture was allowed to solidify at room temperature and the plates incubated at 37°C for 24 h for bacterial and 25°C for seven days for fungal populations. Plates containing 30 to 300 colonies were observed and the number of colonies that grew on each plate was recorded. Microbial load was expressed as colony forming units per ml of sample.

Isolation and storage of bacterial contaminants

Each colony having distinct colonial characteristics such as colour, shape, consistency and elevation, growing on the Nutrient agar count plate was picked and streaked onto freshly prepared Nutrient agar plates and incubated at 37°C for 24 h. The isolated colonies were each stored in an appropriately labeled cryovial and nutrient agar stabs stored at -4°C in a freezer and 4°C in a refrigerator respectively.

Identification of bacterial isolates

The Gram stain procedure was carried out on each isolate to

Table 1. Indications specified for samples of herbal oral liquid products.

Indication	Number percentage (%)
Body and joint pain	4 (8)
Blood infection	8 (16)
Candidiasis	6 (12)
Cough	6 (12)
Diabetes	8 (16)
Erectile dysfunction	2 (4)
Gonorrohea	2 (4)
Hypertension	6 (12)
Infection	2 (4)
Malaria	20(40)
Malaria and typhoid	4(8)
Mouth thrush	4(8)
Pile	12 (24)
Stomach ache	8 (16)
Tonsil infection	2 (4)

determine the shapes and class of the isolates. All Gram positive cocci were streaked onto the surface of over-dried Mannitol Salt agar and the colonial characteristics were noted. Further tests for identification of the Gram positive cocci included catalase and coagulase tests. All Gram negative rods were streaked onto MacConkey agar, Eosine Methylene Blue agar, Salmonella-Shigella agar and Triple Sugar Iron agar. The colonial characteristics were noted and biochemical test which included Indole, Urease, Citrate utilization, M.R.V.P. and oxidase tests were carried out on the isolates which were identified based on the interpretation of results of the biochemical tests (Barrow and Feltham, 1993; Farmer 1999).

Determination of anti-microbial activity of herbal product samples with no microbial contaminants

One of the herbal samples did not show any bacterial growth on Nutrient agar and another did not yield fungal contaminants. Both of them were further examined for antimicrobial activity against selected bacterial and fungal isolates respectively. The sample which did not show any bacterial contamination was tested against reference bacterial strains namely *Bacillus subtilis* NCTC 8236, *E. coli* ATCC 25922, *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 10145 while the sample not yielding fungal contaminants was tested against *Candida albicans* ATCC 24433. A volume of 0.2 ml of overnight broth culture of each test bacterium was seeded into 20ml of sterile molten Nutrient agar at 45°C. The plates were allowed to set and harden before incubating at 37°C for 20 minutes for acclimatization and growth of the inocula. Two holes of 8 mm diameter and equidistant to each other was bored into the plates using a sterile glass cork borer. The bottom of each hole was then sealed with one drop of molten Nutrient agar. Four drops of each of the test samples were placed in each of the holes and the plates were left to stand for 1 h to allow adequate diffusion of the samples. The plates were thereafter incubated at 37°C for 24h. The diameters of the zones of inhibition around each hole in the plates were measured in millimeters. The same procedure was repeated for the test against *C. albicans* ATCC 24433 but using Sarbouraud Dextrose Agar as the test medium and incubation at 25°C for 48 h.

RESULTS

The 50 preparations used in this study were presented by the herb sellers for the treatment of various ailments (Table 1). A total of 48 (96%) of the herbal products were packaged and sold in plastic bottles of which 79.2% were discarded with bottles previously used for packaged water and 20.8% were discarded with alcoholic drinks' containers (Plate 1).

The mean bacterial load of the samples ranged from zero cfu/ml to 2.94×10^{12} cfu/ml while the mean fungal count ranged from zero cfu/ml to a maximum of 3.54×10^{12} cfu/ml. According to the World Health Organization (2007a) and the European Pharmacopoeia (2007), for herbal medicinal products to which boiling water is not added before use, the limits specified for total viable aerobic count are 10^5 bacteria and 10^3 fungi per gram or per millilitre. Only 10% of the samples were therefore of acceptable quality in terms of microbial loads (Table 2).

Identity of contaminants

A total of 85 bacterial isolates were recovered from 49 of the 50 samples and included 36 Gram-positive and 49 Gram-negative organisms. The most frequently isolated contaminants in the tested sample were *Bacillus* species (40%), followed by *Klebsiella* species (31.8%) as shown in Table 3. Other contaminants included *Escherichia coli*, *Staphylococcus* species, *Salmonella* species and *Pseudomonas aeruginosa.* A total of 52% of the samples had one bacterial contaminant each, 26% of the samples had two while 20% had three contaminants. Four contaminants were recovered from one sample.

Plate 1. Picture showing some samples in their packaging (samples were labeled by authors after purchase).

Table 2. Acceptability status of samples.

Quality	Frequency (%)	Remark
Only bacterial load within permissible limit	18	Unacceptable
Only fungal load within permissible limit	4	Unacceptable
Both bacterial and fungal load within permissible limit	10	Acceptable
Bacterial and fungal load beyond permissible limit	68	Unacceptable

One of the samples from which only fungal contaminants were recovered showed marked anti-microbial activity against reference strains of bacteria (Table 4). The other sample from which only bacterial contaminants were recovered exhibited an appreciable antifungal but weak antibacterial activity.

DISCUSSION

According to the WHO report, there is widespread availability and usage of herbal preparations by a large percentage of persons in many developing countries (Robinson and Zhang, 2011). Some reasons for this have been documented by several authors and these include perceived efficacy, safety and absence of side effects (Kennedy, 2005; Clement et al., 2007; Gyasi et al., 2013; Lorenc and Robinson, 2013). One observation made by the authors of this study suggests that the high patronage of herbal medicine peddlers in Ile-Ife may actually be the appearance of a ready capacity for the treatment or management of all manner of communicable and non-communicable diseases. An example is that many of the producers of herbal medicine claim to have products for curing AIDS, a condition yet to have a specific orthodox cure (Gyasi et al., 2013; Lorenc and Robinson, 2013). The analysis of the indications for which the preparations in this study were produced showed that over 80% of the preparations were claimed to be for infectious diseases. This is not surprising as there is a high incidence of infectious disease in developing countries (Krämer et al., 2010) associated with many conditions such as

Table 3. Identity of contaminants present in herbal preparations.

Organism	Number of samples contaminated percentage (%)
Bacillus cereus	7 (14)
Bacillus spp. other than *B. Cereus*	27 (54)
Citrobacter spp.	1 (2)
Enterobacter spp.	5 (10)
Escherichia coli	1(2)
Klebsiella spp.	27 (54)
Pantoea agglomerans	3 (6)
Proteus spp.	6 (12)
Pseudomonas fluorescens	1 (2)
Other *Pseudomonas* spp.	2 (4)
Salmonella species	3 (6)
Coagulase negative *Staphylococcus*	1 (2)
Staphylococcus epidermidis	1(2)

environmental and sanitary conditions that favor the proliferation of infectious disease causing agents (Krämer et al., 2010). Some of these ailments, for example malaria, typhoid, blood infection (septicaemia) and candidiasis are quite serious and may be life-threatening.

Only 10% of the samples assessed in this study were of acceptable microbial quality. As judged by the absence of any form of labels on this class of herbal samples, it was difficult to determine why these were better than the other 90%. The acceptable samples were similar in appearance and packaging and the vendors were located in the same areas as the unacceptable ones. The absence of a label which is one of the characteristics of these unregistered herbal drugs makes it difficult to compare the samples in terms of concentration of herbs or identity of the components of each preparation. The microbial loads of 90% of the samples assessed in this study were beyond the limits stipulated by the regulatory bodies (WHO 2007; European Pharmacopoeia, 2007). Apart from the heavy microbial loads, the presence of unacceptable organisms or pathogens was demonstrated in the herbal samples. The unacceptable organisms recovered included the Gram negative organisms *E. coli* and *Salmonella* species. These are organisms associated with the gastrointestinal tract and indicate the likelihood of faecal contamination (Edberg, 2000). These contaminants could be acquired from the use of water of poor quality for the preparation of the samples and rinsing of containers. Other likely sources are the use of inadequately washed or disinfected plant parts previously exposed to manure. Plant materials such as vegetables have been reported as reservoirs of a wide range of bacteria including enteric pathogens (Holden et al., 2009).The presence of *Escherichia coli* and *Salmonella* spp. has also been stated to be an indication of poor quality of production and harvesting practices (WHO 2007). The recovery of a high number of *Bacillus* species, the frequently predominant aerobic spore-forming bacteria naturally occurring microflora of medicinal plants, supports the fact that vegetative plant parts and roots that have been in contact with the soil or dust are among the components of the preparations. The presence of organisms such as *S. aureus* suggest that contamination could also have occurred through handling by personnel who carry pathogenic bacteria or normal commensals during harvest/collection, post-harvest processing and the manufacturing process. The presence of several contaminants in a single preparation as observed in this study is expected since the preparations usually contain more than one plant or plants parts that have been obtained from multiple harvest sites. The practices of transportation and storage may also cause additional contamination and microbial growth. Proliferation of microorganisms also results from failure to control the temperatures of liquid forms and finished herbal products (WHO, 2007a). Other possible sources of contaminants are the environment and utensils in which the preparation were made as well as the containers used for packaging the preparations. An ideal package should be such that it does not adversely affect the microbial quality of intended preparations. Traditional packaging containers for herbal medicines were small gourds, earthenware pots, tortoise shells, horse hooves, horns of various animals, brass pots, as well as hollow tin rods plugged at both ends or sealed at one end into a conical shape (Sofowora, 2008). These containers were not air tight, so contamination through atmospheric microorganisms was inevitable. One plausible explanation for the absence of these older packaging materials among the samples obtained in this study is that herb sellers may be deliberately packaging their product in a way to improve acceptance by the general public. In addition, the older packaging materials

Table 4. The antimicrobial activity of samples from which either bacteria or fungi was not recovered.

Herbal sample	Test organism / Average zone of inhibition (mm)				
	C. albicans ATCC 24433	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 10145	*B. subtilis* NCTC 8236	*S. aureus* ATCC 29213
Minus bacteria	ND	17	20	18	24
Minus fungi	20	0	0	11	0
Ciprofloxacin 2 mg/ml	ND	32	28	20	28
Ketoconazone 1 mg/ml	30	ND	ND	ND	ND

are now less likely to be available than they were in the past. A total of 48 (96%) of the containers of the samples in this study were plastic bottles of packaged water or carbonated drinks that had been discarded after use. The remaining were bottles of alcoholic beverages and polythene bags. In the environment where this study was carried out, such containers are usually picked up from dump sites or from sites of outdoor parties. Other sources of these discarded bottles are peddlers who purchase these used bottles from bulk sellers. In a case where these containers were usually not washed or simply rinsed with a small quantity of water or water of poor quality, they are potential sources of heavy microbial contamination. The WHO already has specifications for the ideal presentation of herbal products (Patel et al., 2011).

The identity of the contaminants recovered in this study which suggests that possible sources of the contaminants include the environment, raw materials, hands of the producers and the water for production is an indication of a high level of non-adherence to the requirements of Good Manufacturing Practice. This needs to be addressed and producers should be made aware of the benefits of implementing best practice guidelines such as GACP and GMP. In these guidelines, requirements have been described for the raw materials, water for production, preparation utensils and items of equipment, the environment and personnel. The need for hygiene and sanitation of material and environment and the personal hygiene of personnel has been well spelt out to ensure the production of micro-biologically safe preparations (WHO, 2007b)

The instruction given by one of the peddlers to the author to include the antibiotic chloramphenicol in some of the products in order to prolong the shelf life of the products is an indication that the particular peddler knew that the presence of gross contamination of the preparations could lead to the spoilage of the preparation. The presence of antibiotics may partly explain the inability to recover bacterial contaminants from one of the samples which was also later on found to possess strong antibacterial activity against the Gram negative and Gram positive organisms tested in this study. This finding supports an observation made years ago at a workshop organized for traditional herbal practitioners (Ogungbamila and Ogundaini, 1993) and it indicates that the practice is still on. This practice is unethical as it exposes the users' commensal flora or pathogens to sub-inhibitory concentrations of antibiotics, further compounding the already existing problem of antimicrobial resistance in the community.

In conclusion, this study has shown that most of the unregistered herbal oral liquid products available to consumers in Ile-Ife contain unacceptable levels of microbial contamination. In order to benefit from the use of these products, there is the need to ensure that the persons involved with the production and distribution have adequate knowledge. There is an urgent need to implement proper herbal medicines monitoring and quality control for producers and the products. Subjection of raw materials for herbal medicines to appropriate processing will reduce the microbial load and potentially the inclusion of preservatives will help keep the microbial load of the products within standard specification, providing safe medicines to the users.

Conflict of interests

The authors have not declared any conflict of interests.

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Soil bacteriological pollution in pig farm vicinity: Assessment of bacterial dynamics and detection of antimicrobial resistance gene

Dikonketso Shirley-may Matjuda* and Olayinka Ayobami Aiyegoro

Gastro intestinal Microbiology and Biotechnology, Agricultural Research Council- Animal Production Institute, Irene, South Africa.

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Emissions of biological contaminants (microbes) from intensive pig farming may cause environmental problems due to lack of proper waste management. This work was conducted to assess bacteriological pollution of soil in pig farm and to detect the presence of antibiotic resistance gene of the prevailing bacteria. Soil samples were collected from March to August 2013. The method included bacterial enumeration (10^{-1} to 10^{-8}) in Nutrient, Xylose Lysine Deoxycholate (XLD), Eosin Methylene Blue (EMB) and MacConkey agars. Bacteria were identified using API 20E test kit; antibiotic susceptibility test were also determined and identification of resistance gene was carried out using molecular procedures. The viable cells in soil samples ranged from 0 to 2.44×10^{10} cfu/ml. *Pseudomonas luteola, Salmonella choleraesuis* spp *arizonae, Escherichia coli 1, Enterobacter aerogenes,* etc. were the predominant isolates. Sixty-seven percent of isolates were resistant to Penicillin G while 79% were resistant to Spectinomycin. The resistance genes detected in most isolates were *Van A, InuA, Sul2,* bla_{TEM} and *Otr B*. The results showed that bacterial pathogens isolated from pig farm soil were not only diverse but also possessed multiple Antibiotic Resistance Gene (ARG) and this may have possible dire consequences on the environment and public health.

Key words: Microbes, pathogens, pollution, antibiotics, resistance gene, environment.

INTRODUCTION

Mishandling of pig farm waste and animal droppings may impact negatively on the physical environment, especially polluting the soil with bacteriological pathogens. The pollution may consequentially cause serious waterborne and airborne diseases by either as a result of ingestion or direct contact, or inhalation of contaminated aerosols (Ramírez et al., 2005). Applying animal waste to the soil may solve waste disposal issues; unfortunately this can also introduce bacterial pollutants to the soil, groundwater systems, and surface water in the surrounding environment (Obasi et al., 2008). Potential sources of bacterial pollution in pig farms include feedlot pastures,

*Corresponding author. E-mail: mofokengd@arc.agric.za.

treatment lagoons, manure storage, and also land application fields (Hong et al., 2013).

Pathogens can be transported in soil receiving waste through movement with infiltrating water, and surface run-off water and with the movement of sediments and waste particles (Jamieson et al., 2002). The bacteria that are already at the soil surface can act as suspended particles thus trapping even more bacteria which are deposited into the soil (Jamieson et al., 2002). Therefore, the bacteria that are deposited into the soil can travel with mobile water and their cells can interact with air or solid phase, which result in temporary or permanent immobilization (Łuczkiewicz and Quant, 2007). In such conditions, bacteria can also be entrapped in stagnant pore water between gas bubbles (Łuczkiewicz and Quant, 2007). During rain events, connectivity between mobile and immobile water increases, allowing bacteria to migrate with the advancing wetting front, thus allowing the bacteria to reach the ground water through absorption and infiltration and thus contaminate the groundwater (Łuczkiewicz and Quant, 2007).

Agricultural run-offs or seepage of pig farm waste are known to be critical sources of bacterial pollution in soil (Tyrrel and Quinton, 2003). Due to high use of antibiotics in pig farm as growth promoters and also to treat infections and diseases some of these antimicrobial agents are excreted with pig's faeces in an unaltered state **(**Hong et al., 2013). This is because antibiotics are poorly absorbed into the gut of farm animals and therefore can be exposed to natural faunas and floras of soil through faeces and urine of pigs and in soil through surface runoff of pig farm seepage (Kumar et al., 2005). Some of the bacterial pathogens are able to resist the antibiotics in the gut of pigs and can be exposed to soil during defecation, thus introducing bacteria that possesses antibiotic resistance gene (ARG) in the environment (Obasi et al., 2008).

The rapid growth of antimicrobial agents in the environment as a result of the extensive use in pig farms emphasizes the need for intervention (Roberts, 2005). Most antibiotic residues cannot be removed by wastewater treatment plants and these residual antibiotics can be released into the environment and may exert selection pressure on natural soil microorganisms (Heuer et al., 2011). Furthermore, soil may receive inputs of antimicrobials, which can serve to amplify antibiotic resistance genes (ARGs) (Chee-Sanford et al., 2009; Heuer et al., 2011). The use of antibiotics agents can also cause overgrowth of bacterial expressing a resistance gene to the antibiotic agent and therefore may aggregate the evolution of complex genetic vectors encoding, expressing, linking, and spreading the bacteria and other resistance genes (Cheng et al., 2013). In order to further establish the contribution of agricultural practices to the environment, this study was conducted to assess the bacteriological dynamics of soil environment from a pig farm and to detect the presence of antibiotic resistance gene of the prevailing bacteria.

MATERIALS AND METHODS

Study area

The research was conducted at the pig feedlot section situated about 25 km south of Pretoria (25°52′S 28°13′E / 25.867°S 28.217°E / -25.867; 28.217) in Gauteng, South Africa. The section of the farm where this work was conducted housed about 105 pigs, that is, about 70 sows, 10 boars and 25 piglets. The pig farm was divided into sections A and B. The pigs in Section A were kept in semi-intensive range where their enclosure floor was not cemented and therefore were in direct contact with the soil. Section B pigs were housed in intensive unit, namely: Farrowing house, grower house, dry sows house and weaning house. Soil samples were taken from section A of the pig feedlot.

Soil sampling

Top soil samples were collected at the Agricultural Research Council - Animal Production Institute (ARC-API) pig farm section. These samples were collected monthly from March to August 2013 between 07. 00 and 09. 00 hours am on weekly basis. Soil samples of about 2 kg were collected in sterile polythene bags using soil auger at depth of 30 cm (Bhat et al., 2011). The soil samples were collected from 5 sites in the pig farm, that is, pig farm enclosures (Enc S), soil samples from 20 m (Enc S-20 m) and 100 m (Enc S-100 m) away from the pig farm enclosures, soil samples from 20 m (CW S-20 m) and 100 m (CW S-100 m) away from pig farm constructed wetland used for the treatment of pig farm wastewater. Soil samples were placed on ice in a cooler box immediately after sampling and transported to the lab to be analyzed.

Bacteria isolation

Bacteria isolation procedure was adapted from the methods of Ramirez et al. (2005). One hundred milliliter of sterile distilled water was poured into a sterile conical flask (200 mL) and 10 g of the soil sample was weighed and added to the distilled water in the bottle. The flask was tightly capped and mixed thoroughly for 30 min using magnetic stirrer plate. Serial dilution method was adapted where 1 mL of the soil sample supernatant was used in performing the serial dilution from 10^{-1} up to 10^{-8} using sterile 0.9 % (w/v) saline solution (Bezuidenhout et al., 2002). About 1 mL of each dilution was added to 15 mL of agar in test tube, mixed thoroughly and the contents were poured into a petri-dish, allowed to solidify and incubated at 37°C for 2 days. The media that were used included Nutrient agar, MacConkey Agar (for isolation of *Shigella* spp., *Salmonella* spp., *Yersinia* spp., *Providencia* spp., *Serratia* spp.). Xylose Lysine Deoxycholate agar (XLD agar) was used for isolation of *Enterobacter* spp., *Proteus* spp., *Pseudomonas* spp., and Eosin Methylene Blue (EMB) was used for isolation of *Escherichia coli*, *Aerobacter aerogenus*, *Citrobacter* spp., and *Klebsiella* spp.. Viable cells were counted after 2 days of incubation from each petri-dish and isolates were picked and streaked three times on nutrient agar for pure colony. Serial dilutions were done in triplicates

Identification of isolates

Isolates were identified using Analytical Profile Index (API 20E) identification kit (bioMérieux South Africa (Pty) Ltd). Pure isolates were streaked on nutrient agar and incubated at 37°C for 24 h. The

overnight grown cultures were then inoculated in 5 mL of 0.85% (w/v) saline solution and the turbidity of the resulting solution was adjusted to 0.5 McFarland Standard. The manufacturer procedure was followed in inoculating the isolates on the Analytical Profile Index (API 20E) test strips. All reactions were read according to the recommendations of the manufacturer and the seven-digit octal number was calculated, and the organism identity was determined using the apiweb software.

Antimicrobial assays

Antibiotic susceptibility/resistance was determined by the Kirby-Bauer disk diffusion method according to standard procedure outlined by Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2007) and Kumar et al. (2013). The isolates were screened for susceptibility/resistance to a panel of antibiotics using Mueller Hinton agar (Oxoid, UK). Antibiotics tested were Penicillin G (10 µg), Sulphamethaxazole (25 µg), Vancomycin (30 µg), Ampicillin (10 µg), Amoxicillin (25 µg), Apramycin (15 µg), Neomycin (30 µg), Tilmocosin (15 µg), Oxytetracyclin (30 µg), Spectinomycin (25 µg), Lincomycin (15 µg), Trimethoprim (2.5 µg), Nalidixic Acid (30 µg), Gentamycin (10 µg), Tetracycline (30 µg), Ceftadizime (10 µg), Norflaxacin (10 µg), and Nitrofurantoin (300 µg). These antibiotics are used at the pig farm to treat diseases and for growth promotion. Most antibiotics for growth promotions are added to feeds and are given on a regular basis and pigs at the age of 7 months are given a dosage range of 10 to 40 g of growth promoter antibiotics.

E. coli ATCC 25922, *Ps. aeruginosa* ATCC 19429, and *S. marscensce* ATCC 14041 were used as controls. All Antibiotic susceptibility/resistance tests for isolates were assayed in triplicates and incubated at 37°C for 20 h. The Multidrug Resistance Index (MDRI) of each sample was estimated using the following equation: $MDRI = \frac{A}{B\,x\,C}$, where A represents the aggregate antibiotic resistance score of all isolates from the sample, B represents the number of antibiotics, and C represents the number of isolates from the sample (Krumperman, 1983).

Detection of resistance gene in identified isolates

DNA isolation

The isolates were cultured in nutrient broth and incubated for 24 h at 37°C. NucleoSpin Tissue Genomic DNA purification kit (Machery-Nagel, Germany) was used to isolate genomic DNA from the identified isolates. The manufactures procedure was followed for isolation of the genomic DNA (support protocol for bacteria). The purity and yield of the DNA was assessed spectrophotometrically (NanoDrop ND-2000c, Thermo) by calculating the A_{260}/A_{280} ratios and the A_{260} values to determine protein impurities and DNA concentrations.

Polymerase chain reaction (PCR) amplification for detection resistance genes

PCR amplification assays were performed to detect the presence of antimicrobial resistance genes in identified bacterial isolates. The method adopted was that outlined by Hsu et al. (2007). The sequences of 29 primers used for PCR amplification of antibiotic resistance genes are listed in Table 1. Amplification of the DNA was performed in a PCR apparatus with iProof High Fidelity DNA Polymerase (BIO-RAD). The 20 µL reaction mixture contained 0.02U/ µL iProof DNA Polymerase; 1X iProof HF Buffer; 3% DMSO; 700 µM $MgCl_2$; 200 µM dNTPs; 0.5 µM Forward Primer; 0.5 µM Reverse Primer; 1 µg DNA Template and 11.4 µL of nuclease free water. The PCR cycling conditions were as follows: 98°C for 30 s, followed by 35 cycles at 98°C for 10 s, 30 s at the annealing temperature of Primer, 72°C for 30 s, and termination at 72°C for 10 min. *Pseudomonas. aeruginosa* ATCC 19429 were used as control and a reaction mixture with no DNA template was used as blank. Amplified DNA from each sample (10 µL) was mixed with 1 µL of 6x loading buffer dye and loaded on a 1 % horizontal agarose gel containing 0.5 mg/ mL of ethidium bromide. A 100 bp DNA ladder ranging from 100 to 3000 bp (Thermo Scientific) was also added on each gel to confirm the size of amplified DNA bands. All gels were run in 1 X TAE buffer at 5 V cm^{-1} for 30 min, and visualized by UV trans-illumination.

Statistical analysis

Calculation of means and standard deviations for viable counts were performed using Microsoft Excel office 2010 version. Test of significance (two-way ANOVA) were performed using SPSS 17.0 version for Windows program (SPSS, Inc.). All tests of significance were considered statistically significant at P values of < 0.05.

RESULTS

Bacteria population

Results for viable cell counts of pig farm soil samples are shown in Figures 1 to 4. In Nutrient agar (Figure 1), the viable cells ranged from 1.29×10^4 to 1.33×10^{10} cfu/mL, and the results did not reveal any significant variation between months and also between sampling points. On EMB agar (Figure 2), the viable cell counts ranged from 5.00×10^0 to 1.24×10^8 cfu/mL. The results for viable cell on EMB agar did not show significant variation between months and also between sampling points. The viable cell counts ranged from 1.25×10^1 to 1.89×10^8 cfu/mL in XLD agar (Figure 3). The results for viable cell counts on XLD agar varied significantly ($p<0.05$) monthly and did not show significant difference between sampling points. The viable cells ranged from 3.90×10^2 to 7.90×10^8 cfu/mL in MacConkey agar (Figure 4). The results varied significantly ($p<0.05$) monthly and insignificantly at sampling points.

Bacteria identification

The identification of 49 isolates using API20E kit gave the following species: *Pseudomonas Luteola*, *Salmonella choleraesuis* spp *arizonae*, *Escherichia coli 1*, *Enterobacter aerogenes*, *Pasteurella pneumotropica*, *Proteus vulgaris, Pseudomonas Aeruginosa, Burkholderia cepacia, Stenotrophomonas maltophilia*, *Shwenella putrefaciens*, *Klebsiela pneumonia*, *Serratia liquefaciens*, *Enterobacter sakaziki*, *Citrobacter braakii*, *Enterobacter amnigenus 2*, *Enterobacter amnigenus 1*, and *Serratia marcescens*.

The analysis for susceptibility, using 18 different antibiotics, revealed resistant (R), susceptible (S) and intermediate (I) isolates to tested antibiotics are shown in Figure 5. The results showed that 67% of isolates were

Table 1. Primers used for detection of antibiotic resistance genes.

Primers	Sequence (5' to 3')	Annealing temperature	Reference
aadA	F- 5'TGATTTGCTGGTTACGGTCAG'3 R- 5'CGCTATGTTCTCTTGCTTTTG'3	53°C	Vakulenko et al., 2003
aa(6')-Ie-aph(2")-Ia	F-5'CAGGAATTTATCGAAAATGGTAGAAAAG'3 R- 5'CACAATCGACTAAAGAGTACCAATC'3	55°C	Vakulenko et al., 2003
aph(2")-Ib	F- 5'CTTGGACGCTGAGATATATGAGCAC'3 R- 5'GTTTGTACGCAATTCAGAAACACCCTT'3	58°C	Vakulenko et al., 2003
aph(2")-Ic	F- 5'CCACAATGATAATGACTCAGTTCCC'3 R- 5'CCACAGCTTCCGATAGCAAGAG'3	58°C	Vakulenko et al., 2003
aph(2")-Id	F- 5'GTGGTTTTTACAGGAATGCCATC'3 R- 5'CCCTCTTCATACCAATCCATATAACC'3	56°C	Vakulenko et al., 2003
aph(3")-IIIa	F- 5'GGCTAAAATGAGAATATCACCGG'3 R- 5'CTTTAAAAAATCATACAGCTCGCG'3	54°C	Vakulenko et al., 2003
ant(4')-Ia	F- 5'CAAACTGCTAAATCGGTAGAAGCC'3 R- 5'GGAAAGTTGACCAGACATTACGAAACT'3	58°C	Vakulenko et al., 2003
aac(3')-Iv	F- 5'GTCGTCCAATACGAATGGCG'3 R- 5'CAGCAATCAGCGACCTTG'3	55°C	Vakulenko et al., 2003
VanA	(F) CAT GAA TAG AAT AAA AGT TGC AAT A (R) CCC CTT TAA CGC TAA TAC GAT CAA	55°C	Jánošková and Kmeť, 2004
VanB	(F) GTG ACA AAC CGG AGG CGA GGA (R)CCG CCA TCC TCC TGC AAA AAA	58°C	Jánošková and Kmeť, 2004
VanC1	(F) GGT ATC AAG GAA ACC TC (R)CTT CCG CCA TCA TAG CT	54°C	Jánošková and Kmeť, 2004
VanC2/C3	(F) CGG GGA AGA TGG CAG TAT (R) CGC AGG GAC GGT GAT TTT	55°C	Jánošková and Kmeť, 2004
OtrA	(F) GAACACGTACTGACCGAGAAG (R) CAGAAGTAGTTGTGCGTCCG	57°C	Nikolakopoulou et al., 2005
OtrB	(F) CCGACATCTACGGGCGCAAGC (R) GGTGATGACGGTCTGGGACAG	61°C	Nikolakopoulou et al., 2005
bla_{SHV}	(F) ATGCGTTATATTCGCCTGTG (R) TTAGCGTTGCCAGTGCTCGA	53°C	Jiang et al., 2006
bla_{TEM}	(F) ATGAGTATTCAACATTTTCG (R) TTACCAATGCTTAATCAGTG	47°C	Strateva et al., 2007
bla_{OXA}	(F) CGAGCGCCAGTGCATCAAC (R) CCGCATCAAATGCCATAAGTG	56°C	Strateva et al., 2007
bla_{VEB}	(F) CGACTTCCATTTCCCGATGC (R) GGACTCTGCAACAAATACGC	55°C	Strateva et al.,2007

Table 1. Contd.

bla$_{PER}$	(F) AATTTGGGCTTAGGGCAGAA (R) ATGAATGTCATTATAAAAGC	45°C	Strateva et al., 2007
Sul1	F- 5' GGATCAGACGTCGTGGATGT'3 R- 5' GTCTAAGAGCGGCGCAATAC'3	62°C	Faldynova et al., 2013
Sul2	F'- 5' CGCAATGTGATCCATGATGT'3 R'- 5' GCGAAATCATCTGCCAAACT'3	60°C	Faldynova et al., 2013
Inu(A)	(F) GGTGGCTGGGGGGTAGATGTATTAACTGG (R) GCTTCTTTTGAAATACATGGTATTTTTCGA	56°C	Li et al., 2013
Inu(B)	(F) CCTACCTATTGTTTGTGGAA (R) ATAACGTTACTCTCCTATTTC	50°C	Li et al., 2013
Inu(C)	(F) AATTTGCAATAGATGCGGAGA (R) TCATGTGCATTTTCATCA	52°C	Li et al., 2013
Inu(D)	(F) ACGGAGGGATCACATGGTAA (R) TCTCTCGCATAATAACCTTACGTC	55°C	Li et al., 2013
Inu(F)	(F) CACCATGCTTCAGCAGAAAATGATC (R) TTACTTGTTGTGCGGCGTC	55°C	Li et al., 2013

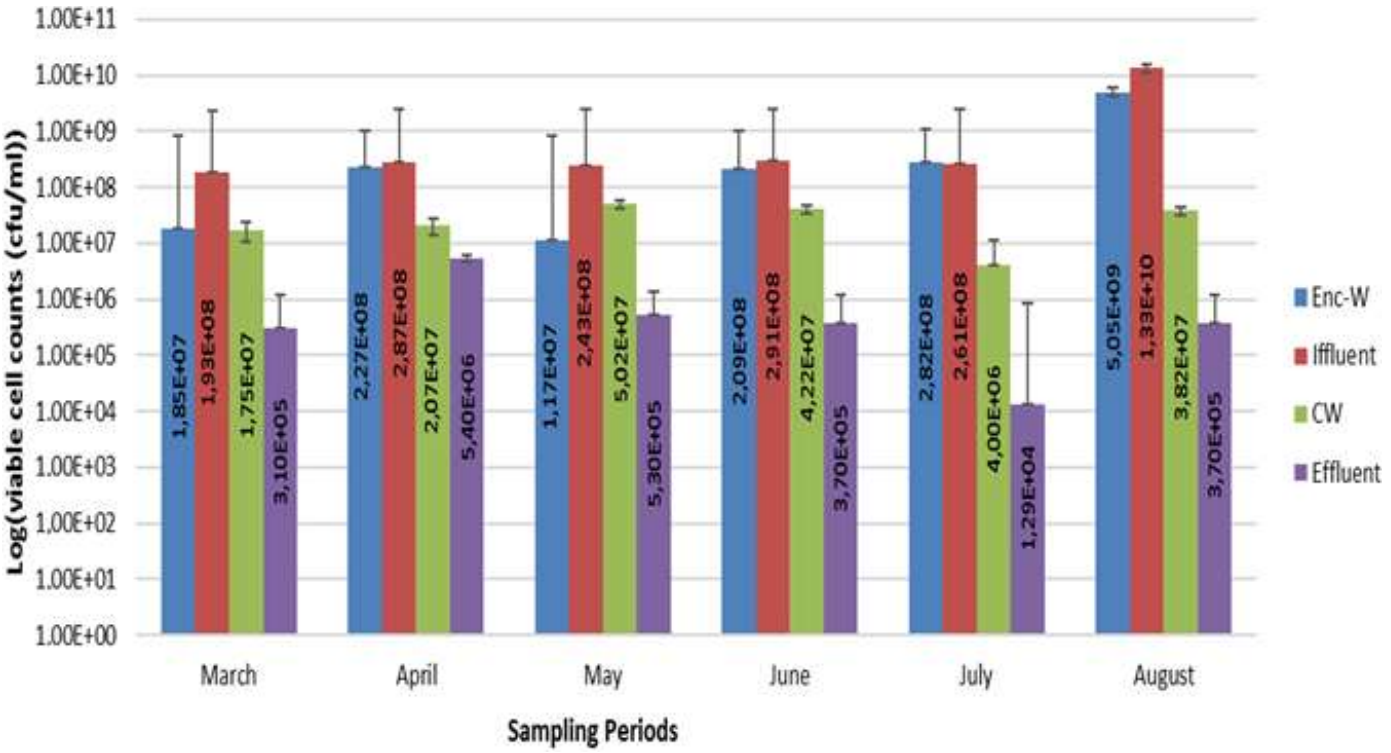

Figure 1. Bacteria population of pig farm soil samples on Nutrient agar. Enc-S = Enclosure soil; Enc S-20 m = Soil 20 m away from enclosures; Enc S-100 m = Soil 100 m away from enclosures; CW S-20 m = Soil 20 m away from constructed wetlands; CW S-100 m = Soil 100 m away from constructed wetlands.

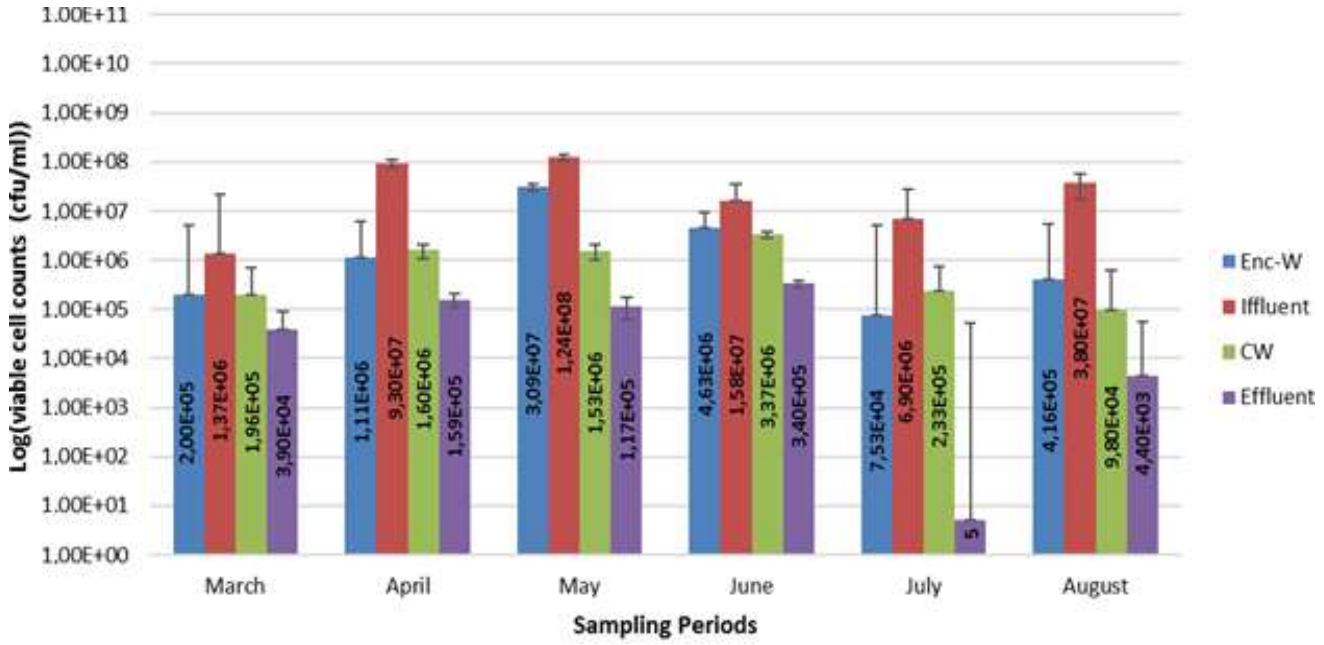

Figure 2. Bacteria population of pig farm soil samples on and Eosin Methylene Blue agar. Enc-S = Enclosure soil; Enc S-20 m = Soil 20 m away from enclosures; Enc S-100 m = Soil 100 m away from enclosures; CW S-20 m = Soil 20 m away from constructed wetlands; CW S-100 m = Soil 100 m away from constructed wetlands.

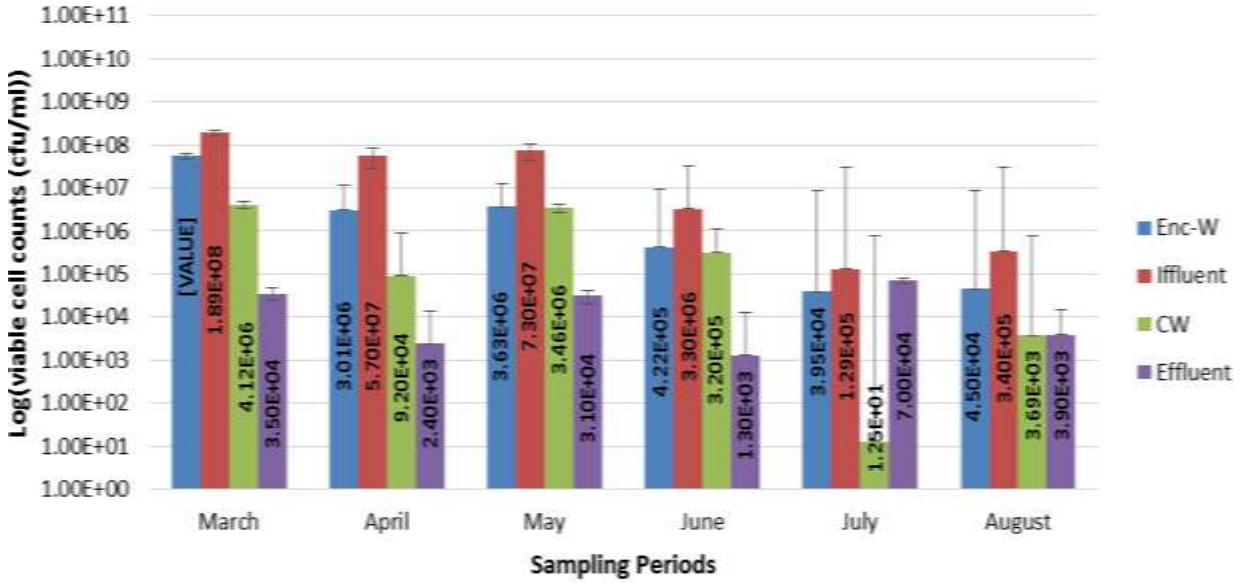

Figure 3. Bacteria population of pig farm soil 30 cm deep samples on Xylose Lysine Deoxycholate agar. Enc-S = Enclosure soil; Enc S-20 m = Soil 20 m away from enclosures; Enc S-100 m = Soil 100 m away from enclosures; CW S-20 m = Soil 20 m away from constructed wetlands; CW S-100 m = Soil 100 m away from constructed wetlands.

resistant to penicillin G, 70 % to vancomycin, 70% to oxytetracycline and 79% to spectinomycin. Among the isolates, 88% were susceptible to norflaxacin 95% to ceftadizime, 72% to Tetracycline, and 60% to nitrofurantoin. In addition, 58% of the isolates were susceptible to neomycin and 51% to nalidixic acid. With ampicillin and gentamycin, the percentage of susceptible isolates (44 and 44% respectively) when compared to those that were resistant to both ampicillin and gentamycin (51 and 49% respectively) did not vary greatly.

The detection of resistance gene showed that most

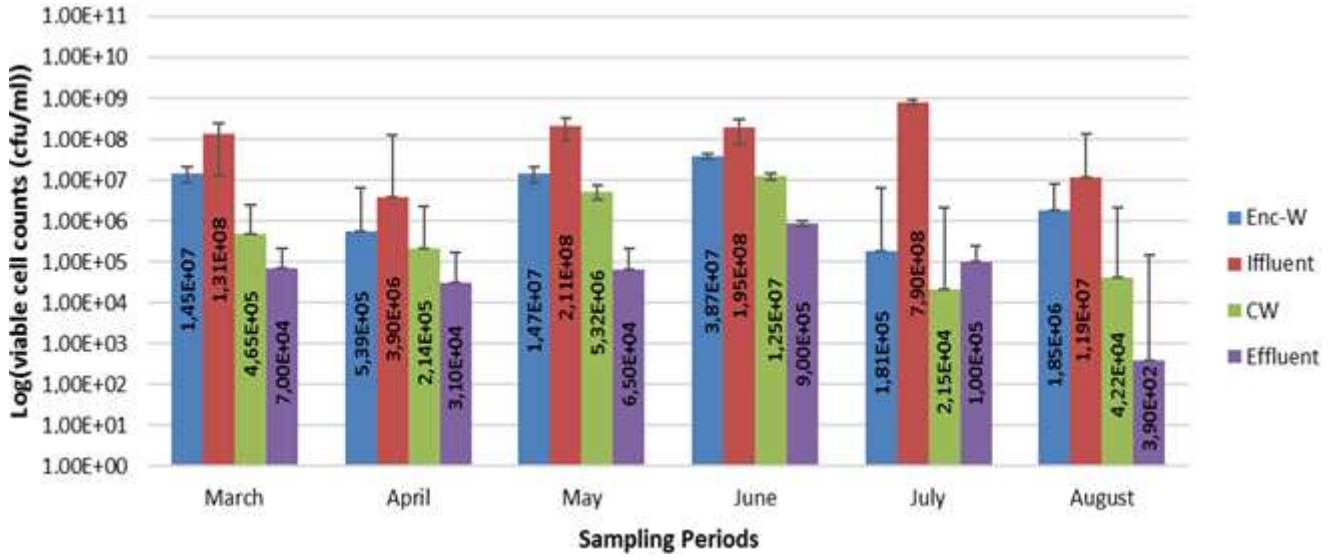

Figure 4. Bacteria population of pig farm soil 30 cm deep samples on MacConkey agar. (Enc-S = Enclosure soil; Enc S-20 m = Soil 20 m away from enclosures; Enc S-100 m = Soil 100 m away from enclosures; CW S-20 m = Soil 20 m away from constructed wetlands; CW S-100 m = Soil 100 m away from constructed wetlands).

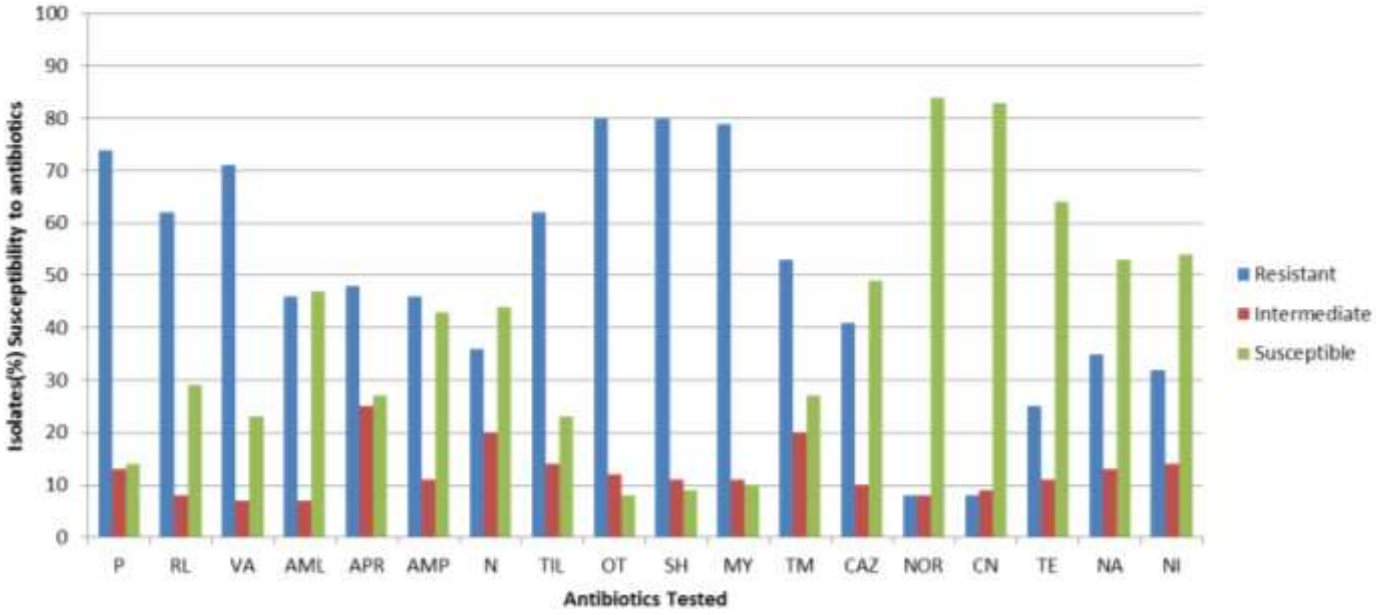

Figure 5. Susceptibility tests of 18 different antibiotics used to test antibiotic sensitivity in isolates. P, Penicillin G; RL, Sulphamethaxazole; VA, Vancomycin; AML, Ampicillin; APR, Amoxicillin; AMP, Apramycin; N, Neomycin; TIL, Tilmocosin; OT, Oxytetracyclin; SH, Spectinomycin; MY, Lincomycin; TM. Trimethoprim; NI, Nitrofurotion; NA, Nadalaxic acid; NOR, Norflaxacin; OT, Oxytetracyclin; TE, Tetracycline; CAZ, Gentamycin; CN, Ceftadizime.

isolates had *VanA*, *VanB, OtrA*, *OtrB* resistance genes (Table 3). The resistant genes; *ant (4')-la*. *AadA*, *ant (4')-la,* and $bla_{VEB;}$ were not detected in all isolates and only *Burkholderia cepacia* and *Enterobacter amnigenus 1* had *aph (2")-lc*. Only *E. coli 1* had *aph (2")-ld* resistance gene. Only *Salmonella choleraesuis* spp *arizonae* had *VanD* resistance gene. As well, only *Serratia liquefaciens* had *VanC* resistance gene and only *Enterobacter amnigenus 1* had *InuD* resistance gene. The gel electrophoresis results for detection of *InuA* resistance gen is shown in Figure 6.

The results for phenotypic antibiotic resistance and Multidrug Resistance Index are shown in Table 2. The most predominate phenotype multiple resistance were P-

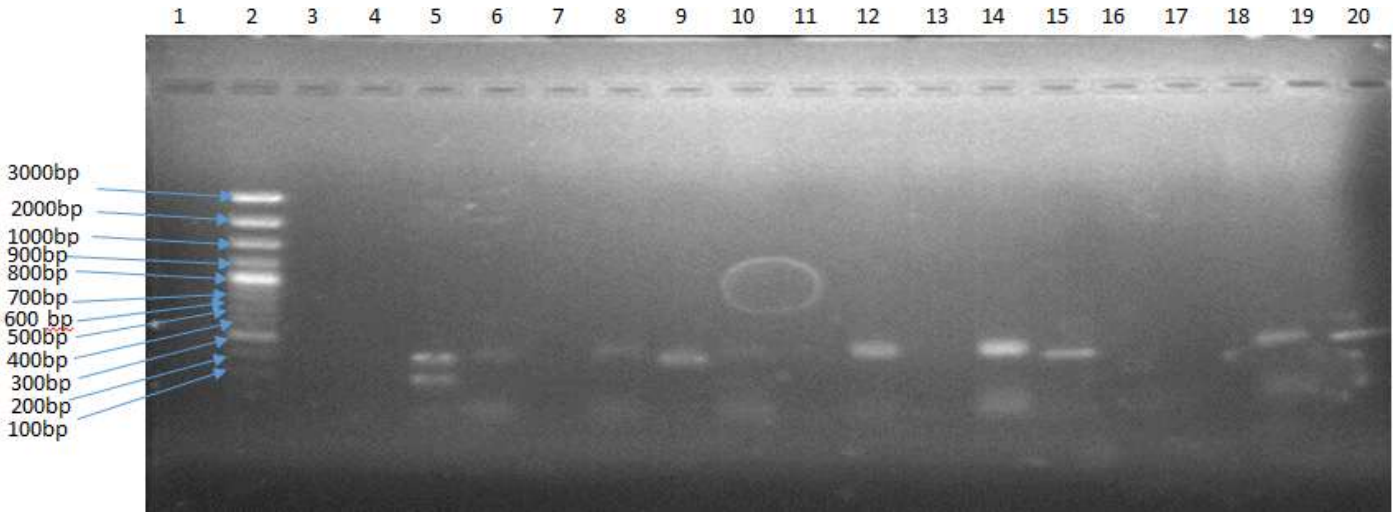

Figure 6. PCR amplification and detection of *Inu A* resistance gene in identified isolates. Lane2: 100 bp DNA Ladder; Lane 12: *Pseudomonas. aeruginosa* ATCC 19429 (positive control); Lane 5: *Enterobacter aerogenes*; Lane 6: *Klebsiela pneumoniae* Lane8: *Enterobacter sakaziki*; Lane9: *Proteus vulgaris*; Lane10: *Burkholderia Cepacia*, lane14: *Pseudomonas. aeruginosa*; Lane15: *Serratia marcescens*, Lane19: *Serratia liquefaciens* Lane20: *Shwenella putrefaciens..* no samples were loaded in lane 3, 4, 7, 11, 13, 14, 15, 16, 17, and 18.

RL-VA-AML-APR-AMP-TIL-OT-SH-MY-NI and P-RL-VA-AML-APR-AMP-N-TIL-OT-SH-MY with 5.1%, respectively, as phenotype antibiotics resistance percentage. About 45 isolates had more than 5 phenotype antibiotic resistance patterns where Penicillin G (P), Sulphamethaxazole (RL), Vancomycin (VA), Ampicillin (AML), Apramycin (AMP), Amoxicillin (APR), Oxytetracyclin (OT), Spectinomycin (SH) were the most predominant.

DISCUSSION

Bacterial contamination has negative impacts on the environment as bacterial pathogens can compete with indigenous soil microorganism for nutrients, transfer antimicrobial resistance genes to indigenous soil microorganism important for soil remediation (Sasákováet al., 2007). Results in this study for viable cell counts were lower as compared to those observed by Cook et al. (2010) where from 2.58×10^{10} to 1.49×10^{11} cfu/mL were obtained from farrowing facilities at pig farm.

Salmonella choleraesuis spp. *arizonae* and *E. coli* 1 were detected in the soil samples from pig enclosures, soil 20 m away from enclosures and soil 20 m away from constructed wetland treating pig farm wastewater. Although *S. choleraesuis* spp *arizonae* presence was confirmed in all soil samples examined, this was occasional as it was not detected in the months of June and July. Still even their small count should be alarming because they can easily spread under favorable conditions and make a serious source of environmental pollution which can lead to airborne diseases (Cook et al., 2010). In addition, the identities of the isolates especially *E. coli* 1 was not performed to strain level and, therefore, the isolate could belong to the serotype E. coli 0157:H4 and E. coli 0104:H4 that have been reported to cause disease in humans (Muniesa et al., 2012). Detection of these bacterial pathogens in soil samples in pig farm may be attributable to the high load of animal excreta in the pig farm seepage and serves as an indicator for possible bacteriological pollution and may have an effect on the soil ecological balance and aquatic life (Ezeronye and Ubalua, 2005). The results obtained in this study was also similar to that observed by Tymczyna et al. (2000) where bacteria such as *Salmonella* spp, *Klebsiella* spp, *Pseudomonas* spp, *Proteus* spp, *Enterobacter aerogenes*, *Citrobacter* spp. etc. were isolated from soil samples from pig farm enclosures and surrounding environment.

The phenotype resistance patterns observed in this study showed that the isolates were highly resistant to more than three antibiotics and the multidrug resistance index (MDRI) of isolates was also high (Table 2). Among 49 phenotype multiple resistance patterns observed, the phenotypes that were mostly observed were P-RL-VA-AML-APR-AMP-TIL-OT-SH-MY-NI, and P-RL-VA-AML-APR-AMP-N-TIL-OT-SH-MY. Some of resistance patterns were not frequently detected, and some of the isolates were found to be resistant to only 1 antimicrobial agent. Multidrug Resistance Index was observed in this study where 5 isolates had an MDRI ranging from 66 to 83% and 8 isolates had MDRI of 61%. In a study conducted by Kotzamanidis et al. (2009), it was observed an AML-CAZ-VA-TE as the most occurring phenotype pattern in isolates from pig farm environment, whereas in this study the most occurring phenotype patterns were P-RL-VA-

Table 2. Predominance of multiple antibiotic resistance phenotypes and multidrug resistant index of isolates.

Phenotype antibiotics resistance			Multidrug resistant index (MDRI)	
Phenotype	**Number(s) of Isolates**	**Percentage (%)**	**Isolates**	**MDRI (%)**
P-RL-VA-AML-AMP-OT-SH-MY-TM-CAZ-NI	1	2.6	ES30-4	61
P-RL-VA-AML-AMP-OT-SH-MY-TM-CAZ-NOR-NI	1	2.6	ES30-6	66
P-RL-VA-AML-APR-AMP-TIL-SH-MY-NA	1	2.6	DMS3	61
P-VA-AMP-TIL-OT-SH-MY-TM-CAZ-NI	1	2.6	ESS14	61
P-VA-AML-APR-TIL-OT-SH-MY-TM-NA	1	2.6	ES30-1	83
P-RL-VA-AML-APR-AMP-N-TIL-OT-SH-MY	2	5.1	ESS5	67
P-RL-VA-AML-APR-TIL-OT-SH-MY-TM-NI	1	2.6	DMS11	72
P-RL-VA-AML-APR-AMP-TIL-SH-MY-TM-CAZ-NOR-TE-NA	1	2.6	DMS7	61
P-VA-AML-APR-AMP-N-TIL-OT-SH-MY-TE-NA	1	2.6	DMS6	61
P-VA-APR-TIL-OT-SH-MY-TM-CAZ-NA	1	2.6	ES30-2	83
P-RL-VA-AML-APR-AMP-TIL-OT-SH-MY	1	2.6	ES30-3	61
P-RL-VA-AML-APR-AMP-N-TIL-OT-SH-MY-TM-CAZ	1	2.6	ES30-14	61
P-RL-VA-AML-AMP-TIL-OT-SH-MY-TM-CAZ	1	2.6	ES30-12	61
P-RL-VA-AML-AMP-N-TIL-OT-SH-MY-TM-CAZ-CN-NA-N	1	2.6	DMS13	65
P-RL-VA-AML-APR-AMP-OT-SH-MY-TM-CAZ	1	2.6	ESS9	71
P-RL-VA-AML-APR-AMP-TIL-OT-SH-MY-NI	2	5.1	ES30-19	61

AML-AMP-SH-MY and P-RL-VA-AML-APR-SH-MY. The phenotype pattern, also, differed from those observed by Kainer et al. (2007) where the most occurring phenotype pattern was P-RL-VA-APR-N-TIL-OT-SH-MY. However, Werner et al. (2008) observed phenotype patterns VA-SH-TM and SH-MY-TM which were also detected in this study.

The result observed in this study for antibiotic susceptibility/resistance test (Figure 4) showed that these organisms have been well exposed to the tested antibiotics and they have developed mechanisms to evade or avoid the effects of these antibiotics. This high antimicrobial resistance is of concern because antibiotic resistance genes can be transferred from pathogens to non-pathogenic (indigenous) microorganism. These observations were also similar to those observed by Kainer et al. (2007) and Werner et al. (2008) where resistance to similar antibiotics was reported. The increased use of antibiotics in livestock industry can introduce a selective pressure which leads to the development of resistance or even multi-resistance characteristics in some of the bacterial populations (Chen and Jiang, 2014).

Since soil bacteria like *Burkholderia Cepacia* are usually used in bioremediation of soil, the acquiring of antibiotic resistance gene renders these soil bacteria unsafe for bioaugmentation application (Krista et al., 1996). Bacterial pathogens, such as *E. coli*, *Proteus* spp., *Salmonella* spp., *Enterobacter* spp., were observed to have multiple resistance genes to most of the antibiotics tests. Contamination of the natural environment by these bacteria may accelerate the growth of algae, deplete dissolved oxygen in water systems, cause eutrophication, and emit toxins that can kill fish and other animals (Pandey et al., 2014).

The results showed that most isolates possessed *aa (6')-le-aph (2")-la gene*, *aph (3")-llla* genes for aminoglycosides resistance, *Sul1* gene and *Sul2* gene for Sulphamethaxazole resistance, *VanA*, *VanB* and *VanC2/C3* resistance genes for vancomycin, *Inu A* and *Inu C* resistance genes for lincomycin, *OtrA* and *OtrB* resistance genes for oxytetracyclines and bla_{TEM} and bla_{PE} resistance gene for beta-lactamase resistance. *AadA*, *ant (4')-la,* and bla_{VEB} resistant genes were not detected in all isolates and only *Burkholderia Cepacia* and *Enterobacter amnigenus 1* had *aph*

Table 3. Detection of resistance genes in isolates.

Isolate	Antibiotic resistance genes																										
	aadA	***aa(6')-Ie-aph(2")-Ia***	***aph(2")-Ib***	***aph(2")-Ic***	***aph(2")-Id***	***aph(3")-IIIa***	***ant(4')-Ia***	***VanA***	***VanB***	***VanC***	***VanC2/C3***	***VanD***	***InuA***	***InuB***	***InuC***	***InuD***	***InuF***	***bla$_{TEM}$***	***bla$_{SHV}$***	***bla$_{OXA}$***	***bla$_{VEB}$***	***bla$_{PER}$***	***OtrA***	***OtrB***	***aac(3')-Iv***	***Sul1***	***Sul2***
Pseudomonus. luteola	-	-	-	-	-	-	-	+	+	-	+	-	-	-	+	-	-	+	-	-	-	+	-	+	-	-	-
Salmonella choleraesuis spp *arizonae*	-	+	-	-	-	+	-	+	+	-	+	+	-	-	-	-	-	-	-	+	-	-	+	+	+	+	+
E. coli 1	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+	-	-	+
Enterobacter aerogenes	-	-	+	-	-	+	-	+	+	-	+	-	-	-	+	-	-	+	-	-	-	-	+	+	+	+	+
Pasteurella pneumotropica	-	+	-	-	-	-	-	+	+	-	+	-	+	-	+	-	-	+	-	+	-	-	+	-	-	-	+
Proteus vulgaris	-	+	-	-	-	+	-	+	+	-	+	-	+	-	+	-	+	-	-	-	-	+	-	+	+	-	+
Pseudomonus. aeruginosa	-	-	-	-	-	-	-	+	+	-	+	-	-	-	+	-	-	-	-	+	-	+	+	+	-	-	+
Burkholderia Cepacia	-	+	+	+	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	+	+	-
St. maltophilia	-	+	-	-	-	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	+	+
Shwenella putrefaciens	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	+	-	+	-
Klebsiela pneumonia	-	-	-	-	-	-	-	+	+	-	+	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	+
Serratia liquefaciens	-	-	+	-	-	+	-	-	+	+	-	-	+	-	-	-	+	+	-	-	-	-	+	-	-	+	-
Enterobacter sakaziki	-	-	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	+	+	-	+	+
Citrobacter braakii	-	+	-	-	-	+	-	-	+	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-
Enterobacter amnigenus 2	-	-	-	-	-	+	-	+	+	-	-	-	-	+	+	-	+	+	-	-	-	+	+	+	-	+	-
Enterobacter amnigenus 1	-	+	-	+	-	-	-	+	+	-	+	-	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-
Serratia marcescens	-	-	-	-	-	+	-	+	+	-	-	-	+	-	-	-	-	+	-	-	-	-	+	+	+	-	+
Number of isolates tested	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17	17
Total number of isolate possessing tested ARG	**0**	**8**	**4**	**2**	**1**	**9**	**0**	**11**	**13**	**1**	**9**	**1**	**7**	**1**	**8**	**1**	**5**	**10**	**2**	**4**	**0**	**7**	**13**	**12**	**6**	**9**	**10**

+, Antibiotic resistant gene detected; -, no antibiotic resistance gene detected; ARG, antibiotic resistance gene.

(2")-Ic. Only *E. coli 1* had *aph (2")-Id* resistance gene, only *Salmonella choleraesuis* spp *arizonae* had *VanD* resistance gene, only *Serratia liquefaciens* had *VanC* resistance gene and only *Enterobacter amnigenus 1* had *InuD* resistance gene. *Enterobacter amnigenus 1* and *Burkholderia Cepacia* were the only 2 isolates to possess *aph (2")-Ic resistance gene,* while Enterobacter *sakaziki* and *E. coli 1* were the only 2 isolates to poses *bla$_{SHV}$* resistance gene.

Sul resistance genes have been reported as the most frequently detected ARGs in pig farm seepage by Zhu et al. (2013). Equally, from this study, *Sul* resistance gene (Table 3) was detected in most of isolates from soil samples from pig farm. In a study conducted by Chee-Sanford et al. (2001), *OtrA* resistance gene was the most abundant and detected in surface soil (depth between 0-30 cm) in pig farm and this was consistent with the current observations where *OtrA* resistance gene was observed in most

surface soil sample isolates.

In a study conducted by Faldynova et al. (2013), *sul1* and *aadA* resistance genes were very abundant in pig farm soil and seepage while *sul2* resistance gene was less observed in isolates from pig farm surrounding environment. The observations from this study differed from those observed by Faldynova et al. (2013) because *aadA* resistance gene was not detected in all isolates and *sul1* and *sul2* resistance genes were more or less equally observed in isolates from soil samples in pig farm. In a study conducted by Li et al. (2013), they observed an abundance of *InuF* resistance gene in all soil samples collected in pig farm and this was not the case in this study as *InuF* resistance gene was less detected in soil samples from pig farm. Similar results reported by Li et al. (2013) were also reported by Cheng et al. (2013) where widespread of *InuF* resistance gene was also observed in soil samples proximal to pig farm.

Conclusion

This study revealed that the soil at pig farm was contaminated with bacterial pathogens as bacteria such as *E. coli*, *E. aerogenes, K. pneumonia, P. vulgaris* etc. were detected especially with their ARGs in all soil samples from pig farm. The presence of this bacterial pathogens in soil sampled in the pig farm in this study was observed not only to be diverse but were also abundant and this may threaten the quality of the surrounding natural environment. In addition, this study also revealed that this prevailing bacterial pathogens isolated from pig farm soil had multiple ARG. The presence of ARG in soil causes horizontal gene transfer and may form a critical zone for the species-rich environmental microbiota and antibiotic-resistant microorganism to exchange genetic material with indigenous soil microorganism (Schulz et al., 2012).

Conflicts of Interests

The authors have not declared any conflict of interests.

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