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Mitigation of drought in rice by a phyllosphere bacterium *Bacillus altitudinis* FD48

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Phyllosphere bacteria plays important role in mitigating biotic and abiotic stress. This study aimed to use phyllosphere bacterium of rice to enhance drought tolerance. Bacterial isolates obtained from rice phyllosphere were investigated for their plant growth promoting activities and role in alleviation of drought stress in rice. The isolates were identified as *Bacillus altitudinis* FD48*, Bacillus pumilus* FS20 and *Bacillus aquimaris* MD02 based on 16S rRNA gene sequence. *B. altitudinis* FD48 was found to produce indole acetic acid (IAA) (2.82 µg/ml) compared to other two isolates even under PEG induced drought conditions. However, under normal conditions, *B. altitudinis* FD48 produced 8.0 µg/ml. Quantitative estimation of 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity was found to be 192 n moles α-ketobutyrate mg^{-1} h^{-1} and positive for *accD* gene. *B. altitudinis* FD48 increased the root and shoot length of rice under *in vitro* conditions and also improved the germination percentage of rice seeds at different concentration of PEG 6000. Inoculation of rice with plant growth promoting, drought tolerant *B. altitudinis* FD48 increased relative water content, chlorophyll stability index and membrane stability index compared to control (uninoculated plants) when the plants were subjected to drought by discontinuing water for 8 days after 30 days of germination. Similarly, rice treated with *B. altitudinis* increased proline content, phenolics content, catalase activity and reduced malondialdehyde (MDA) content in plants. Ethylene emission was significantly reduced by *B. altitudinis* FD48 inoculation under drought condition when compared with control. This study suggests that the isolate *B. altitudinis* FD48 may be used at field level to mitigate drought stress in rice.

Key words: Proline content, drought, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, plant growth promoting bacteria, phenolics content, indole acetic acid (IAA).

INTRODUCTION

Rice is a cereal crop which shapes the culture and economy of India and is cultivated in about 43.5 M ha area but with relatively low average productivity. Abiotic factors like drought, flood and salinity have adverse effect on rice production (Singh et al., 2016). Drought is one of the major abiotic stresses affecting the yield of the crop. Being a drought sensitive crop, rice exhibits deleterious effects when exposed to drought at critical growth stages

such as panicle initiation, anthesis and grain filling (Weisburg et al., 1991). The responses of plants to drought are complex and affect several components. It not only affects cell water potential but also induces closure of stomata, decrease in photosynthesis, nitrate assimilation and various anabolic enzyme reactions (Zhang et al., 2010). It also induces the generation of active oxygen species which in turn cause lipid peroxidation and consequently membrane injury, protein degradation, enzyme inactivation, pigment bleaching and disruption of DNA strands (Pompelli et al., 2010).

Various tolerance mechanisms have been suggested on the basis of biochemical and physiological changes related to drought (Shukla et al., 2012). Recent studies have indicated that plant associated bacteria can help to withstand abiotic stresses more efficiently (Chauhan et al., 2015). If such microorganisms have the ability to promote growth, they become extra beneficial. Plant growth promoting bacteria (PGPB) can mitigate the impact of abiotic stresses on plants through a process called induced systemic tolerance (IST), which includes bacterial production of cytokinins, production of antioxidants and degradation of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) by bacterial ACC deaminase. Rhizosphere colonizing bacteria were well studied for their role in stress tolerance (Sandhya et al., 2011), but few studies were focused on phyllosphere bacterial amelioration of abiotic and biotic stress in plants. Most of the research on phyllosphere bacteria has been paying attention on individual bacterial isolates using culture dependent methods with respect to their role in plant protection by pathogen suppression (Delmotte et al., 2009) and in plant growth promotion (PGP) (Papen et al., 2002) or through the production of beneficial substances such as 1-aminocyclopropane-1-carboxylate deaminase (ACCD). Phyllosphere colonizing *Methylobacterium* were reported to produce phytohormones like cytokinin and auxins (Madhaiyan et al., 2005) and stress response enzyme ACC deaminase (Chinnadurai et al., 2009). ACCD is responsible for the cleavage of the plant ethylene precursor, ACC, into ammonia and α-ketobutyrate (Honma and Shimomura, 1978). By decreasing ACC levels in plants, ACC deaminase producing organisms reduce plant ethylene level. During stress condition, ethylene is present in high concentration and may cause damages to plant (Glick et al., 2007).

Bacillus species is a major inhabitant of rhizosphere region which also exist as epiphytic microorganisms with plant growth promoting activities (Idris et al., 2004) and stress tolerance ability (Chandramohan and Mahadevan, 1968). Rice inoculated with *Bacillus* spp. was found to alleviate oxidative damage by improving plant growth and activating antioxidant defense systems, thereby improving the stability of membranes in plant cells under drought condition (Gusain et al., 2015). The objective of this study was to identify the role of *Bacillus* spp. in alleviating drought stress by altering the growth and biochemical parameters of rice.

MATERIALS AND METHODS

Organism source and molecular characterization

The isolates FD48, FS20 and MD02 used in the study were obtained from Department of Agricultural Microbiology, Directorate of Natural Resources Management, Tamil Nadu Agricultural University, Coimbatore. The aforementioned isolates were previously isolated from the phyllosphere of rice. The genomic DNA from the three isolates was extracted using the standard protocol of hexadecyl-trimethyl ammonium bromide (CTAB) method as given by Melody (1997) with minor modifications. Nearly-full length of 16S rRNA gene was amplified from the genomic DNA from the three isolates using FD1 (5'AGA GTT TGA TCC TGG CTC AG 3') and RP2 (5'ACG GCT ACC TTG TTA CCA CTT 3') primers (Weisburg et al., 1991). A total of 20 µL of reaction volume contains 50 ng of genomic DNA, 0.2 mM of each dNTP, 1 µM of each primer, 2.5 mM of MgCl$_2$ and 1 U of Taq DNA polymerase (all from Bangalore Genei, India) and the buffer supplied with the enzyme. PCR amplification was performed in a thermocycler (Eppendorf Master Cycler, Germany) using conditions as follows: initial denaturation at 95℃ for 10 min; 35 cycles consisting of 94℃ for 1 min (denaturation); 55℃ for 1 min (annealing); 72℃ for 1 min (primer extension) and final extension at 72℃ for 10 min. The cloned product was sent for sequencing to Bioserve technologies and sequenced through using ABI prism terminator cycle sequencing ready reaction kit and electrophoresis of the products were carried out on an Applied Biosystems (Model 3100) automated sequencer.

The identity of 16S rDNA sequence was established by performing a similarity search against the GenBank database (website: http://www.ncbi.nih.gov/BLAST). The phylogenetic tree was constructed by neighbour-joining method (Saitou and Nei, 1987) using MEGA6 software.

Plant growth promoting activities of the isolates

Indole acetic acid (IAA) production

One millilitre of the culture at exponential stage was inoculated in 100 ml LB medium containing filter sterilized L-tryptophan (0.01% w/v) (Chandramohan and Mahadevan, 1968). The flasks were incubated at room temperature for 7 days. The cells were harvested by centrifugation at 10,000 rpm for 5 min and the supernatant was collected and concentrated to 25 ml. The culture filtrate was adjusted to pH 2.8 with 1N HCl. Equal volume of ice cold (4°C) diethyl ether and culture filtrate was added in a separating funnel, shaken well and allowed to stand in dark for 4 h with intermittent shaking. The aqueous phase was separated from organic phase and the extraction was repeated 3 times. Discarding the aqueous phase, the organic phases were pooled and evaporated to dryness in the dark. The residue was dissolved in 2 ml of absolute methanol and analyzed in HPLC (Thermo, Spectrasystem UV 2000). The quantity of IAA produced was estimated using Salper's reagent (1 ml of 0.5N FeCl$_3$ mixed in 50 ml of 35% perchloric acid) (Gorden and Paleg, 1957).

ACC deaminase activity

1-Aminocyclopropane 1-carboxylate deaminase (ACCD) activity was assayed according to a modified method (Honma and Shimomura, 1978) which measures the amount of α-ketobutyrate

produced when the enzyme ACCD cleaves ACC to α-ketobutyrate. The bacterial culture grown in LB medium for 24 h were transferred to minimal medium (DF salt medium) (Dworkin and Foster, 1958) with ACC as the sole source of nitrogen. Cultures were incubated overnight in a shaking water bath at 200 rpm at room temperature. The biomass accumulated was harvested by centrifugation at 8000 rpm for 10 min at 4°C. The supernatant was removed and the cells were washed with 5 ml DF salts minimal medium. Following an additional centrifugation for 10 min at 8000 rpm at 4°C, the cells were suspended in 7.5 ml DF salts minimal medium in a fresh culture tube. Just prior to incubation, 45 µl of 0.5 M ACC (filter sterilized) was added to the cell suspension to obtain a final concentration of 3.0 mM. The bacterial cells were returned to the shaking water bath at room temperature for overnight incubation. The bacterial cells were harvested by centrifugation at 8000 rpm for 10 min at 4°C. The supernatant was removed and cells were washed by suspending the cell pellet in 5 ml of 0.1M Tris-HCl, pH 7 and transferred to a 1.5 ml microcentrifuge tube. The contents were centrifuged at 16000 rpm for 5 min and the cell pellet was suspended in 600 µl of 0.1M Tris-HCl, pH 8.5. Thirty microlitres of toluene was added to the cell suspension and vortexed at the highest setting for 30 s and 100 µl aliquot of the toluenized cells was set aside and stored at 4°C for protein assay at a later time. The protein concentration of toluenized cells was determined (Chakraborty et al., 2013). The remaining toluenized cell suspension was used for ACCD activity assay. Two hundred microlitres of the toluenized cells were placed in a fresh 1.5 ml microcentrifuge tube and 20 µl of 0.5M ACC was added to the suspension, briefly vortexed and then incubated at 30°C for 15 min. Following the addition of 1 ml of 0.56 M HCl, the mixture was vortexed and centrifuged for 5 min at 16000 rpm at room temperature. One millilitre of the supernatant was vortexed with 800 µl of 0.56 M HCl and 300 µl of the 2, 4-dinitrophenyl hydrazine reagent was added to the glass tube, the contents were vortexed and then incubated for 30 min at 30°C. Following the addition and mixing of 2 ml of 2N NaOH, the absorbance was measured at 540 nm.

ACC deaminase gene amplification

The screening of ACC deaminase containing isolates from rice phyllosphere was done based on the amplification of ACC deaminase gene. Presence of *accD* gene (700 bp) in the phyllosphere isolates was detected (Saravanakumar and Samiyappan, 2007) using the primers *ACCDF* 5' ATG AAC CTG CAA CGA TTC 3'and *ACCDR* 5' TCA GCC GTC TCG AAA GAT 3'.

Drought tolerance level of the isolates

The effects of drought on the growth of isolates were studied using polyethylene glycol MW 6000 (PEG) (M/S Hi-Media, Mumbai, India) at different concentration ranging from 0 to 25%. The isolates were inoculated in LB broth containing different concentration of PEG (10, 15, 20, and 25%) and incubated at 28°C for two days. The bacterial growth was measured spectrophotometrically at OD 660 nm (Abdel-Salem et al., 2010).

IAA production under stress condition

LB broth amended with L-tryptophan was prepared at different level of PEG concentrations (10, 15 and 20%) and inoculated with the isolates. The production of IAA by the isolates was measured under increasing PEG concentration (Uma Maheshwari et al., 2013). The supernatant was collected by centrifuging at 10,000 rpm for 20 min. Two millilitres of supernatant was mixed with two drops of

Orthophosphoric acid and 4 ml of Salkowski reagent. Tubes were incubated at room temperature for 25 min and OD value was measured at 535 nm (Gorden and Paleg, 1957).

PEG induced drought stress on seed germination of rice

The rice seeds were surface sterilized in 0.1% $HgCl_2$ for 2 min rinsed five times with sterile water. The seeds were then treated with overnight grown cultures of FD48, FS20 and MD02. *Methylobacterium* species (Pink Pigmented Facultative Methylotrophs, PPFM) was maintained as standard and seeds treated with sterile water as control. The seeds were then placed on a filter paper in Petri dishes, moistened with 10, 15 and 25% of PEG (MW 6000) concentration corresponding to water potential of -1.4, -2.9 and -6.7 bars. The Petri plates were sealed with Parafilm to prevent evaporation and kept at room temperature. Seeds were considered germinated when the radical had extended for at least 2 mm. Germination percentage and root length were measured after 24, 48 and 72 h (Muscolo et al., 2014).

Paper towel culturing for rice

Overnight water soaked paddy seeds (cultivar ADT 36) were surface sterilized with sodium hypochlorite with 3% for 3 min followed by several washes with sterile distilled water. The seeds were placed on 2% sucrose agar plates spread with bacterial culture (T_1 - control, T_2 - MD02, T_3 - PPFM, T_4 - FD48, T_5 - FS20) and incubated at room temperature for germination. The sprouted seeds (10 numbers) were placed in germination paper towels and kept in sterile polythene pouches, in a stand. The whole experimental set up was provided with light and dark at 12 h intervals. Watering was done using sterile distilled water at 4 days interval. Along with ACC deaminase producing bacterial inoculants as cell suspensions in distilled water (10^9 cfu per ml) was sprayed at 7, 14, and 21 days. After 25 days of inoculation, the root length, shoot length, root dry weight and shoot dry weight of the seedlings were recorded.

Pot-culture experiment

To assess the impact of bacterial inoculant on growth and drought tolerance of rice, an experiment was carried out in the green house of Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore. Wetland soil used for pot culture studies was sterilized and filled in pots at 5 kg pot^{-1}. The bacterial culture was grown in LB broth and *Methylobacterium* spp. (PPFM) in AMS medium till the population reached to 10^{10} cells ml^{-1}. Overnight water soaked paddy seeds (cultivar ADT 36) were surface sterilized with sodium hypochlorite for 3 min followed by several washes with sterile distilled water. Surface sterilized seeds were imbibed with above said bacterial inoculants for 1 h and shade dried before sowing. Intermittent foliar spraying of bacterial culture was also carried out. Paddy seeds were sown directly in the pot, maintaining three plants per pot and the treatments were replicated seven times in a completely randomized block design. Soil water content (SWC) was estimated (Cha-um et al., 2012) using the formula: (FW-DW/AvFW-DW) $\times$ 100, where FW is the fresh weight of soil, DW is the dry weight of soil (at 110°C), AvFW is the average fresh weight of the soils on day 0 of the experiment (Ogbaga et al., 2014). After 30 days of germination, drought stress was induced by discontinuing watering for eight days (at 45% of water holding capacity). Observations were taken from non-stressed and drought stressed plants after 8 days of drought stress. Treatment details are T1, Control; T2, *Bacillus altitudinis* (FD48) and T3, *Methylobacterium* spp. (PPFM).

Relative water content (RWC)

Relative water content was determined (Schonfeld et al., 1988) where fresh weights for twenty discs from the youngest fully expanded leaf were determined within 2 h after excision. Turgid weight was obtained after soaking the discs for 16 to 18 h in distilled water. After soaking, discs were quickly and carefully blotted dry with tissue paper prior to determination of turgid weight. Dry weight was obtained after drying the discs sample for 72 h at 70°C. Relative water content was calculated from the following equation:

RWC = [(fresh weight - dry weight) / (turgid weight - dry weight)] × 100

Chlorophyll stability index

Two clean glass tubes were taken and 5 g of representative leaf sample was placed in them with 20 ml distilled water. One tube was then subjected to heat in water bath at 56°C for exactly 30 min. Other tube was kept as control. The leaves were then ground in a mortar for 5 min with 10 ml of 80% acetone. The contents were centrifuged at 3000 rpm for 10 min and the supernatant was made up to 25 ml using 80% acetone. The total chlorophyll content was measured at 652 nm in a spectrophotometer (Murthy and Majumdar, 1962):

$$\text{Total Chlorophyll} = \frac{\text{O.D. value at 652 x 1000x V}}{34.5 \times 1000 \times W}$$

and expressed as mg total chlorophyll per gram of fresh weight, V=Final volume of acetone extract, and W=Fresh weight in gram

The chlorophyll stability index is the ratio of total chlorophyll content of the treated sample to the untreated samples and expressing in percentage.

$$CSI = \frac{\text{Total chlorophyll content (Treated)}}{\text{Total chlorophyll content (Control)}} \times 100$$

Membrane stability index

Membrane stability index (MSI) of fresh leaves was determined (Bailly et al., 1996; Kaya et al., 2003). The conductivity of solution was measured using a conductivity bridge meter using the formula:

MSI =1- C1/C2

Where, C1- conductivity at 40°C and C2- conductivity at 100°C.

Measurement of biochemical parameters

Assessment of biochemical (proline, MDA, total phenolics, and Catalase activity) responses of rice were carried out on fresh plant material that was immediately extracted and assayed according to the appropriate methods listed subsequently.

Proline content was determined by the method of Bates et al. (1973). Total phenolics were estimated (Malik and Singh, 1980) using Folin reagent and the absorbance was measured at 660 nm against each blank. Catalase activity (CAT; EC 1.11.1.6) was determined (Gopalachari, 1963). The activity of enzyme was expressed as µg of H_2O_2 g^{-1} min^{-1}. For estimating MDA content, a

quantity of 250 mg plant sample was homogenized in 5 ml 0.1% Trichloro acetic acid (TCA). The homogenate was centrifuged at 10000 g for 5 min. To 1 ml aliquot of the supernatant 4 ml of 20% TCA containing 0.5% thio barbituric acid (TBA) were added. The mixture was heated at 95°C for 30 min, quickly cooled in an ice bath and centrifuged at 10000 rpm for 10 min. The absorbance of the supernatant was read at 532 and 600 nm (Heath and Packer, 1968). For the estimation of ethylene, the plants were uprooted after 40 days, washed with sterile distilled water until free from soil and ethylene production was measured by gas-chromatography (GC) (Chinnadurai et al., 2009). The whole plant was packed in 120 ml vials and the vials were capped with a rubber septum and following 4-h incubation, 1 ml of headspace was sampled for each vial and the ethylene content was measured in a GC packed with poropak-N column at 70°C, equipped with a flame ionization detector. The carrier gas was N_2 at the flow rate of 60 ml min^{-1} and the combustion gas was H_2 at the flow rate of 50 ml min^{-1} with the combustion-supporting gas air at the flow rate of 500 ml min^{-1}. The amount of ethylene detected through GC were manipulated and expressed as µmol of ethylene evolved per g of fresh plant in 1 h.

Statistical analysis:

Data were subjected to analysis of variance (ANOVA) using a statistical computer package AGRES to determine whether the treatments effects were significant. The treatment and variety means were separated using the least significant differences (LSD) test.

RESULTS

Molecular characterization of rice phyllosphere isolates

Phylogenetic tree was generated by using the phylogeny program and neighbor-joining method. The 16S rRNA sequences from *Bacillus* genus were used for diversity studies (the bacterial species and accession number are given in Figure 1).

Plant growth promoting activities of the isolates

Among the isolates, the highest amount of IAA was produced by *B. altitudinis* (FD48) (8.14 µg/ml) followed by *Bacillus pumilus* (FS20) (5.64 µg/ml) and *Bacillus aquimaris* (MD02) (4.43 µg/ml). PEG treatment at 15, 20 and 25% reduced the level of IAA production in all isolates compared to control (without PEG). Among the three isolates tested, FD48 produced IAA (2.82 µg ml^{-1}) at higher concentration of PEG (25%) whereas isolates FS20 and MD02 did not produce detectable amount of IAA at same level (Figure 2).

Among the three phyllosphere isolates, *B. altitudinis* FD48 was able to grow on DF minimal broth supple-mented with 3 mM ACC as sole nitrogen source, whereas *B. pumilus* FS20 and *B. aquimaris* MD02 showed no growth. All the three isolates showed growth on DF minimal broth supplemented with 0.2% $(NH_4)_2SO_4$. ACC deaminase activity of *B. altitudinis* FD48 was quantified
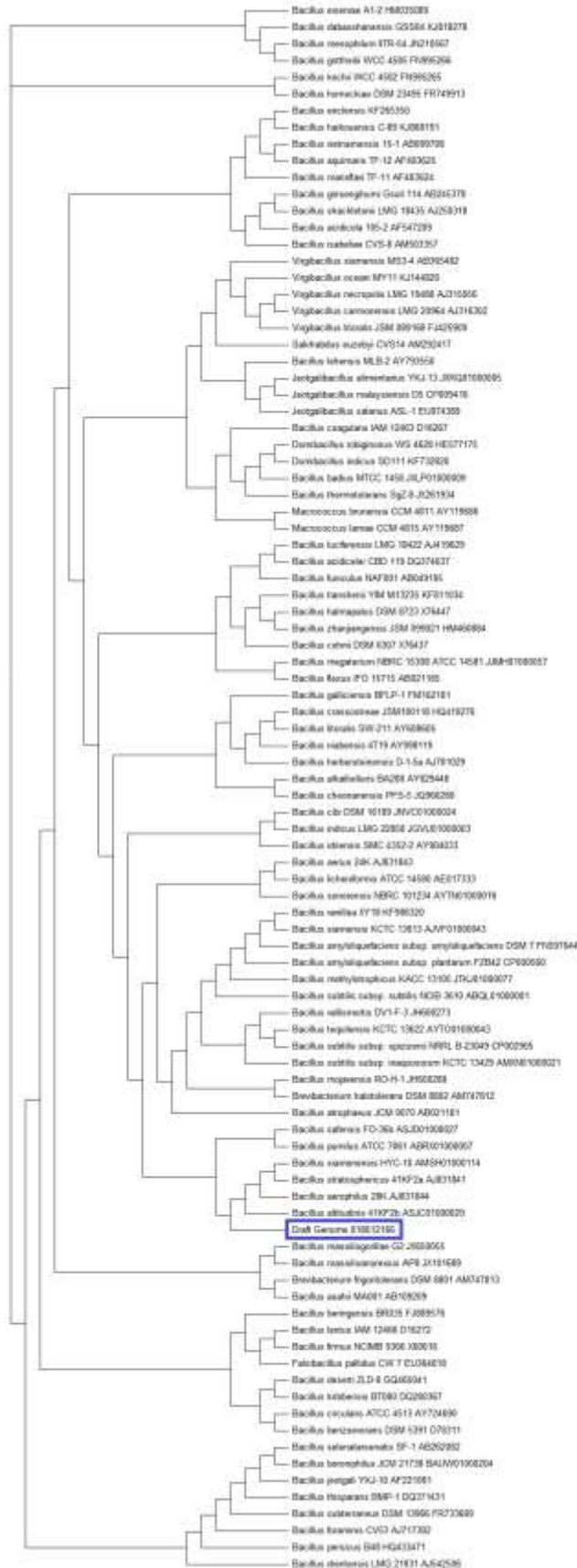
Figure 1. Phylogenetic tree of obtained isolates.
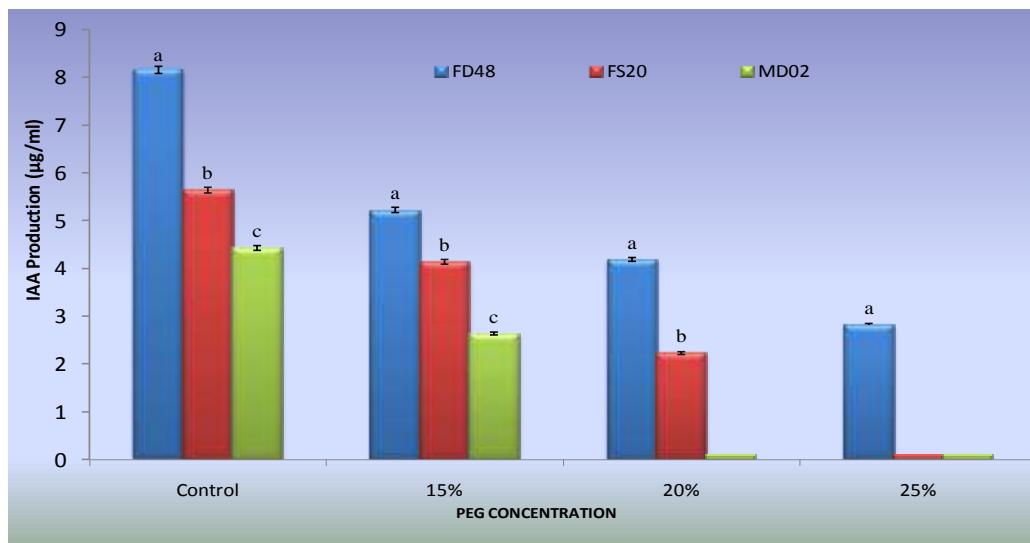
Figure 2. Effect of PEG 6000 concentrations on the production of indole acetic acid (IAA) by phyllospere isolates.

Table 1. Effect of PEG 6000 concentrations on the growth of rice phyllosphere isolates (OD at 660 nm).

Isolate	Control	10% PEG	15% PEG	20% PEG	25% PEG
Bacillus altitudinis (FD48)	1.32 (±0.015)	0.83 (±0.01)	0.78 (±0.009)	0.61 (±0.007)	0.55 (±0.006)
Bacillus pumilus (FS20)	1.05 (±0.012)	0.61 (±0.007)	0.44 (±0.005)	0.29 (±0.003)	0.10 (±0.001)
Bacillus aquimaris (MD02)	0.99 (±0.011)	0.52 (±0.006)	0.36 (±0.004)	0.10 (±0.001)	0.11 (±0.001)

Values are mean ± standard error of seven replicates. PEG: Polyethylene glycol.

and found to produce 192.5 n moles α-ketobutyrate mg^{-1} h^{-1}. An amplification of 700 bp of *accD* gene was observed. This PCR based screening confirmed the presence of *accD* gene in *B. altitudinis* FD48.

Drought tolerance level of isolates

The effect of drought towards the growth of all the three isolates were studied using LB broth supplemented with PEG. All the isolates grew well in LB broth without PEG. As the concentration of PEG increased, the growth decreased. The isolate *B. altitudinis* FD48 was able to grow at 25% PEG 6000 (0.55 OD) whereas the isolates *B. pumilus* FS20 (0.44 OD) and *B. aquimaris* MD02 (0.36 OD) were able to grow only upto 15% PEG 6000 concentration (Table 1).

Germination percentage and root length of rice seeds treated with phyllosphere bacterial cultures

Rice germination was decreased as the concentration of PEG increased, that is, 0 to 25%. However, the effect of PEG was greatly reduced by treating rice seeds with

bacterial cultures viz., *B. altitudinis* FD48, *B. pumilus* FS20, *B. aquimaris* MD02 and *Methylobacterium* spp. (PPFM) (Table 2). Among the isolates tested, *B. altitudinis* FD48 greatly enhanced the germination percentage at 25% levels of PEG compared to uninoculated control. Root length was decreased as the concentration of PEG increased, that is, 0 to 25%. However, culture treated seeds recorded better root growth than the uninoculated control at all concentration of PEG. At higher concentration of PEG (25%), *Ba. altitudinis* FD48 treated seeds showed 0.56 cm root length followed by *Methylobacterium* spp. (PPFM) treated seeds (0.42 cm) whereas uninoculated control showed only 0.22 cm root length (Table 2).

Effect of phyllosphere isolates on biometric characteristics of rice seedlings (cultivar- ADT 36) after 25 days of inoculation

Phyllosphere bacterial isolates with plant growth promoting activities were selected for gnotobiotic studies. Among the three isolates the highest shoot length was observed in seedlings treated with *B. altitudinis* FD48 (9.5 cm) which was significantly superior to *Methylobacterium*

Table 2. Germination percentage of rice seeds treated with phyllosphere bacterial isolates at different concentration of PEG 6000 after 72 h.

Isolate	Germination (%)				Root length (cm)			
	Control	10% PEG	15% PEG	25% PEG	Control	10% PEG	15% PEG	25% PEG
Bacillus altitudinis (FD48)	98	88	68	52	1.96	1.14	0.74	0.56
Bacillus pumilus (FS20)	98	72	50	32	1.08	0.80	0.54	0.32
Bacillus aquimaris (MD02)	96	60	40	26	1.08	0.60	0.42	0.30
Methylobacterium sp. (PPFM)	98	80	60	42	1.12	0.94	0.60	0.42
Control	96	50	22	14	0.98	0.56	0.29	0.22
SEd	4.24	3.77	2.92	2.01	0.08	0.07	0.05	0.05
CD (0.05)	8.86	7.88	6.09	4.19	0.17	0.16	0.12	0.10

Values are mean of five replicates. SEd: Standard error of deviation; CD: critical difference at 5% level.

Table 3. Effect of phyllosphere bacterial isolates on root length, shoot length, root dry weight and shoot dry weight of rice grown in paper towel method for 25 days.

Isolates	Root length (cm)	Shoot length (cm)	RDW (mg)/plant	SDW (mg)/plant
Bacillus altitudinis (FD48)	15.23	9.5	3.77	5.11
Bacillus pumilus (FS20)	12.06	7.27	3.13	3.86
Bacillus aquimaris (MD02)	8.87	6.92	2.9	3.42
Methylobacterium sp. (PPFM)	14.01	8.51	3.37	4.45
Control	6.76	5.23	1.75	2.89
SEd	0.46	0.44	0.14	0.16
CD (0.05)	0.94	0.90	0.29	0.33

Values are mean of five replicates. SEd: Standard error of deviation; CD: critical difference at 5% level. RDW: Root dry weight; SDW: shoot dry weight.

spp. (PPFM) (8.51 cm). The uninoculated control recorded the lowest shoot length (5.23 cm) while higher root length was recorded in *B. altitudinis* FD48 treated seedlings (15.23 cm) followed by *Methylobacterium* spp. (PPFM) treated seedlings (14.01 cm). The least root length was observed in control (6.76 cm). The root dry weight was the highest in *B. altitudinis* FD48 treatment (3.77 mg) followed by *Methylobacterium* spp. (PPFM) treatment (3.37 mg). The least RDW was observed in control (1.75 mg). *B. altitudinis* FD48 (5.11 mg) showed the highest shoot dry weight followed by *Methylobacterium* spp. (PPFM) (4.43 mg). The control recorded least shoot dry weight (2.89 mg) (Table 3).

Crop response study to evaluate the efficiency of phyllosphere bacteria in alleviation of drought stress under *in vitro* conditions

The pot culture experiment was carried out at Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore to evaluate the efficiency of phyllosphere bacteria on the growth of rice (cultivar- ADT 46) under drought condition. Relative water content (RWC) of plants decreased in response to drought condition. However, culture treated plants were observed to have more RWC compared to control under induced drought condition. *B.altitudinis* FD48 treated plants showed 69.38% RWC followed by *Methylobacterium* spp. (PPFM) treated plants (68.61%) whereas the control recorded the lowest RWC (60.53%) (Table 4). Chlorophyll stability index (CSI) of plants decreased in response to drought condition. Drought stressed plants inoculated with *B. altitudinis* FD48 showed 69.23% CSI followed by *Methylobacterium* spp. (PPFM) inoculation (68.32%). The chlorophyll stability index of control plants were the lowest (55.4%) under drought condition (Table 4).

Proline content was significantly influenced by both drought stress and culture treatments. A substantial increase in the amount of free proline was observed in all treatments due to drought stress (Table 4). However, it was interesting to note that *B. altitudinis* FD48 treated rice plants produced the highest concentration of proline (5.73 μ mol g^{-1} fresh weight) relative to *Methylobacterium* spp. (PPFM) treated plants (5.11 μ mol g^{-1} fresh weight) and control (3.16 μ mol g^{-1} fresh weight). The phenolics content of plants in all treatments increased from well watered condition to drought condition. Highest phenol content under drought stress was observed in treatment *Bacillus altitudinis* FD48 (83.57 μg g^{-1} fresh weight) followed by

Table 4. Relative water content, chlorophyll stability index, total phenolics and proline content of bacterial treated rice plants grown under drought stress.

Culture	Relative water content (%)		Chlorophyll stability index (%)		Total Phenolics (µg g^{-1} fresh weight)		Proline content (µ mol g^{-1} fresh weight)	
	NS	DS	NS	DS	NS	DS	NS	DS
Control	87.05	60.53	55.31	78.93	55.31	78.93	1.03	3.16
Bacillus altitudinis (FD48)	88.77	69.38	68.46	83.57	68.46	83.57	1.16	5.73
Methylobacterium spp. (PPFM)	89.29	68.61	67.14	82.65	67.14	82.65	1.09	5.11
SEd	0.24	0.32	0.56	0.41	0.56	0.41	0.01	0.18
CD (0.05)	0.51	0.67	1.18	0.86	1.18	0.86	0.03	0.39

Values are mean of seven replicates. SEd: Standard error of deviation; CD: critical difference at 5% level. NS: non stressed; DS: drought stressed.

Table 5. Catalase activity, Malondialdehyde content, membrane stability index and ethylene emission level of bacterial treated rice plants grown under drought stress.

Culture	Catalase activity (µg of H$_2$O$_2$ g^{-1} min^{-1})		Malondialdehyde content (n moles/g fresh weight)		Membrane stability index (%)		Ethylene (µmol/g fresh weight/h)	
	NS	DS	NS	DS	NS	DS	NS	DS
Control	6.47	4.88	87.36	60.42	87.36	60.42	32.2	97.5
Bacillus altitudinis (FD48)	6.27	3.51	89.21	69.32	89.21	69.32	27.1	72.2
Methylobacterium spp. (TNAU1)	6.39	4.01	88.54	68.55	88.54	68.55	28.6	75.8
SEd	0.19	0.17	0.40	0.26	0.40	0.26	0.75	0.42
CD (0.05)	0.40	0.36	0.84	0.55	0.84	0.55	1.57	0.90

Values are mean of seven replicates, SEd: Standard error of deviation; CD: critical difference at 5% level; NS: non stressed; DS: drought stressed.

Methylobacterium spp. (PPFM) (82.65 µg g^{-1} fresh weight). The control plants had the lowest phenol content (78.93 µg g^{-1} fresh weight) (Table 4).

Catalase activity did not differ significantly among the treatments under non stressed condition which indicated the reduced activity of catalase enzyme. The catalase activity increased under drought condition with *B. altitudinis* FD48 treated plants with significantly higher activity (3.51 µg of H$_2$O$_2$ g^{-1} min^{-1}) followed by *Methylobacterium* spp. (PPFM) treated plants (4.01 µg of H$_2$O$_2$ g^{-1} min^{-1}). The least catalase activity was observed in control (4.88 µg of H$_2$O$_2$ g^{-1} min^{-1}) (Table 5). MDA content was significantly influenced by drought and culture treatment. The MDA content was higher in untreated (control) plants under drought condition (6.91 n mol/g fresh weight). *Methylobacterium* spp. (PPFM) treatment recorded 5.81 n mol/g fresh weight of MDA under stressed condition. The accumulation of MDA content was lower in treatment *B. altitudinis* FD48 (4.66 n mol/g fresh weight) revealing reduced accumulation of lipid peroxides under drought stress (Table 5).

Limiting watering caused a loss in membrane stability in untreated rice plants and treated plants (Table 5). However, *B. altitudinis* FD48 treated plants significantly improved membrane stability (69.32%) compared to *Methylobacterium* spp. (PPFM) treated plants (68.55%) and control (60.42%) (Table 5). The whole plant of rice was used to quantify ethylene emission by gas chromatography. The quantity of ethylene estimated from the plant was expressed by µmol per gram fresh weight of the plant per hour and presented in Table 5. Ethylene emission level was low under non stressed condition and under drought condition the level of ethylene emission increased. However, *B. altitudinis* FD48 treated plants resulted in low ethylene emission (72.2 µmol/g fresh weight) compared to control (97.5 µmol/g fresh weight) under stressed condition.

DISCUSSION

Plants are constantly exposed to abiotic stress, such as drought, which is one of the most serious problems associated with plant growth and development. Hence, inoculation with PGPB is most effective under drought-stress environment to increase productivity (Chanway and Holl, 1994). Growth promotion by the PGPB may be attributed to mechanisms such as production of Plant Growth Promoting hormones and other PGP activities (Glick, 1995). The phyllosphere, the interface between the aerial parts of terrestrial plants and the air, represent the largest biological habitat on earth (Delmotte et al., 2009). These bacterial populations are thus sufficiently large to have an important impact on the global biogeochemical processes of nutrient elements (e.g., carbon and nitrogen cycles) as well as the health and physiological characteris-

tics of the plants.

The isolated phyllosphere bacteria were identified based on 16S rRNA gene sequencing. Further, the multiple plant growth promoting traits and tolerance level to drought stress conditions were examined. Influences of phyllosphere bacterial inoculation on growth of rice under drought condition were also studied *in vitro* conditions.

Occurrence of *Bacillus* spp. in the phyllosphere of vegetables was reported earlier (Zhang et al., 2010). Presence of leaf associated culturable *Bacillus* spp. on tobacco leaves and their quorum quenching ability has been reported (Ma et al., 2013). *B. altitudinis* isolated from rice phyllosphere, showed antagonistic activity against *Rhizoctonia solani* (De Costa et al., 2008).

It was reported that IAA production can increase the epiphytic fitness of bacteria (Manulis et al., 1998). At low concentrations, IAA plays an important role in loosening cell wall (Vanderhoff and Dute, 1981) and stimulating the release of saccharides, which serves as nutrient source for epiphytic bacteria and supports their growth (Fry, 1989; Goldberg, 1980). The results revealed that the *accD* gene positive isolates could produce a partial amplification (700 bp) of *accD.* This PCR based detection is relatively easy to screen the isolates than the enzyme assay. Among 3 isolates, only *B. altitudinis* FD48 showed positive to *accD*. In earlier studies, the rhizobacteria were reported to be the predominant group of ACC deaminase producers in plants including *Enterobacter cloacae* (Penrose and Glick, 2001), *Rhizobium* (Ma et al., 2003), *Pseudomonas, Alcaligenes*, *Bacillus*, etc. The ACC deaminase producing *Methylobacterium* was first isolated from stem tissues of rice and designated as *Methylobacterium oryzae* (Madhaiyan et al., 2007). The presence of ACC deaminase producing *Methylobacterium* spp. in rice phyllosphere was reported (Chinnadurai et al., 2009). In this study, ACC deaminase activity of *B. altitudinis* FD48 was found to produce 192.5 nmoles α-ketobutyrate mg^{-1} h^{-1}. This result was similar to the findings of Chinnadurai et al. (2009) who reported ACC deaminase activity of *Methylobacterium* spp. from rice phyllosphere in the range of 190 to 400 nmoles α-ketobutyrate mg^{-1} h^{-1}.

The inoculated rice seeds showed higher root and shoot growth than the control. FD48 treated seeds performed better when compared with other isolates. This finding is in agreement with earlier studies conducted by Ghosh et al. (2003), Jiang and Lafitte (2007), and Madhaiyan et al. (2006) in different plant species. The increase in root and shoot length and their dry weight may be due to the plant growth promoting activities of the isolates. The isolate *B. altitudinis* FD48 also supported the germination of rice seeds under different PEG concentration. It has been suggested that production of betaine, an osmolyte by certain bacteria provides a barrier against dehydration (Sleator and Hill, 2002). It is not known, however, how the interaction of plants with such microorganisms improves plant response to drought.

B. altitudinis FD48 also showed growth on increasing concentration of PEG 6000 (which induces stress) and proved their ability to withstand drought stress. The results are in agreement with Upadhyay et al. (2012) who reported the growth of *Bradyrhizhobial* isolates under increasing PEG concentration. *B. altitudinis* FD48 reported IAA production at 25% PEG concentration, whereas other isolates did not produce any detectable quantities of IAA with increasing PEG concentration. These results are in confirmation with Uma Maheshwari et al. (2013) and Marulanda et al. (2009). Moreover, the ability of these strains to increase the production of IAA as much as the increased osmotic stress (PEG) in the growing medium would account for their osmotic tolerance. In addition to general plant growth, IAA stimulates stress tolerance because of physical and chemical changes in plant caused by these Plant Growth Promoting Bacteria (Mayak et al., 2004). IAA can improve the root proliferation and help plants to accumulate water from the surrounding environment, thereby improving the response to drought stress.

The phyllosphere isolates showed increased content of proline, total sugars and total amino acid under PEG induced drought stress condition when compared with non-stressed condition. But the protein content was less in all isolates under drought stressed condition. *B. altitudinis* FD48 performed better than other two isolates under drought condition. These results are in accordance with Sandhya et al. (2010) and Singh et al. (2016). Under stress conditions energy flow of the cells is directed towards protection mechanisms to synthesize osmolytes (sugars, proline, etc) to protect them against fluctuations in osmotic conditions (Timmusk, 2003) and these osmolytes accumulate to higher levels to alleviate stress effects (Rasanen et al., 2004). The accumulated osmolytes enhance the stability of proteins and membrane under water-limiting environments (Kogut and Russell, 1987). However, the concentration of protein was reduced significantly under stress indicating the degeneracy under stress conditions. Trehalose accumulation was also found in *B. altitudinis* FD48 which is a compatible osmolyte and may result in providing stress tolerance to the isolate. Trehalose accumulation in *Azospirillum brasilense* improved drought tolerance and biomass in maize treated with the culture (Rodriguez-Salazar et al., 2009).

The inoculation of phyllosphere isolate *B. altitudinis* FD48 improved the plant growth under drought condition by maintaining relative water content, increased accumulation of proline and phenolics and by enhancing the activity of antioxidant enzymes when compared to control. Under drought stress Relative water content (RWC) declined in inoculated and uninoculated seedlings. However, bacterial inoculation did help seedlings to maintain their Relative water content (RWC) during drought periods. Similar report was made on the use *Pseudomonas* spp. inoculation to help the maize plants

to maintain their relative water content under drought condition (Sandhya et al., 2010). The mechanism behind the increased Relative water content (RWC) when treated with PGPB is yet to be elucidated. Some studies predict that this may be a result of bacterial absicisic acid which results in closure of stomata (Casanovas et al., 2002). Drought stress caused a disturbance in membrane permeability and expressed by an increase in solute leakage (Premchandra et al., 1990; Deshmukh et al., 1991). The results on MSI showed a decreasing trend as the time without water prolonged. The leakage was higher in untreated plants than *B. altitudinis* FD48 treated plants indicating severe membrane damage in the former under drought stress. *Bacillus* spp. treated maize plants reduced the MDA content under stress condition compared to control (Sandhya et al., 2010). The higher leakage of solutes was probably due to enhanced H_2O_2 accumulation and lipid peroxidation under oxidative stress (Dionisio-Sese and Tobita, 1998). The plasma membrane is generally protected from desiccation-induced damage by the presence of membrane-compatible solutes, such as sugars and amino acids. Therefore, a link may exist between the capacity for osmotic adjustment and the degree of membrane protection from the effect of dehydration. Accumulation of antioxidant enzymes may also result in protecting membrane stability.

The inoculation also increased proline content under drought stress compared to control which may be due to up regulation of proline biosynthesis pathway to keep proline in high levels, which helps in maintaining cell water status, protects membranes, and proteins from stress (Yoshiba et al., 1997). Proline accumulation by repressed catabolic pathway under oxidative stress helps the plants to decrease oxidative damage (Nayer and Reza, 2008). The results obtained were in confirmation with several authors (Ruiz-Sanchez et al., 2011; Sandhya et al., 2010; Sandhya et al., 2011). *Azospirillum* and arbuscular mycorrhizal inoculation increased the shoot proline content in rice under drought condition when compared to control (Ruiz-Sanchez et al., 2011).

In the present study, total phenolics were positively influenced by FD48 treatment and were significantly higher than control plants under stress condition. This result was in agreement with the report that inoculation of rice with *Trichoderma* spp. increased phenolics content under drought condition (Shukla et al., 2012). The phenolic compounds, besides having antifungal, anti-bacterial and antiviral activities also possess antioxidant properties and thus act as scavengers of activated free radicals (Malik and Singh, 1980).

High temperature causes membrane collapse, which leads to chlorophyll degradation in the plant. This results in the loss of chlorophyll pigment by stress, which ultimately results in the decline in the rate of photosynthesis and finally crop yield. Since Chlorophyll stability index is a function of temperature, the property of

chlorophyll pigments can be correlated with drought tolerance/susceptibility of the crop plants. Prolonged drought stress reduced the chlorophyll stability index in all treatments. But FD48 treated plants showed more Chlorophyll Stability Index (CSI) when compared to *Methylobacterium* spp. (PPFM) and control.

B. altitudinis FD48 and *Methylobacterium* spp. (PPFM) treated rice plants showed more catalase activity than control under drought condition. This result was in agreement with Shukla et al. (2012), Sandhya et al. (2011) and Gusain et al. (2015) who reported that under conditions of environmental stress, when ROS such as H_2O_2 are produced, catalase enzyme triggered by the bacteria act as scavenging enzymes and play a central role in protecting the cell from oxidative damage. Catalase and exopolysaccharides producing ability of rhizobacteria has been reported (Hussain et al., 2014).

The MDA, which is indicative of oxidative stress, increased as drought increased in plants. Our findings showed an increase in MDA in both treated and untreated plants though increase was less evident in *B. altitudinis* FD48 treated rice plants. Degree of accumulation of MDA content has been reported to be indicative of the rate of lipid peroxidation due to drought stress (Bailly et al., 1996). Our results are in agreement with Gusain et al. (2015) and Shukla et al. (2012) where bacterial treatment reduced the MDA content of rice under drought condition.

Ethylene emission increased when rice plants were subjected to drought stress, but *B. altitudinis* FD48 and *Methylobacterium* spp. (PPFM) treated plants reported less ethylene emission when compared to control plants. This might be due to suppression of the stress-induced accelerated synthesis of ethylene by the ACC deaminase activity of the bacteria in the inoculated plants. Sharp increases in ACC levels and, consequently, ethylene synthesis in plants under drought stress conditions has been frequently reported (Asghar et al., 2002; Mayak et al., 2004). Therefore, the inhibitory effects of ethylene induced by drought stress might have been eliminated through the ACC deaminase activity of the isolates. Our results were supported by Zahir et al. (2008) who reported the reduced emission of stress ethylene by rhizobacteria treated peas under drought condition and the alleviation of water stress in chick pea by ACC deaminase producing rhizobacteria under axenic conditions (Sharma et al., 2013).

Conclusion

The present study shows that ACC deaminase producing *B. altitudinis* FD48 was capable of withstanding PEG induced drought by synthesizing osmolyte like proline. *B. altitudinis* FD48 treated plants withstand drought by accumulating proline, enhancing the activity of antioxidant enzymes and by reducing MDA content. Furthermore, reduction in ethylene was also reported in

B. altitudinis FD48 treated plants under drought condition. It seems that *B. altitudinis* FD48 influenced the biochemical parameters of rice and helped them improving tolerance to water stress. *B. altitudinis* FD48 proved to have a promising role in improving plant performance under drought condition.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Serotypes and antimicrobial resistance profiles of *Salmonella* isolated from fresh beef processing and chilled fresh beef samples produced and marketed in the metropolitan region of Cuiabá, in the State of Mato Grosso, Brazil

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Salmonella spp. in food products of animal origin can cause foodborne infections, and when antimicrobial-resistant strains are present, can evolve to severe disease. This study aims to determine the occurrence of *Salmonella* and identify the serotypes present in the beef production chain of the metropolitan region of Cuiabá, State of Mato Grosso, Brazil, and evaluate their antimicrobial resistance profile. The presence of *Salmonella* was determined by the ISO-6579:2002 method and evaluation of resistance to 17 antibiotics was performed by the CSLI 2014 method. The following samples were analyzed: (1) environmental samples: corral swab, bleeding, skinning and deboning knives, ribbon saw, boning bench; (2) animal samples: anal swab, internal and external carcass sponge, organ pool, boning shavings and by-products in the slaughterhouse; and (3) ground meat samples from retail sales from 10 butcher shops and permanent fair stalls. *Salmonella* was present in ten of the 182 samples (5.5%), five of which were *Salmonella* Panama detected in the corral, boning tables, meat from the butcher's shop and permanent market. These strains were sensitive to all antibiotics characteristics and similar to *Salmonella enterica* rough, and being isolated from the ribbon saw used in the production chain. However, *Salmonella* Anatum and *Salmonella* Infantis isolated from carcasses, bone meal and viscera presented resistance to sulfonamides, trimethoprim, sulfametazol/ trimethoprim and nitrofurantoin in relation to the use of antibiotics in animal production and the selective pressure of multiresistant *Salmonella* spp. in products derived from these animals.

Key words: Serotyping, bovine meat, slaughterhouse, butcher, antibiotic resistance.

INTRODUCTION

Salmonella is the most frequently isolated bacteria responsible for diagnosed cases of foodborne diseases worldwide (Puig Peña et al., 2011). *Salmonella* presents more than 2600 serotypes among which *Salmonella* Typhi is pathogenic to humans (Grimont and Weill, 2007; Issenhuth-Jeanjean et al., 2014).

However, some nontyphoidal serotypes considered as pathogenic to humans also exist which are frequently isolated from food infection cases as well as from systemic infections, such as meningitis and bacteremia (Zaidi et al., 2006). These include *Salmonella* Enteritidis and *Salmonella* Typhimurium, although *Salmonella* Infantis, *Salmonella* Anatum, and *Salmonella* Panama have also been cited as infectious agents and/or have been detected in food products involved in cases of food or systemic infections (Hendriksen et al., 2011; Huang et al., 2013; Capalonga et al., 2014).

Serotypes *S.* Infantis, *S.* Panama, and *S.* Anatum are among the 15 main *Salmonella* serotypes considered agents of human infection and food contaminants occurring in Brazil, in both the southeastern (São Paulo) and southern (Rio Grande do Sul) regions in the 1990s and between 2007 and 2012, respectively (Tavechio et al., 2002; Huang et al., 2013). Globally, the distribution of these serotypes is diverse, with *S.* Infantis found in Africa, North America and Latin, Asia, Europe and Oceania; *S.* Anatum reported in Africa, Latin America and Oceania and, *S.* Panama present only in Asia and Latin America. These serotypes frequently range from the fifth to the fourteenth position as agents of human infection (Hendriksen et al., 2011).

Worldwide, meats, their derivatives, and foods produced from mechanically deboned meat are more frequently involved in food infection cases caused by *Salmonella* (Puig Peña et al., 2011). In Brazil, the food products involved in these cases have been reported, mainly, as eggs, chickens and their by-products. However, reports of cases, where red meat and its by-products are implicated, are on the rise, especially regarding *S.* Infantis and *S.* Panama (Capalonga et al., 2014).

The emergence of Multi-Resistant Drug (MDR) *Salmonella* is a worldwide concern. This is a consequence of the extensive use of antibiotics in humans and animals, being a risk condition that increases the severity of the disease and hospitalization rates, as well as the possibility of death (Ribeiro et al., 2007; Huang et al., 2013). The antimicrobial resistance of *Salmonella* currently, represents a worldwide problem for both the veterinary and public health sectors. An increase in the incidence of antibiotic-resistant *Salmonella* strains in production animals and their by-products is observed, especially in Latin America, particularly in cattle, although studies in this regard are still scarce (Perez-Montaño et al., 2012; Capalonga et al., 2014). Therefore, more information is needed on the occurrence of MDR *Salmonella* in this matrix to assist in the control of the indiscriminate use of antibiotics.

Brazil is one of the largest beef producers in the world, with a production of 9.56 million tons in 2015. Of this total, 19.63% was exported, and 81% was consumed in the local market (ABIEC, 2016). In this context, the State of Mato Grosso leads the slaughter capacity among all Brazilian States, slaughtering 35.466 cattle a day (ABIEC, 2016). Hence, the importance of *Salmonella* prevalence investigation in this state contributes significantly to the Brazilian food safety and public health surveillance. The present study aimed to investigate the occurrence of *Salmonella* and identify the serotypes and their antimicrobial resistance profiles, by evaluating the beef production chain, from the production environment to the wholesale market in the slaughterhouse, as well as retail sales from butcher shops and municipal market stalls, in the metropolitan region of Cuiabá, Mato Grosso, Brazil.

MATERIALS AND METHODS

Sampling

A total of 182 samples were evaluated; 156 slaughterhouse samples and 26 butcher shop samples. The slaughterhouse samples were collected during the processing of 13 batches of animals from different cities and farms. Environmental samples were obtained from the corral and boning tables (I, II and III), utensils (bleeding, skinning and boning knives) and equipment (ribbon saw). Animal samples were obtained from anal swabs, internal and external carcass sponges, organ pool (esophagus, diaphragm, and masseter muscle), boning shavings and bone and viscera meal.

All environmental and anal region samples from the animals were harvested with 3M ™ Sampler swabs containing Buffered Peptone Water (BPW). 3M™ Sponge-Sticks containing buffered peptide water were used for sample collection from the neck, thorax, abdomen and hindquarter regions, using a sterilized field boundary bracket (10 cm by 10 cm, or 10 0 cm^2). The tissue fragments were packed in sterile plastic bags. Subsequently, for convenience, another 26 samples consisting of portions of 500 g of chopped meat (*quadriceps femoris*) were obtained from five butcher's shop in the city districts, and five-meat stall at a permanent fair, 12 and 14 samples, respectively. The samples from both the slaughterhouses and retailers were collected under refrigeration and immediately taken to the laboratory and analyzed, from April to July 2015, in the Cuiabá metropolitan region, latitude: 15°35'46 "S; Longitude: 56°05'40 "W, the State of Mato Grosso, Brazil.

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Isolation and identification of *Salmonella* species

The isolation method was based on the protocol recommended by the International Standardization Organization (ISO-6579:2002). Briefly, 25 g of the sample were inoculated in Buffered Peptone Water (Himedia®, Mumbai, India), incubated at 37°C for 24 h, enriched in Rappaport-Vassiliadis Broth (Oxoid®, United Kingdom), incubated at 42°C for 24 h and then in Muller Kauffmann Novobiocin Tetrathionate Broth (Himedia®, Mumbai, India) at 37°C for 24 h, with subsequent plating on Xylose Lysine Deoxycholate Agar (Himedia®, Mumbai, India) and Rambach Agar (Merck, Darmstadt, Germany), incubated at 37°C for 24 h.

The typical colonies were selected, purified on Nutrient Agar and subsequently inoculated on API 20E (BioMérieux®, Lyon, France). The strains that showed a typical *Salmonella* reaction were subjected to serum-agglutination by the anti-salmonella polyvalent O serum.

DNA extraction

The strains biochemically identified as *Salmonella* were inoculated in 10 mL of BHI Broth (Brain Heart Infusion) and incubated at 35°C for 24 h. A 1.5 mL aliquot was then centrifuged at 14,000 x g for 5 min; the pellet was dissolved in 500 µL Mili-Q water, and heated at 100°C for 10 min on a heating plate (BioGPRO, Brazil), then cooled at 4°C for 10 min. The lysate was then centrifuged at 14,000 x g for 5 min, and 200 µL of the supernatant was removed, maintained in a freezer and subsequently subjected to multiplex PCR.

Multiplex-PCR

The reaction was performed in a total volume of 25 µL containing 1U Taq Polymerase (Invitrogen®), 1x Taq buffer (5 mM KCl Tris-HCl, pH 8.5) 1.5 mM $MgCl_2$, 0.1 mM dNTP's (Promega®), 0.9 MM primer Inv-A, and 0.4 µM of IE-1 and Flic-C primers (Invitrogen®). The conditions were based on the study performed by Paião et al. (2013).

The m-PCR assay was performed with an initial denaturation for 5 min at 95°C, followed by 30 cycles one min at 95°C, 1 min at 58°C, and 30 sec at 75°C, with a final extension step at 72°C for 7 min. The PCR product was analyzed by electrophoresis on 1.5% agarose gels, TBE buffer (45 mmol L^{-1} Tris pH 8.3, 45 mmol L^{-1} borate, and 2 mmol L^{-1} EDTA) as the running buffer. The gels were then stained with Gel Red (Invitrogen®) and photo documented (MiniBis-Pro DNT, Bio-Imaging Systems®).

Antimicrobial susceptibility test

The isolates were submitted to antimicrobial susceptibility tests by the disc diffusion method employing 17 antimicrobials: ampicillin, 10 mcg; aztreonam, 30 mcg; cephalothin, 30 mcg; cefoxitin, 30 mcg; ceftiofur, 30 mcg; chloramphenicol, 30 mcg; florfenicol, 30 mcg; streptomycin, 300 mcg; gentamicin, 10 mcg; nalidixic acid, 30 mcg; ciprofloxacin, 5 mcg; enrofloxacin, 5 mcg; tetracycline, 300 mcg; sulfamethoxazole trimethoprim. 25 mcg; sulfonamide, 300 mcg; trimethoprim, 5 mcg and nitrofurantoin, 300 mcg (Cecon®, Brazil). The discs were distributed equidistantly in plates which were then incubated at 35°C for 16 to 20 h (CLSI, 2014).

Inhibition halos were measured, and the results were compared to standards contained in the Clinical and Laboratory Standards Institute (Patel and CLSI, 2016), and classified as sensitive, intermediate or resistant. Screening of strains suggestive of Extended-Spectrum Beta-lactamase (ESBL) producers was also carried out, using the β-Lactams breakpoint criterion for *Enterobacteriaceae* that detects all resistance mechanisms,

including ESBL and plasmid-mediated AmpC. According to Cavalieri et al. (2005) strains with halos ≤22 mm for ceftazidime (30 mcg), and ≤27 mm for aztreonam (30 mcg) are potential β-lactamase-producing strains suggestive of ESBL.

Salmonella spp. serotyping

Salmonella serotype identification was carried out at the National Reference Laboratory Diagnosis of Enteric Bacteria, Oswaldo Cruz Institute, Oswaldo Cruz Foundation (FIOCRUZ), by detection of somatic and flagellar antigens, using polyvalent and monovalent antisera, with or without flagellar phase induction (Voss-Rech et al., 2015).

RESULTS AND DISCUSSION

Among the 182 bovine samples (from the environment, carcass, and byproducts), 5.5% (10/182) were positive for *Salmonella* in the biochemical, serological and molecular tests (multiplex PCR).

The presence of this bacterium in the 5.1% (8/156) of the slaughterhouse samples, 8.3% (1/12) of the butcher shop samples and 7.1% (1/14) of the permanent fair samples (Figure 1), is in disagreement with the Brazilian legislation, which advocates its absence in bovine meat (BRASIL, 2001). Studies carried out in Mato Grosso, in the city of Barra do Garças, in ground butchered meat indicated the occurrence of 17% *Salmonella* (Sousa et al., 2012). This microorganism was also detected in 12.5% of fresh meat samples evaluated before and after deboning in Cuiabá butcher shops (Sigarini et al., 2006). These percentages are higher than those detected in the present study, although the strain serotypes were not determined in the studies above. This high detection may be because only butcher shops, not slaughterhouses, were included in these evaluations.

Salmonella was found in environmental samples in the evaluated slaughterhouse: serotype *S.* Panama was detected in the corral (1 strain) and at deboning (2 strains), while *Salmonella enterica* subspecies *enterica* rough was observed in the ribbon saw strains isolated from four different lots (2, 4, 6 and 11) during different weeks. Serotypes *S.* Anatum and *S.* Infantis were isolated from carcasses and bone and viscera meal in slaughterhouse samples (Table 1). Two strains of *S.* Panama were isolated from samples of chopped meat (*quadriceps femoris*) collected one at the neighborhood butcher's shop and another at the meat stall of a permanent fair in Cuiabá (Table 1).

Usually, contamination by these bacteria occurs due to inadequate hygienic and handling conditions in the slaughterhouse. In this context, microbiological evaluations performed on utensils, environment, and carcasses of cattle, pigs, and sheep in the slaughterhouse of Australia (Bakhtiary et al., 2016), in Mato Grosso (Santos et al., 2017) and Rio de Janeiro (Cabral et al., 2014), Brazil have been reported. In these studies, the occurrence of microorganisms was observed
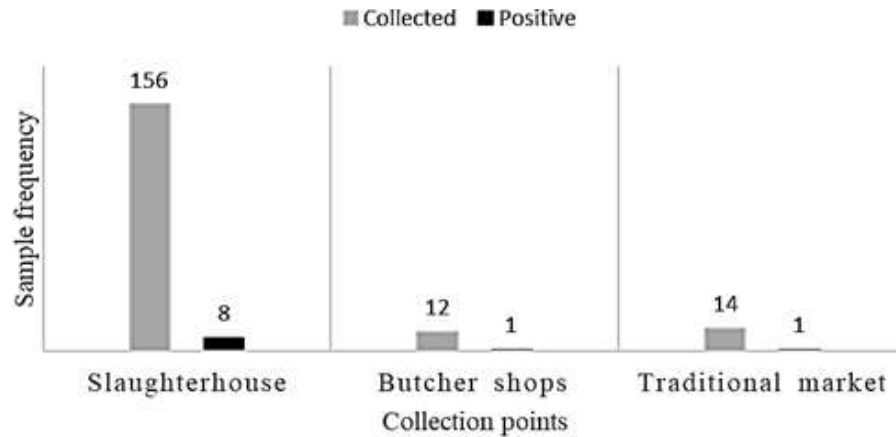
Figure 1. Distribution of the evaluated samples by collection points, and their positivity frequency for *Salmonella* in beef from Mato Grosso, Brazil.

Table 1. Frequency of the *Salmonella* serovars and antibiotic resistance profile of 10 strains isolated from a bovine slaughterhouse and butcher shops in the State of Mato Grosso, Brazil.

Serotype *Salmonella*	Number of strains	Samples	Antimicrobial resistance
S. Anatum	2	Carcass sponge	Tri, Sul
		Bone meal	Sut, Tri, Sul
S. Infantis	2	Carcass sponge	Nit, Sut, Tri, Sul
		Viscera meal	Sut, Tri, Sul
S. enterica rough	1	Ribbon saw swab	-
	5	Corral Swab (1)	-
S. Panama		Boning stand (2)	-
		Chopped meat (2)	-

Nit, nitrofurantoin (300 mcg); Tri, trimethoprim (5 mcg); Sut, sulfamethoxazole/trimethoprim (25 mcg); Sul, sulfonamide 300 mcg.

which are indicators of fecal contamination, belonging to the *Enterobacteriaceae* family, species such as *Escherichia coli*, and *Salmonella enterica* in utensils, environment, and carcasses indicating hygiene problems in the slaughter process.

S. Anatum and *S.* Infantis were observed both in the bovine carcasses and by-products (bone meal and viscera). *S.* Anatum is commonly found in cattle and has frequently been detected in feces, skin, lymph nodes, meat fluids and bovine carcasses of both dairy and beef in the southern United States (Kunze et al., 2008), as well as in beef from Mexico (Varela-Guerrero et al., 2013), Namibia (Shilangale et al., 2015) and South Africa (Madoroba et al., 2016). Serotypes *S.* Infantis and *S.* Panama were also reported in carcasses and processed meat from four slaughterhouses in the State of Jalisco, Mexico (Perez-Montaño et al., 2012). *S.* Anatum *S.* Panama and *S.* Infantis have also been detected in ground beef from three cities in the same Mexican State (Cabrera-Diaz et al., 2013).

In Brazil, only *S.* Panama and *Salmonella enterica* subspecies *enterica* rough were found in a salami production line in the State of Paraná, with *S.* Panama being the most frequently detected (Ribeiro et al., 2007). In the present study, these serotypes were detected in the slaughterhouse as environmental contaminants present in the corral, deboning tables and ribbon saw. *S.* Panama was present in the ground meat samples (*quadriceps femoris*) from the retail trade, butcher shops and the permanent fair stalls in Cuiabá. This data is similar to reported for South Korea, where *S.* Panama was detected in beef, although those samples were marketed wholesale (Hyeon et al., 2011). Serotypes *S.* Panama and *S.* Infantis were also detected in beef and other animals (poultry and pork) in the retail trade in São Paulo, Brazil (Jakabi et al., 2004). In Italy, serotype *S.* Panama was found in pork, while *S.* Infantis occurred in pork and poultry and *S.* Anatum was detected in pork and beef (Busani et al., 2005).

The presence of serotypes *S.* Anatum and *S.* Infantis in

carcasses from the slaughterhouse and *S.* Panama in samples from the butcher and permanent fair samples are concern from public health point of view. These serotypes have also been detected in processed beef implicated in cases of food poisoning in southern Brazil (Capalonga et al., 2014). In Yucatan, Mexico, a study on *Salmonella* as an agent of food infections observed that serotypes *S.* Anatum, *S.* Infantis, and *S.* Panama were isolated from both patients and food, including poultry, beef and pork (Gutiérrez-Cogco et al., 2000). Epidemiological studies have shown that serotypes *S.* Anatum, *S.* Infantis, and *S.* Panama are frequently isolated from contaminated foods and human infections in southeastern Brazil since the 1990s (Tavechio et al., 2002), and in southern Brazil from 2007 to 2012 (Capalonga et al., 2014). In Mexico, the *S.* Anatum serotype was isolated more frequently from products of non-human origin, whereas *S.* Infantis and *S.* Panama serotypes were isolated from humans (Gutiérrez-Cogco et al., 2000). The distribution of *Salmonella* serotypes isolated from humans, animals, and food worldwide indicates that serotypes *S.* Anatum and *S.* Infantis are frequently isolated in certain countries in Africa and Oceania, while *S.* Anatum, *S.* Infantis, and *S.* Panama have been reported in Asia, Latin America and Europe and *S.* Infantis is among the most frequently isolated serotypes in North America (Hendriksen et al., 2011).

In the present study, *S.* Anatum and *S.* Infantis displayed resistance to antibiotics belonging to the antifolate (sulfonamide, trimethoprim, and sulfamethoxazole/trimethoprim) and nitrofuran classes, while *S.* Panama and *Salmonella enterica* subspecies *enterica* rough strains were sensitive to all tested antimicrobials (Table 1). *S.* Infantis displaying resistance to trimethoprim/sulfamethoxazole has been observed in carcasses and *in natura* bovine meat from Mexico, in strains isolated from slaughterhouses (Cabrera-Diaz et al., 2013), as well as *S.* Anatum from retail markets (Perez-Montaño et al., 2012). *S.* Panama sensitive to all tested antibiotics have also been detected in slaughtered carcasses (Cabrera-Diaz et al., 2013; Varela-Guerrero et al., 2013), corroborating the present findings. *S.* Panama strains resistant to ampicillin, chloramphenicol, streptomycin, gentamicin, tetracycline, and sulfamethoxazole/trimethoprim have also been observed in ground beef samples from the State of Jalisco, Mexico (Cabrera-Diaz et al., 2013). In the present study, no difference in the antimicrobial susceptibility profile was observed among the *S. Panama* strains from the slaughterhouse and in the retail trade.

Non-typhoid *Salmonella* strains, including serotypes *S.* Anatum, *S.* Infantis, and *S.* Panama, can cause gastroenteritis, which can progress to systemic infections (bacteremias and meningitis) (Huang et al., 2013; Rowlands et al., 2014). These strains may display an invasive capacity, a characteristic of multi-resistance to antimicrobial drugs, which are genes encoded capacity that may potentiate their persistence in the organism and hence, their pathogenicity (Ribeiro et al., 2007; Huang et al., 2013).

Studies suggest that treatment in cases of contamination by antibiotic-resistant *Salmonella* Typhimurium strains can promote their permanence within the host, thus increasing the virulence, transmissibility, and spread of the disease (Diard et al., 2014). This leads to the possibility that the serotypes *S.* Anatum and *S.* Infantis, present in carcass, bones and viscera meal may display higher pathogenic potential than serotypes *S.* Panama and *S. enterica* rough, present in the slaughterhouse environment and in retail meat since they display sensitivity to all evaluated antibiotics.

Conclusions

We verified that *Salmonella* serotypes *S.* Anatum, *S. enterica* subspecies *enterica* rough, *S.* Infantis and *S.* Panama were present in the meat production chain from the metropolitan region of Cuiabá, in the State of Mato Grosso, Brazil.

For some serotypes of *Salmonella* detected in this, productive chains were mentioned in other regions of Brazil, and the world as the causal agent of foodborne diseases, which was transmitted by the meat. Isolated from carcasses and animal by-products (*S.* Anatum and *S.* Infantis) were resistant to the antifolate and nitrofuran classes. This suggests the need to use antibiotics cautiously in veterinary medicine and human to limit the resistance of these microorganisms to antimicrobials. For these, results may be related to the use of antibiotics in animal production and selection of multiresistant *Salmonella* spp. that persists in animal products.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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African Journal of Microbiology Research

Full Length Research Paper

Prevalence, risk factors and multidrug resistance profile of *Staphylococcus aureus* isolated from bovine mastitis in selected dairy farms in and around Asella town, Arsi Zone, South Eastern Ethiopia

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A cross-sectional study was carried out from November 2012 - May 2013 to estimate the prevalence of multi drug resistance *Staphylococcus aureus* from bovine mastitis and to assess its associated risk factors in selected dairy farms in and around Asella town, Arsi Zone, Ethiopia. A total of 384 fresh raw milk samples from dairy cows were examined for mastitis. Milk samples collected from 251 mastitic lactating cows were subjected to bacteriological examinations for isolation and identification of *S. aureus*. *S. aureus* isolates were identified from 44.62% (112/251) mastitic milk samples. Higher prevalence rates of *S. aureus* isolates were recorded in subclinical mastitis (45.78%, 103/225) compared to clinical cases (34.62%, 9/26), however, no statistically significant difference (p > 0.05) in prevalence of *S. aureus* between subclinical mastitis and clinical cases. Multivariate logistic regression analysis of the effect of different risk factors on the prevalence of *S. aureus* revealed that; cross breed (OR = 2.501, 95%CI: 1.173 - 5.334), late stage of lactation (OR = 4.260, 95%CI: 1.9309.402), previous mastitis record (OR = 2.553, 95%CI: 1.3324.894), large sized herd (OR = 15.824, 95%CI: 6.36839.320) and poor udder hygiene (OR = 2.040, 95%CI: 1.184,3.514) were more likely to be infected with antimicrobial resistance *S. aureus*. All the isolates of *S. aureus* were subjected to antimicrobial susceptibility testing. The highest rate of susceptibility was to chloramphenicol (97.5%) followed by gentamycin (95.3%), vancomycin (92.7%), and clindamycin (90.9%). Whereas, the highest rate of resistance among the isolates was against penicillin G (87.3%) followed by tetracycline (82.2%), trimethoprim-sulfamethoxazole (69.1%), oxacillin (56.4%), ampicillin (55.1%) and cefoxitin (58.1%). The results of the present study reveal that 65.18% of the isolates were found to be multiple antibiotic resistance phenotypes. Regular antimicrobial sensitivity testing and best practices for achieving hygienic milking should be established.

Key words: Milk, bovine mastitis, multidrug resistance, prevalence, risk factors, *Staphylococcus aureus*, Assella.

INTRODUCTION

Staphylococcus aureus is a truly diverse pathogen which is capable of causing a wide variety of illnesses in both humans and animals (Jensen and Lyon, 2009). It is a common cause of mastitis in dairy cows (Virgin et al., 2009; Sharma et al., 2015); a primary reason for antibiotic use on farms (Groot and van't Hooft, 2016). Aside from causing bovine mastitis, *S. aureus* may be implicated in mastitis in humans, wound infections, toxic shock syndrome, bacteremia, scalded skin syndrome, osteomyelitis and meningitis, among other syndromes (Kloos et al., 1995; Lowy, 1998). *S. aureus* is also a major cause of non-fatal food poisoning due to the production of highly stable extra cellular enterotoxins, which are powerful emetics (Genigeorgis, 1989; Leloir et al., 2003).

The emergence of pathogenic microorganisms resistant to commonly used antibiotics is a worldwide concern of the 21st century. Antibiotic resistance seems to be increasing, and multiple antibiotic resistant strains have started to emerge (Otter and French, 2010). Infections with antibiotic resistant bacteria have been known to be associated with frequent treatment failure and increased severity of the disease (Finch and Hunter, 2006; Swartz, 2004; Angulo et al., 2004). One of the most important bacteria in this regard is *S. aureus*, in particular its methicillin-resistant strains (Cosgrove et al., 2003; von Eiffet al., 2001). Both human and non-human antimicrobial usage may result in increased occurrence of bacterial resistance (Anderson et al., 2003). The problem with *S. aureus* became more complicated when it was found that it quickly developed resistance and was capable of producing many antibiotic resistant strains (Farzana et al., 2004; Cookson and Phillips, 1998). Development of resistance has been attributed to the indiscriminate and extensive use of antibiotics for therapeutic or as growth promoters in food animal production (Normanno, 2005). Isolates of *S. aureus* are frequently resistant to β-lactam antibiotics (Deurenberg and Stobberingh, 2008). The resistance to β-lactam antibiotics to *S. aureus* is mediated by the mecA gene which resides on a staphylococcal chromosomal cassette (SCCmec) that encodes a modified penicillin-binding protein (PBP), the PBP2a or 2', which shows reduced affinity to penicillins, such as methicillin and oxacillin and for all other β-lactam antibiotics (Kwon et al., 2006; Pantosti et al., 2007).

The description "methicillin-resistant *S. aureus* (MRSA)" was first used in 1961, based on the discovery of a human *S. aureus* infection in the United Kingdom that was resistant to methicillin (Fitzgerald et al., 2001).

Since that time, MRSA has emerged as a significant problem worldwide, and the term has evolved to include resistance to additional ß-lactam antibiotics. Currently, the term MRSA is often used to describe multi-drug resistant *S. aureus* (Leclercq, 2002; Garipcin and Seker (2015).

The first case of MRSA in veterinary species was identified in the milk of a cow with mastitis (probably of human origin) in 1972 (Duquette and Nuttall, 2004; O'Mahony et al., 2005). *S. aureus* is a significant cause of mastitis in cows and small ruminants (Vanderhaeghen et al., 2010; Unal et al., 2012). In dairy cows, *S. aureus* is a common bacterial cause of mastitis and MRSA is known to cause mastitis as well. Antimicrobial resistance has been detected in *S. aureus* isolates collected from bovine intramammary infection (IMI) at frequencies which vary widely by compound and region sampled. A seven-year survey of Michigan dairy herds found that 49.6% of the *S. aureus* isolates tested were resistant to penicillin and ampicillin, but that resistance to other compounds such as tetracycline, pirlimycin, erythromycin and oxacillin was low, ranging from 8.5% down to less than 1% (Erskine et al., 2002). *S. aureus*-related bovine mastitis is a common reason for therapeutic and/or prophylactic use of antibiotics on dairy farms (Vanderhaeghen et al., 2010; Kumar et al., 2010).

Methicillin resistant *S. aureus* is emerging as a zoonotic and veterinary bacterial pathogen of public health importance (Smith et al., 2009; Springer et al., 2009). It is a zoonosis that has garnered the attention of scientists and the public in recent years. Several reports suggest that animals may serve as reservoirs for Methicillin Resistant *S. aureus (*MRSA) infection of humans (Loeffler and Lloyd, 2010). The advent of antibiotic-resistant staphylococci poses additional potential food safety and occupational health concerns. Resistance genes of *S. aureus* can disseminate from animals to humans by direct contact or through the food chain (Kluytmans, 2010; Loeffler and Lloyd, 2010; Cuny et al., 2015).

The indiscriminate and intensive use of antibiotics in veterinary medicine might be associated risk factors attributed to the increasing occurrence of antibiotic resistant strains of *S. aureus* in cows with mastitis (Hawkey, 2003). Current management practices employed for milk production and individual cow factors might be other contributing factors associated with the dissemination of antibiotic-resistant bacterial strains (Acar and Moulin, 2006). The *staphylococci* have adapted to survive in the udder; they usually establish chronic, subclinical, infection and are shed in the milk which serves as a source of infection for other health cows during the milking process (Radostits et al., 2007). The

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main source of infection for *S. aureus* mastitis is the udder of infected cows which is transferred via milker's hands, utensils, towels and the environment (floor) in which the cows are kept (Radostits et al., 2007).

Antibiotics on dairy operations are used to treat highly prevalent infections, such as subclinical mastitis, and as a preventive measure during dry cow therapy (Zadoks et al., 2002). Monitoring the emergence of resistant pathogens in animal reservoirs is important particularly for those with zoonotic potential (Normanno et al., 2005). There is a lot of interest in recent years in bovine *S. aureus* resistance to multiple antibiotics because of comparison with MRSA (multiple/methicillin resistant *S. aureus* infections) in humans, including nosocomial infections in hospitals and nursing homes all over the world (Van Loo et al., 2007).

A number of reports indicated that multi drug resistant *S. aureus* is the predominant organisms isolated from bovine mastitis. Several studies have been conducted worldwide (Levy, 1998; Green and Bradley, 2004; Kumar et al., 2010; Wang et al., 2013) to investigate the prevalence of *S. aureus* in milk.

Recently, there have been several studies conducted on multidrug resistance profile of *S. aureus* isolated from bovine mastitis in various parts of Ethiopia (Sori et al., 2011; Daka et al., 2012; Abebe et al., 2013; Tassew et al., 2016). However, prevalence, risk factors and multidrug resistance profile of *S. aureus* isolated from bovine mastitis has been insufficiently investigated in the study area. Moreover, to date there is no published data on its status, magnitude and distribution in Arsi zone in general and in and around Asella town in particular. Therefore, the objective of this study was: (i) to isolate and identify *S. aureus* and establish its prevalence; (ii) to determine multi drug resistance profile of *S. aureus* isolates from mastitic cows' milk, and (iii) to assess potential risk factors associated with the prevalence of *S. aureus*.

MATERIALS AND METHODS

Study area

The study was conducted in and around Asella town of Arsi zone, Oromia Regional State, South Eastern Ethiopia. Asella is located at a distance of 175km south east of Addis Ababa at 7°57'N and 39°7'E with an altitude of 502 to 4130 m above sea level. The annual rainfall of the study area ranges from 200 to 400 mm with mean annual temperature of 22.5°C. Agricultural production system of the study area is mixed crop and livestock farming. Dairy farming using improved breeds is a common practice in and around Asella town. The study area is known by the abundance of dairy farms that constituted the known milk sheds (Land O'Lakes, 2010).

Study animals

The study animals were lactating dairy cows in and around Asella town. The breeds of animals were local zebu and zebu crossbred with Holstein-Friesian. The status of multidrug resistance profile of *S. aureus* isolated from bovine mastitis in and around Asella district

was unknown since no study had been conducted in the two districts before. The animals included in the study consisted of 384 lactating dairy cows, 86 (22.4%) indigenous zebu and 298 (77.8%) Holstien-zebu crosses (proportional allocation), selected by simple randomly from dairy farms in the study area. The farms were categorized in to large (> 10 dairy cattle), medium (5-10 dairy cattle) and small (<5 dairy cattle) according to the guideline of ILRI (1996).

Study design

A cross sectional type of study supported by laboratory tests was carried out to determine multi drug resistance profile of *S. aureus* isolates from mastitic cows' milk, to isolate and identify methicillin resistant *S. aureus* (MRSA) and to assess potential risk factors associated with the disease from October 2012 to May 2013 on dairy cows in and around Assella, Arsi Zone, South Eastern Ethiopia. Relevant individual animal biodata and farm level information were collected using a semi-structured questionnaire.

Sampling method and determination of sample size

The sampling was undertaken using a two level approach, choosing primarily individual farms with mastitis history and then sampling randomly individual cows from each farm. Greater proportions of cows (62%) were sampled from smallholder farms (small herd size) while the remaining (38%) were from medium and large herd size in and around the study district.

Since there was no reasonable research done in this area so far; the sample size was calculated by the formula recommended by Thrusfield (2007), with 95% confidence interval, at 5% desired absolute precision and expected prevalence of 50%. Hence, the total numbers of sample required for this study was 384 lactating dairy cows. Proportionality of incorporating cows in the sample was applied as per the population size of each herd.

Questionnaire survey of risk factors

Data was collected using a semi-structured questionnaire. The questionnaire was administered with the primary objective of elucidating the multifactorial background of mastitis. Data collected include intrinsic factors such as age, breed, parity, stage of lactation, previous history of mastitis and body condition. Extrinsic factors such as dry cow therapy, herd size, udder hygiene and floor type were also recorded.

Sample collection and bacteriological examination

Collection of milk samples

Milk samples were collected according to the National Mastitis Council, NMC (2004). Firstly, the quarters were thoroughly washed with clean water and wiped dry. Teats were then disinfected with 70% ethyl alcohol. Approximately 10 ml of raw milk was then collected aseptically from clinical and subclinical (CMT positive) mastitic cows into sterile universal bottles after discarding the first three milking streams. The samples were transported under cold chain to Asella regional diagnostic laboratory. The samples were then stored in ice at 4°C until cultured on standard bacteriological media (Quinn et al., 2004).

Clinical examination and California mastitis test

Clinical examination of the udder was based on the method previously indicated (Radostits et al., 2007). The clinical findings considered include abnormalities of the secretion, abnormalities of

the udder and teat, and systemic reaction. The California Mastitis test was performed according to previously established method (Quinn et al., 2004). It is used to determine the prevalence of sub-clinical mastitis and also as screening test for selection of samples to be cultured for the cows under study. A small milk sample (approximately ½ teaspoon) from each quarter was collected in to a plastic paddle that has 4 shallow cups marked A, B, C and D. An equal amount of California Mastitis Test reagent was added to the milk. The paddle was rotated to mix the contents. The CMT result was interpreted as negative (0), trace (T), weak positive (+1), Distinct positive (+2) and strong positive (+3) as per the recommendation given by Quinn et al. (2004).

Bacterial isolation and identification

The collected raw milk samples were cultured on 5% sheep blood and Mackonkey agar (Oxoid, UK) and the plates were incubated aerobically at 37°C and examined after 24 h of incubation for growth of bacterial colonies. The colonies were conditionally isolated based on Gram's stain reaction, cellular morphology, colony morphology, pigmentation and hemolytic feature on blood agar and other environment from which the bacterium were isolated. The representative colonies were sub cultured on nutrient agar (Oxoid, UK) and incubated at 37°C for 24 h (to obtain pure isolates for further identification). The isolated colonies from nutrient agar were subjected to catalase test, slide or tube coagulase test. Manitol salt agar, Purple Agar Base media plate with 1% of maltose and Voges Proskauer tests were done on the coagulase positive Staphylococci to identify *S. aureus* (Quinn et al., 2004; NMC, 2004). All of the bacterial isolates were cryopreserved in brain heart infusion broth (BHI, Becton, Dickinson and Company, Sparks, MD, USA) with 20% glycerol at -20°C for further analyses.

Antimicrobial susceptibility testing

The *S. aureus* isolates were subjected to antimicrobial susceptibility testing by Kirby-Bauer disc diffusion method (Carter and Chengappa, 1991; Quinn et al., 2004; CLSI, 2008). Antimicrobials of animal and human health significance were taken into consideration. Antimicrobial agents from various classes were employed. The following antibiotics (Oxoid, Hampshire, England) were used for testing: Ampicillin (10 µg), vancomycin (30 µg), gentamycin (10 µg), erythromycin (15 µg), clindamycin (10 µg), tetracycline (30 µg), oxacillin (1 ug), amoxacillin (25 µg), chloramphenicol (30 µg), trimethoprim-sulfamethoxazole (25 µg), cefoxitin (30 µg), and penicillin G (10 µg). In brief, the isolates were inoculated in tryptone soya broth (TSB) and incubated at 37°C for 24 h. The turbidity of the suspension was adjusted to obtain turbidity visually comparable with that of 0.5 McFarland standards. Muller-Hinton Agar (MHA) plate was prepared and a sterile cotton swab was dipped into the suspension and swabbed on the surfaces of Muller-Hinton Agar plate. Then, the antibiotic discs were placed on the agar plate using sterile forceps and pressed gently to ensure the complete contact with the agar surface. The plates were read 24 h after incubation at 37°C under aerobic condition. The inhibition zones of antimicrobial discs were recorded in millimeters, interpreted and classified according to procedures established by CLSI (2008) as susceptible, intermediate or resistance. Intermediate results were regarded as resistant (Huber et al., 2011). Multiple antibiotic resistant (MAR) phenotypes were documented for isolates revealing resistance to three and more antimicrobials (Rota et al., 1996).

Data analysis

All data from laboratory investigations and questionnaire survey were entered into computer using Microsoft Excel and transferred to STATA version 11.0 for Windows (Stata Corp. College Station, TX, USA) for analysis. Prevalence was calculated as a percentage value. The association between the explanatory and response variables was analyzed using the Chi-square test (χ2). Multivariate logistic regression analyses were used to analyze the effects of different potential risk factors on the prevalence of *S. aureus* mastitis. The independent or explanatory variables considered in the model were those that showed statistical significance (<0.2). Odds ratio (OR) was used to evaluate the degree of association between putative risk factors with prevalence of *S. aureus* mastitis. The 95% confidence interval and a p-value < 0.05 was considered statistically significant.

RESULTS

A total of 384 lactating dairy cows with either clinical or subclinical (CMT positive) mastitis were examined for the involvement of *S. aureus*. The overall prevalence of mastitis was 65.36% (251/384). Out of 251 mastitis positive cows, 6.78% (26/384) and 58.59% (225/251) were found to be clinical and subclinical mastitis, respectively. *S. aureus* was isolated from 34.62% (9/26) and 45.78% (103/225) of the clinical and sub-clinical cases, respectively. The overall prevalence of *S. aureus* was 44.62% (112/251). There was no statistically significant difference (p > 0.05) in prevalence of *S. aureus* between clinical and subclinical mastitis as indicated in Table 1.

A Chi-square analysis revealed that prevalence of *S. aureus* isolates was significantly associated with the age groups, breed and parity (p<0.05); stage of lactation (P<0.001), mastitis record (P<0.001), herd size (p<0.001), udder hygiene (P<0.01) and floor type (p<0.05) (Table 2).

Multivariate logistic regression analysis of the effect of different risk factors on the prevalence of *S. aureus* is presented in Table 3. Hence, multivariate analysis revealed that; cross breed (OR = 2.501, 95%CI: 1.173, 5.334), late stage of lactation (OR = 4.260, 95%CI: 1.930, 9.402), previous mastitis record (OR = 2.553, 95%CI: 1.332, 4.894), large sized herd (OR = 15.824, 95%CI: 6.368, 39.320) and poor udder hygiene (OR = 2.040, 95%CI: 1.184, 3.514) were more likely to be infected with *S. aureus.*

All the isolates of *S. aureus* were tested for antimicrobial susceptibility as illustrated in Table 4. Of the entire antibiotics used in this study, the highest rate of susceptibility was to chloramphenicol (97.5%) followed by gentamycin (95.3%), vancomycin (92.7%), and clindamycin (90.9%). Whereas, the highest rate of resistance among the isolates was against penicillin G (87.3%) followed by tetracycline (82.2%), trimethoprim-sulfamethoxazole (69.1%), oxacillin (56.4%), ampicillin (55.1%), and cefoxitin (58.1%). From the total isolates tested, 55.5% were susceptible, 4.7% intermediate and 39.8% resistance to antimicrobials discs.

Multiple antibiotic resistance phenotypes were determined for the *S. aureus* isolates as depicted in Table 5.

Table 1. *Staphylococcus aureus* isolated by the mastitis type.

Form of mastitis	No. positive samples	*S. aureus* (%)	P value
Clinical	26	9 (34.62)	
Subclinical	225	103 (45.75)	0.527
Overall	251	112 (44.62)	

Table 2. Chi-square analysis of intrinsic and managemental factors with prevalence of *S. aureus*.

Factor	Category	No. examined	No. positive	Prevalence (%)	P value
Age (years)	≤ 5	108	22	20.37	0.018
	> 5	276	90	32.61	
Breed	Local	86	14	16.28	0.003
	Cross	298	98	32.89	
Parity	Primiparous	55	9	16.36	0.024
	Multiparous	329	103	31.31	
Stage of lactation	Early (< 3months)	75	13	17.33	
	Mid (3-5 months)	203	43	21.18	0.000
	Late (> 5 months)	106	56	52.83	
Mastitis record	No	312	74	23.72	0.000
	Yes	72	38	52.78	
Herd size	Small	238	41	17.23	
	Medium	104	39	37.50	
	Large	42	32	76.19	0.000
Udder hygiene	Poor	156	57	36.53	0.009
	Good	228	55	24.12	
Floor type	Soil	258	84	32.55	0.036
	Concrete	126	28	22.22	

Table 3. Multivariate logistic regression analysis of associated risk factors with prevalence of *S. aureus* isolates.

Factor	Category	Positive (%)	COR (95%CI)	AOR (95%CI)	P value
Age (years)	≤ 5	22 (20.37)	1	1	0.127
	> 5	90 (32.61)	1.891 (1.112, 3.219)	1.848 (0.839, 4.071)	
Breed	Local	14 (16.28)	1	1	0.018
	Cross	98 (32.89)	2.520 (1.354, 4.691)	2.501 (1.173, 5.334)	
Parity	Primiparous	9 (16.36)	1	1	0.237
	Multiparous	103 (31.31)	2.329 (1.099, 4.938)	1.794 (0.682, 4.721)	
Stage of lactation	Early (< 3 months)	13 (17.33)	1	1	
	Mid (3-5 months)	43 (21.18)	1.282 (0.645, 2.546)	1.013 (0.471, 2.182)	0.000
	Late (> 5 months)	56 (52.83)	5.342 (2.628, 10.855)	4.260 (1.930, 9.402)	
Mastitis record	No	74 (23.72)	1	1	0.005
	Yes	38 (52.78)	3.595 (2.113, 6.114)	2.553 (1.332, 4.894)	
Herd size	Small	41 (17.23)	1	1	
	Medium	39 (37.50)	2.883 (1.713, 4.851)	2.786 (1.568, 4.951)	0.000
	Large	32 (76.19)	15.376 (7.008, 33.735)	15.824 (6.368, 39.320)	
Udder hygiene	Good	55 (24.12)	1	1	0.01
	Poor	57 (36.53)	1.811 (1.160, 2.827)	2.040 (1.184, 3.514)	
Floor type	Concrete	28 (22.22)	1	1	0.655
	Soil	84 (32.55)	1.690 (1.031, 2.770)	1.174 (0.581, 2.370)	

COR, Crude odds ratio; AOR, Adjusted odds ratio; CI, Confidence interval; 1, Reference.

Table 4. Antimicrobial resistance profiles of *S. aureus* isolated from mastitic milk (N = 112).

Antibiotics tested	Susceptible (%)	Intermediate (%)	Resistance (%)
Ampicillin	36.4	8.5	55.1
Vancomycin	92.7	-	7.3
Gentamycin	95.3	4.7	-
Erythromycin	69.5	12.7	17.8
Clindamycin	90.9	7.3	1.8
Tetracycline	16.8	-	83.2
Oxacillin	43.6	-	56.4
Amoxicillin	40.2	6.5	53.3
Chloramphenicol	97.5	2.5	-
Streptomycin	65.9	6.4	27.7
Trimethoprim-sulfamethoxazole	21.6	9.3	69.1
Cefoxitin	40.7	1.2	58.1
Penicillin G	10.9	1.8	87.3

Table 5. The predominant MAR phenotypes for *S. aureus* isolated from mastitic milk (N=112).

MDR pattern	Phenotypes	Number observed	Percentage
Three	PG-TE-STR	4	3.57
	PG-TE-Ox	21	18.75
Four	PG-TE-TMX-Ox	7	6.25
	PG-TE-CXT-Ox	6	5.36
Five	PG-TE-TMX-CXT-Ox	8	7.14
Six	PG-TE-TMX-CXT-AP-Ox	17	15.18
	PG-TE-TMX-CXT-AML-Ox	5	4.46
Seven	PG-TE-TMX-CXT-AP-Ox-E	2	1.79
Eight	PG-TE-TMX-CXT-AP-STR-Ox-VA	2	1.79
	PG-TE-TMX-CXT-AML-STR-Ox-DA	1	0.9

The predominant multiple antibiotic resistance phenotypes for the isolates in the study area were PG-TE-Ox and PG-TE-TMX-CXT-AP-Ox in 18.75 and 15.18% of the isolates, respectively. It is thus evident that MAR *S. aureus* were isolated from mastitic milk sample. Out of the total *S. aureus* isolates recovered from the study district, 65.18% of the isolates develop multiple antibiotic resistance phenotypes. Among all MAR phenotypes of *S. aureus* isolates, 52.05% of them were resistant to three or four antibiotics and 41.10% were resistant to five or six antimicrobials. Furthermore, 6.85% of them were resistance to seven or eight antibiotics.

Percentages of the phenotypes were calculated by dividing the number of a particular MAR phenotypes by the total number of isolates identified in the study area. PG, penicillin G; TE, tetracycline; Ox, oxacillin; AP, ampicillin; AML, amoxicillin; TMX, trimethoprim-sulfamethoxazole; CXT, cefoxitin; VA, vancomycin; E, erythromycin and DA, clindamycin.

DISCUSSION

The bacteriological examination done for this study using different techniques revealed an overall prevalence of *S. aureus* in bovine mastitic milk was 44.62%. This is comparable with previous findings of Workineh et al. (2002), Kerro and Tareke (2003), Mekibib et al. (2010) and Abo-shama (2014) who reported 39.2% *S. aureus* isolates at Addis Ababa, 40.3% at Southern Ethiopia and 47% from dairy farms of Holeta town, Ethiopia and 40% at Sohag governorate, Egypt, respectively. However, the present finding is higher than the reports of Abebe et al. (2013) who reported 15.5% at Addis Ababa. Furthermore, other studies undertaken in various corners of the country at different times disclosed lower prevalence rates than the present study (Sisay et al., 2012; Sori et al., 2011). On the contrary, higher prevalence rates than the current result was recorded in recent times from Ethiopia (Alemu, 2010; Gebremichael et al., 2013). The differences in the

prevalence rates of *S. aureus* in mastitic cows from various findings could be due to variability in farm management practices, breeds of targeted cows, level of production and variations in the study methods and materials employed by the investigators. Based on the observations made during the collection of samples, improper hygiene and poor farm management practices contributed to the high prevalence rates of *S. aureus* in the milk. *S. aureus* is a contagious pathogen which can spread from one animal to another or personnel by contact with cows during unhygienic milking procedures (Rowe, 1999).

Prevalence of *S. aureus* isolates was higher in subclinical mastitis, 45.78% (103/225) compared to clinical mastitis, 34.62% (9/26), however, with no statistical significant difference (P > 0.05) in prevalence of *S. aureus* between the two forms of mastitis. The predominance of *S. aureus* in subclinical mastitis than the clinical cases is similar with previous studies that proved *S. aureus* is the principal causative agent of subclinical mastitis (Radostits et al., 2007; Andrew et al., 2004; Garedew et al., 2015). Moreover, *S. aureus* has acclimatized to dwell in the teat resulting in chronic and subclinical diseases. From there it could release into the milk, which serves as a source of infection for healthy cows during the milking process (Radostits et al., 2007).

Current result revealed that prevalence of *S. aureus* isolates was significantly varied with the age categories and parity. Significant association of age and parity with mastitis was reported by other authors (Moges et al., 2011; Zeryehun et al., 2013). Cows with many calves (>7) have about 13 times greater risk (62.9%) of developing an udder infection than those with fewer (3) calves (11.3%) (Biffa et al., 2005). The increased prevalence of *S. aureus* in older animals in this study can be related to increased susceptibility of pathogenic organisms in udder relaxed sphincter muscles of teats. According to Erskine et al. (2002) primiparous cows have more effective defense mechanism than multiparous cows.

Higher prevalence rates of *S. aureus* were recorded in cross breed than local zebu. In multivariate logistic regression analysis, cross breed were identified as risk factors (OR = 2.501; p = 0.018). According to Radostits et al. (2007), this could be correlated with variations in anatomical and physiological features of the mammary gland, as well as high milk yielding of the cows. Furthermore, increase in milk yield from genetic selection might be related with genetic vulnerability to mastitis.

The results of the current study disclosed that incidence of *S. aureus* was significantly varied with stage of lactation. Late stage of lactation had shown to have a significant effect (p<0.001, OR=4.260, 95% CI: 1.930, 9.402) on the prevalence of *S. aureus* when compared to early stage. This finding was in harmony with the report of Abera et al. (2010) from Adama town. The current result might be due to the fact that chronic mastitis, most

often subclinical, is more frequent later during the lactation. *S. aureus* is a predominant cause of subclinical mastitis (Radostits et al., 2007; Garedew et al., 2015).

Cows with previous history of mastitis had higher *S. aureus* prevalence (P<0.001) compared to cows with no previous history of mastitis. Multivariate logistic regression analysis also showed significant effect of previous mastitis record (OR=2.553, 95%CI=1.332, 4.894, p<0.01) with prevalence of *S. aureus*. The present investigation was in agreement with the report of Abera et al. (2012). *S. aureus* is the most prevalent bacteria isolated from mastitis (Rall et al., 2013). The current result implies that treatment of cows for mastitis may not be effective in eradicating the pathogens and the disease may be carried over from previous lactations to the next. Moreover, investigation by Firaol et al. (2013) recorded antimicrobial resistant agents among pathogens which cause mastitis in Ethiopia.

The highest prevalence of *S. aureus* (76.19%) was observed in a large size herd categories followed by medium and small scale. Increase in prevalence with increased herd size was observed with highly significant association with prevalence of *S. aureus* (p<0.000). Significantly higher risk was observed in large sized herds (OR=15.824, 95%CI =6.368, 39.320) than corresponding herds. *S. aureus* have adapted to survive in the udder; known by their contagious nature and are shed in the milk which serves as a source of infection for other health cows during the milking process. It is generally observed that large herds are characterized by increased stocking density and increased risk of exposure to infection (Radostits et al., 2007).

Prevalence of *S. aureus* was significantly associated (p<0.01) with poor udder/teat hygiene. Poor udder hygiene (OR = 2.040, 95%CI: 1.184, 3.514) were more likely to be infected with *S. aureus*. The present observation was in line with the report of Mulugeta and Wassie (2013). Sanitary milking habits are important to avoid the spreading of bacteria or their proliferation. In this study, owners who did not wash teats before and after milking found to have high prevalence of *S. aureus* mastitis than owners who used to. Radostits et al. (2007) documented that udder preparation both before and after milking influence the spread of mastitis pathogens. The predominant source of the infection is the udder of infected cows transmitted through milker's hands, utensils, towels and the environment (floor) in which the cows are kept (Radostits et al., 2007).

The present study disclosed that cows kept in houses with soil floor had higher prevalence rates of *S. aureus* than those managed on concrete floor. Houses with soil floor increased the risk of *S. aureus* infection. The association between soil floor and high prevalence of *S. aureus* revealed in our finding was in agreement with the result of Abera et al. (2010). This could be attributed to the favorable environment created for survival and multiplication of mastitis bacterial pathogens. Cows that

were kept in dirty and muddy shelters favor the proliferation and spread of mastitis pathogens.

In vitro antimicrobial susceptibility patterns of *S. aureus* isolates revealed that highest rate of susceptibility among the isolates was recorded against chloramphenicol (97.5%) followed by gentamycin (95.3%), vancomycin (92.7%) and clindamycin (90.9%). Gizat (2004) also reported good efficacy of chloramphenicol (100%). Gebreyohanes (2008) reported susceptibility to chloramphenicol (84%). A result finding reported from South Africa showed that all of the isolates of *S. aureus* (100%) from two commercial farms were susceptible to vancomycin (Ateba et al., 2010). This antibiotic is no longer used for treatment of animal diseases in many countries (Pace and Yang, 2006), which might be contributed for the the current findings recorded. De Neeling et al. (2007) reported that the tested livestock - associated MRSA isolates were highly susceptible to most classes of antimicrobial drugs, except β-lactams and tetracyclines, the latter of which has been attributed to its high usage in animal husbandry.

On the other hand, *S. aureus* isolates of mastitic milk were most resistance among others to penicillin G (87.3%) followed by tetracycline (82.2%), trimethoprim-sulfamethoxazole (69.1%), cefoxitin (58.1%), oxacillin (56.4%), ampicillin (55.1%). The current investigation was in harmony with the report of Abebe et al. (2013) who recorded resistance of *S. aureus* to penicillin G and tetracycline found to be 94 and 73.8%, respectively, around Addis Ababa. Moreover, the present report was comparable with the result of Daka et al. (2012) who found resistance of *S. aureus* isolates to penicillin G (67.9%); ampicillin (67.9%); oxacillin (60.3%) and amoxicillin (30.8%). Gianneechini et al. (2002) recorded high resistance of *S. aureus* isolates against trimethoprim/sulphamethoxazole (90%, 100%), oxytetracycline (85%, 98%) and penicillin (87%, 94%). Alekish et al. (2013) reported 87.4% of the isolates were resistant against trimethoprim/sulphamethoxazole, 84.5% against penicillin, and 77.7% against oxytetracycline. The variability in susceptibility result could partly arise on how frequent a drug was in use in the study area. The beta-lactams, tetracycline, sulfonamides and some aminoglycosides have become the first line of antimicrobial agents used for treatment of bovine mastitis in Ethiopia. *S. aureus* are frequently resistant to other antibiotic agents in clinical use, including β-lactams, fluoroquinolones, aminoglycosides, rifampin, and mupirocin (Carbon, 2000). The resistance of *S. aureus* to penicillin G may be attributed to the production of beta lactamase enzyme that inactivates penicillin and closely related antibiotics. Resistance to penicillin G is used as a marker to assess the susceptibility of *S. aureus* isolates against other beta-lactam antibiotics (Waage et al., 2002; Pace and Yang, 2006). This correlates with the present findings. In the study area, the beta-lactams were the drugs of choice for therapy of intramammary infections,

such that frequent and often inadequate use of these medications has probably contributed to emergence of resistant bacteria in the herds. Similar finding was reported by Jaims et al. (2002) that the emergence of antimicrobial resistant strain is nearly always as a result of repeated therapeutic and/or indiscriminate use of them. Probably around 50% of mastitis caused by *S. aureus* strains produce beta-lactamase and there is evidence that these strains are more difficult to cure with all antibiotics (Levy, 1998; Green and Bradley, 2004).

It was observed that large percentages of cefoxitin (58.1%) resistant *S. aureus* were isolated from the study area. Disc diffusion testing using cefoxitin disc is far superior to most of the currently recommended phenotypic methods like oxacillin disc diffusion and is now an accepted method for the detection of MRSA by many reference groups including CLSI (Skov et al., 2003). Therefore, one can easily conclude that these are Methicillin resistant *S. aureus* (MRSA). CLSI recommends usage of cefoxitin instead of oxacillin when using the disk diffusion method to determine resistance against methicillin for *S. aureus* (CLSI, 2008). Significant proportion of *S. aureus* isolates were resistant to cefoxitin (58.1%), implying they were methicillin resistant *S. aureus* (MRSA). Resistance to cefoxitin is a marker for methicillin resistance *S. aureus* (Broekema et al., 2009). Methicillin resistant *S. aureus* is the term applied for any strain of *S. aureus* that has showed resistance to β-lactam antibiotics (de Medeiros et al., 2011; Swenson et al., 2009; Batabyal et al., 2012 and Zutic et al., 2012).

The antimicrobial susceptibility tests revealed that the isolates had the characteristics of general multidrug resistance pattern (penicillin, tetracycline, trimethoprim/ sulfamethoxazole, cefoxitin, oxacillin, ampicillin andamoxicillin). This is comparable with the report of Gebreyohanes (2008) and Huber et al. (2009). This could be attributed to the erratic and extensive use of antibacterial drugs without prior antimicrobial susceptibility testing. Such antimicrobial resistant organisms can pose serious health related hazards to animals as well as human beings. In recent times, an increasing antimicrobial resistance rate has been reported in *S. aureus* from bovine mastitis (Saini et al., 2012; Wang et al., 2013; Sharma et al., 2015).

Conclusions

The present study revealed that multidrug resistant *S. aureus* isolates is prevalent in dairy farms in the study area. Breed, stage of lactation, previous mastitis record, herd size and udder hygiene were found to be risk factors significantly related to *S. aureus* prevalence. It was observed that *S. aureus* isolates were highly sensitive to chloramphenicol (97.5%) followed by gentamycin (95.3%), vancomycin (92.7%) and clindamycin (90.9%). Whereas, the highest rate of resistance among the

isolates was against penicillin G (87.3%) followed by tetracycline (82.2%), trimethoprim-sulfamethoxazole (69.1%), oxacillin (56.4%), ampicillin (55.1%), and cefoxitin (58.1%). *S. aureus* from mastitic cows showed multidrug resistance to a great extent to commonly used antibiotics ensuring that the right use of antibiotics of choice is very important in line of treatment and control of the infections caused by *S. aureus*. Moreover, it is suggested that advanced molecular methods should be employed to characterize these isolates for the presence of antibiotic resistance determinants which may provide data to support conclusions.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Isolation of indigenous microorganisms from soil contaminated with metal scraps for the uptake of selected heavy metals in constituted growth media

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Indigenous bacteria and fungi were isolated from soil obtained from a metal scrap dumpsite in Ibadan, Nigeria. Soil sample analyses showed presence of calcium, iron, magnesium, zinc, manganese and copper. A selective isolation of microorganisms was done using heavy metal constituted growth media at concentration of 25 mg/L. *Lactobacillus casei* was isolated from cadmium and lead composed growth media, *Corynebacterium xerosis* and *Corynebacterium kutsceri* from nickel composed growth media. *Aspergillus niger* and *Histoplasma capsulatum* were isolated from growth media composed of nickel and lead respectively at a 25 mg/L concentration. Growths were observed for all isolates at 50mg/L, 100mg/L and 400mg/L composed growth media. A 7 day bio-treatment process with isolates for uptake of heavy metals from growth media solution at 50 mg/L concentration of heavy metal was done. *Histoplasma capsulatum, Aspergillus fumigatus* and *Aspergillus niger* reduced the heavy metal concentrations in lead, nickel and cadmium to 6, 18.12 and 12.45 mg/L respectively. *Lactobacillus casei, and C. xerosis* reduced the heavy metal concentration of cadmium, lead and nickel to 13.55, 33, 25, 22.38, 15.45 and 29.11 mg/L respectively. These microorganisms reduced the initial concentration of heavy metals and could thus be used for bioremediation processes.

Key words: Bioremediation, biotreatment, bacteria, fungi.

INTRODUCTION

Heavy metals refer to any metallic element that has relatively high density and at low concentration is toxic or poisonous (Lenntech, 2004). The major functions of a soil are its ability to protect water and air quality, sustain plant and animal productivity, and to promote human health (Doran and Parkin, 1994; Chen and Mulla, 1999). Heavy metals, include lead (Pb), cadmium (Cd), Zinc (Zn), mercury (Hg), arsenic (As), silver (Ag), chromium (Cr), copper (Cu), iron (Fe), and the platinum group elements while cadmium and lead are not readily absorbed by

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microorganisms (Kris, 2012).

Heavy metals occur as natural constituents of the earth crust, and are persistent environmental contaminants since they cannot be easily degraded or destroyed and can enter into the body system through food, air and water, and bio-accumulate over a period of time (Lenntech, 2004; United Nations Environmental Protection/Global Program of Action, 2004; Draghici et al., 2010). Cadmium is released as a by- product of zinc (and occasionally lead) refining; lead is emitted during its mining and smelting activities, from automobile exhausts (by combustion of petroleum fuels treated with tetraethyl lead antiknock) and from old lead paints while mercury is emitted by the degassing of the earth's crust (Lenntech, 2004).

With the surface dumping of the metals to air and rain, acid mine drainage (AMD) can be generated. When agricultural soils are polluted, these metals are taken up by plants and consequently accumulate in their tissues (Trueby, 2003). Animals that graze on such contaminated plants and drink from polluted waters, as well as marine lives that breed in heavy metal polluted waters also accumulate such metals in their tissues, and milk, if lactating (Habashi, 1992; Garbarino et al., 1995; Horsfall and Spiff, 1999; Peplow, 1999). Lead is the most significant toxin of the heavy metals, and the inorganic forms are absorbed through ingestion by food, water, and inhalation (Ferner, 2001).

It is evident that the presence of heavy metals in our environment poses a great threat to life processes in the soil. The contamination of heavy metals in our environment is an unavoidable occurrence as a result of human activities. Although certain naturally occurring processes such as phytoremediation by plants and microbial processes can reduce their concentrations, it is therefore of utmost importance to study and understand these microorganisms. It is important to also note that certain microbes have evolved over time, capable of utilizing these metals more efficiently as nutrient source. The objectives of the study are to determine heavy metals present in the soil sample, isolate and identify bacteria and fungi and use these microorganisms for the uptake of selected heavy metals from compounded medium of nutrients and heavy metals.

MATERIALS AND METHODS

Collection of sample

Soil samples contaminated with heavy metal were collected from a scrap yard well over 40 years located at Gate, Ibadan, Oyo state, Nigeria in May, 2016 and brought to the laboratory for analysis

Identification of heavy metals present in the soil sample

The calibration plot method was used for the analysis of heavy

metal concentration with the Atomic Absorption Spectroscopy (AAS). For each element, the instrument was auto-zeroed using the blank (distilled water) after which the standard was aspirated into the flame from the lowest to the highest concentration. The corresponding absorbance was obtained by the instrument and the graph of absorbance against concentration plotted. The samples were analyzed in duplicates with the concentration of the metals present being displayed in milligram per kilogram (mg/L) after extrapolation from the standard curve (Greenberg et al., 1985).

Isolation of fungi

This was carried out according to the method Joshi et al. (2011) with potato dextrose agar (PDA) containing 25 mg/L of Pb, Ni, Cr, and Cd, separately. The 1000 mg/L stock solutions of Pb, Ni, Cr and Cd were made in double distilled water using $Pb(NO_3)_2$, $NiCl_2 \cdot 6H_2O$, $CdCl_2$, and $K_2Cr_2O_7$. The stock solution of heavy metals were sterilized separately through bacteriological filters and added to sterilize PDA medium to reach a concentration of 25 mg/L.

Serial dilution of the sample was made up to 10^6 and 1 ml each of dilution 10^4 and 10^6 was added into sterilized Petri plates in duplicate. 20 ml of PDA medium containing 25 mg/L of, one of these heavy metals was poured in these Petri plates and incubated at 28°C for 72 h. The colonies of fungi were isolated and purified.

Identification of fungal isolates

The fungal isolates were identified according to Alexopoulus et al. (2002) by observing their morphology under x100 magnification of a compound microscope and their cultural characteristics.

Screening of fungal isolates for heavy metals tolerance

Further screening of the fungal isolates (25 mg/L) for heavy metals (Pb, Ni, Cr and Cd) tolerance were carried out using the following concentrations - 50, 100 and 400 mg/L in PDA medium. The fungal isolates alone were streaked on PDA medium and served as control. The plates were incubated for 72 h and afterwards observed for growth.

Isolation of bacteria

This was carried out according to the method by Joshi et al. (2011), using Nutrient agar (NA) containing 25 mg/L of Pb, Ni, Cr, and Cd separately. The 1000 mg/L stock solutions of Pb, Ni, Cr and Cd was made in double distilled water using $Pb(NO_3)_2$, $NiCl_2 \cdot 6H_2O$, $CdCl_2$, and $K_2Cr_2O_7$. The stock solution of heavy metals were sterilized separately through bacteriological filters and added to sterilized NA medium to make the concentration 25 mg/L.

A serial dilution of each sample was made up to 10^6 and 1 ml each of dilution 10^4 and 10^6 was added into sterilized Petri plates in duplicates. 20 ml of NA medium containing 25 mg/L of one of these heavy metals was poured in these Petri plates and incubated at 37°C for 48 h. The bacterial isolates were picked and purified.

Identification of bacterial isolates

Microscopic tests such as the Gram staining and spore staining tests were carried out. Some biochemical tests were also carried out on the isolates as described by Barrow and Feltham (1993) and identified following the Bergey's Manual of Determinative

Bacteriology according to Bergey and John (2000).

Screening of bacterial isolates for heavy metals tolerance

The further screening of bacterial isolates (25 mg/L) for heavy metals (Pb, Ni, Cr and Cd) tolerance were carried out using the following concentrations - 50, 100 and 400 mg/L in Nutrient Agar medium. The bacterial isolates alone were streaked on NA medium and served as control. The plates were incubated for 48 h at 37^{0}C and afterwards observed for growth.

Uptake of heavy metals in solution of growth media

This was done using the modified method Tsekova et al. (1998) by compounding the growth media for bacteria and fungi with specific heavy metals (cadmium, lead and nickel) of 25 mg/L. The solution was sterilized at 121°C for 15 min. Bacterial inoculum was incubated for growth at 37°C for 24 h and the fungal inoculum at 28°C for 72 h. The inocula were standardized with reference to the Mcfarland standard.

Statistical analysis

Statistical analysis was done using one way analysis variance (ANOVA) and significance difference accepted at P≤0.05.

RESULTS

10 metals were isolated from the soil sample obtained from the metal scrap dumpsite. Table 1 shows the analyses of the metals present in the soil sample which included calcium, iron, magnesium, nickel, cadmium, lead, zinc, magnesium and copper. The metal with the highest concentration was iron at 89,900.0 mg/L followed by magnesium - 12580.0 mg/L and calcium 9255.0 mg/L. Lead was 919.0 mg/L, copper 550 mg/L and manganese 535.0 mg/L. The metals with the lowest concentrations were cadmium and chromium at 0.60 and 0.20 mg/L, respectively.

Table 2 shows the bacteria isolated from the heavy metal constituted with nutrient media at 25 mg/L. *Lactobacillus casei* was isolated from cadmium constituted media and lead constituted growth media, *Corynebacterium xerosis* from lead and nickel constituted growth media while *Corynebacterium kutsceri* from nickel constituted media. The fungal isolates obtained at 25 mg/L of the respective heavy metals are shown in Table 3. The three fungi were *Aspergillus niger* from cadmium (CdF.1) which had septate hyphae with black spores and creamy white mycelium. The conidia were arranged in chains on the small vesicle with a row of phialides, *Aspergillus fumigatus* from nickel (N1F.1) and *H. capsulatum* from Lead (PbF.1), which was characterized by the presence of large, rounded and tubercula temacro conidia formed on short hyaline undifferentiated conidiophore with the mycelium showing white pink

Table 1. Analysis of metals present in the soil sample.

Metals	Concentration (mg/L)
Calcium	9255.00
Iron	89900.00
Magnesium	12580.00
Nickel	43.10
Cadmium	0.60
Lead	919.00
Chromium	0.20
Zinc	1065.00
Manganese	535.00
Copper	550.00

Table 2. Identification of bacterial isolates from selective growth media constituted with heavy metals at 25 mg/L.

Isolate code	Probable organism
Cd1	*Lactobacillus casei*
Pb1	*Lactobacillus casei*
Pb2	*Corynebacterium xerosis*
N1.1	*Corynebacterium xerosis*
N1.2	*Corynebacterium kutsceri*
N1.3	*Corynebacterium kutsceri*

Key: Cd1= Isolate from cadmium enriched media; Pb1-Pb2 = Isolates from lead enriched media; NI.1-NI.3 = Isolates from nickel enriched media.

coloration.

The Table 4 shows the analyses of the final heavy metal concentrations, obtained after a 7 day bio-treatment of 50 mg/L concentration of the heavy metal in Nutrient medium and Potato dextrose agar by the indigenous bacterial and fungal isolates, respectively. *H. capsulatum, A. fumigatus* and *A. niger* were observed to reduce the heavy metal concentrations of lead, nickel and cadmium to 6.00, 18.12 and 12.45 mg/L, respectively. The bacterial isolates – *L. casei* reduced the initial heavy metal concentration (50 mg/L) of cadmium to 13.55 and 33.00 mg/L, respectively. *C. xerosis* reduced the initial 50 mg/L concentration of lead to 25.00 and 22.38 mg/L, respectively while *C. kutsceri* also reduced the initial heavy metal concentration (50 mg/L) nickel to 15.45 and 29.11 mg/L, respectively. This shows the uptake of the heavy metals by the bacterial isolates.

The bio-treatment of lead using a co-culture of *L. casei* and *C. xerosis* reduced the initial 50 mg/L concentration to 32.5 mg/L while another co-culture bio-treatment of nickel using *C. xerosis* and *C. kutsceri* reduced the initial 50 mg/L concentration to 0.20 mg/L. At P≤0.05 there was

Table 3. Identification of fungal isolates from soil sample at 25 mg/L heavy metal concentration.

Isolate code	Description	Probable organism
CdF.1	Septate hyphae with black spores and creamy-white mycelium. The conidia were arranged in chains on the small vesicle with a row of phialides.	*Aspergillus niger*
N1F.1	Septate hyphae with brownish spores, the conidia were columnar with uniseriate heads, the conidiophores were conical shaped bore on a small vesicle. The mycelium was observed to be creamy-white	*Aspergillus fumigatus*
PbF.1	Characterized by the presence of large, rounded, tuberculate macro conidia formed on short hyaline undifferentiated conidiophores. The Mycelium showed a white-pink coloration.	*Histoplasma capsulatum*

Key: CdF.1: Cadmium enriched media; N1F.1: Nickel enriched media; PbF.1: Lead enriched media.

Table 4. Analysis of heavy metal concentrations after treatment using indigenous bacterial and fungal isolates.

Isolate code	Initial concentration of heavy metal before treatment (mg/L)	Final concentration of heavy metal (mg/L)	Reduction (%)
Lead			
PbF.1	50±0.007	6.00±0.0000[a]	88.00
Pb1,2	50±0.007	32.50±0.0707[b]	35.00
Pb1	50±0.007	33.00±0.1414[c]	34.00
Pb2	50±0.007	33.50±0.007[d]	33.00
Cadmium			
Cd1	50±0.007	13.55±0.007[b]	72.90
CdF.1	50±0.007	12.45±0.007[a]	75.10
Nickel			
N1F.1	50±0.007	18.12±0.007[c]	63.76
N1.1	50±0.007	22.38±0.007[d]	55.24
N1.2	50±0.007	15.45±0.007[b]	34.55
N1.3	50±0.007	29.11±0.007[e]	41.78
N1.1, 1.2, 1.3	50±0.007	0.20±0.007[a]	99.60

Key: a-e: superscripts indicating comparison of means; PbF.1= *H. capsulatum* used for biotreatment of lead constituted growth media; Pb1,2= *L. casei* and *C. xerosis* as a consortium for biotreatment of lead constituted media; Pb1 = *L. casei* used for biotreatment of lead constituted media; Pb2 = *C. xerosis* used for biotreatment of lead constituted media; CdF.1 = *A. niger* used for biotreatment of cadmium constituted media; Cd1 = *L. casei* used for biotreatment of cadmium constituted media ; N1F.1 = *A. fumigatus* used for biotreatment of nickel constituted media; N1.1, N1.2 and N1.3 = *C. xerosis* and *C. kutsceri* respectively used for biotreatment of nickel constituted media.

a statistical significant difference between the final concentrations observed in the bio-treatment of lead with bacterial and fungal isolates. Bio-treatment with *Histoplasma capsulatum* showed the highest reduction followed by *L. casei*, co-culture of *L.casei* and *C. xerosis* and lastly *C. xerosis*. At P≤0.05 there was a statistical significant difference between the final concentrations observed in the bio-treatment of cadmium with bacterial and fungal isolates.

Bio-treatment with *A. niger* showed the highest reduction followed by *L. casei*. At P≤0.05, there was a statistical significant difference between the final concentrations observed in the bio-treatment of nickel with the bacterial and fungal isolates. Bio-treatment with a co-culture of *C. xerosis* and *C. kutsceri* resulted in the highest reduction followed by *C. kutsceri*, (N1.2), *A. fumigatus, C. xerosis* and lastly *C. kutsceri* (N1.3).

DISCUSSION

Heavy metals can be found occurring naturally in the environment as they are by nature constituents of the environment. The indiscriminate use for human purposes

has altered their geochemical cycles and biochemical balance. This has however resulted in the excessive release of heavy metals such as cadmium, copper, lead, nickel, zinc etc. into natural resources like soil and even aquatic environments. A prolonged exposure and accumulation of such heavy metals can have deleterious health effects on human life and aquatic biota. The role of microorganisms in the biotransformation of heavy metals into non-toxic forms and the accumulation of heavy metals by bacteria has numerous applications for the bioremediation of heavy metal-contaminated sites (Ruchita et al., 2015).

Heavy metal ions are present in natural and industrial disposed wastewater. These metallic ions present on the surface and underground water resulted in soil contamination. Many conventional techniques have been employed to eliminate heavy metal ions including physical (membrane separation, ion exchange) and chemical (neutralization, precipitation) techniques (Yan and Viraraghavan, 2003). However, these methods are only efficient to eradicate mass of heavy metal present at high or moderate concentration but ineffective at diluted or low concentration of metal ions (Guibal et al., 1992).

According to Iram et al. (2013), the biotreatment of heavy metals would be possible with the aid of microorganisms (bacteria and fungi) isolated from metal contaminated soil. The isolation of *L. casei, C. xerosis* and *C. kutsceri* in this study, using cadmium, lead and nickel, respectively is supported by the study carried out by Sumaryati et al. (2015), who reported the bioremediation of cadmium with lactic acid bacteria. A review by Gosa (2015) reported species of *Corynebacterium* as, capable of the remediation process of heavy metals. Fungal isolates: *A. niger, A. fumigatus* and *H. capsulatum* were also isolated from growth media constituted with cadmium, nickel and lead, respectively which agrees with the results obtained from the study carried out by Barros et al. (2003), who reported the biosorption of cadmium using *A. niger.*

Iram et al. (2013) reported the potential of *A. fumigatus* in nickel bioremediation while Halttunen et al. (2007) reported the removal of cadmium by *L. casei* from 61.8±3.3 to 74.5±3.3% after 4 h of incubation and *L. fermentum* resulted in 81.2% reduction. This supports the finding of the study of cadmium reduction by *L. casei.* According to Halttunen et al. (2007) and Akhmetsadykova et al. (2013), there was a considerable reduction in the concentration of lead in solution after biotreatment with *L. casei*. Halttunen et al. (2007) reported up to 97% of lead removal from solution with an initial metal concentration of 100 and 1000 ug/L. The research findings indicate that bioremediation using screened indigenous microorganisms present in contaminated soil can reduce the concentration of heavy metals in given media. The research can be further implemented in a larger scale on site contaminated with heavy metals.

Conclusion

The isolation of authochthonous bacteria and fungi from soil contaminated with heavy metals showed that, these microorganisms may be capable of heavy metal bioremediation. The presence of some microbial species that are indigenous to the soil environment as revealed in this study shows adaptation of these organisms to the presence of pollutants such as heavy metals, and as a result have developed mechanisms to utilize them as part of their metabolism. Further studies needs to be carried out on the development of processes for the use of these species in the removal of heavy metals from the environment.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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