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Full Length Research Paper

Probiotic potential *Streptomyces* species from the grains of pearl millet (*Pennisetum glaucum*)

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Actinomycetes have been demonstrated for plant growth-promotion, antagonistic against plant pathogens and insect pests and biofortification traits in many agriculturally important crops. The present investigation was conducted to characterize probiotic properties of actinomycete(s) isolated from pearl millet flour and batter samples. A media selective and specific were used for isolation, actinomycetes isolation agar (AIA), and the most prominent actinomycete (found abundantly in the AIA plate) was isolated and maintained on AIA slants at 4°C for further investigation. The most prominent actinomycete was characterized for traits including Gram staining, morphology (such as color, margin, size, shape, elevation, form and surface), biochemistry (such as urease, catalase, oxidase, hydrogen sulphide, nitrogen reduction, gelatin liquefaction, starch hydrolysis and carbohydrate utilization), IMViC tests (such as indole, methyl red, Voges Proskauer and citrate utilization), probiotic potentials (such as acid [pH 2, 3], bile [0.5%], NaCl [6 and 9%], phenol tolerance [0.4%]), antibiotic tolerance (such as tetracycline, streptomycin, kanamycin, chloramphenicol, ciprofloxacin, ampicillin, penicillin, erythromycin and vancomycin) and antimicrobial activities against human pathogens (such as *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhi*). It was possible to isolate only one probiotic actinomycete based on the properties. The sequences of 16s rDNA gene of the actinomycete was matched with *Streptomyces* species in BLAST analysis. The sequences of the *Streptomyces* spp. were submitted to NCBI and accession number obtained. This study indicated that the selected *Streptomyces* spp. could be used to develop new probiotic foods.

Key words: Probiotics, pearl millet, *Streptomyces* species, product development.

INTRODUCTION

Nutritional quality of food is important not only for maintaining human health but also for physical well-being and this is attained mainly by eating cereals. Wheat and rice are the important staple food for people across the world that lead not only to an array of emerging life style diseases but also challenging nutrition and human health. Hence, there is an urgent need for recommending diversity in diets through inclusion of other

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cereals such as millets in order to enhance the nutritional status and address malnutrition across the world. Millets are not consumed widely as a staple, except by people inhabiting the semi-arid tropics in Asia and Africa, though these are widely used as animal or bird feed. Pearl millet (*Pennisetum glaucum*) is one of the important foods for people living in Asia and Africa, being a major source of calories and a vital component of food security (Amadou et al., 2013). Pearl millet is well-adapted to cultivation systems characterized by high temperature, low rainfall (below 600 mm), low soil fertility and resistance to pests and diseases and thus is suited to the semi-arid tropics. It is often ground into flour and consumed as porridge, roti and beverages (Obilana and Manyasa, 2002; Amadou et al., 2011). It also serves as the source of prebiotics (such as total oligosaccharides, resistant starch, total dietary fiber and β-glucan) for functional food (Awika and Rooney, 2004; Ige, 2013). Any food or food ingredients in association with probiotic microbes either influence beneficial effect on the host or reduce the risk of chronic diseases are referred as functional foods (Huggett and Schliter, 1996; Charalampopoulos et al., 2002; Hassan et al., 2014). Probiotic foods normally contain a single or mixture of probiotic microorganisms that improve the health of the host by improving intestinal microbial balance (Fuller, 1989). Probiotic microbes are being used for preparation of dairy food for thousands of years. Recently, non-dairy based probiotic drinks utilizing pearl millet are also reported (Syal and Vohra, 2014; Mridula and Sharma, 2015). Probiotic microbes associated with cereals such as wheat, rice and sorghum are reported widely but not much information is available for pearl millet cultivars (Badau, 2006).

Actinomycetes are a group of Gram-positive bacteria, with high G + C content belonging to the order Actinomycetales, found commonly in compost and rhizospheric soil. They play an important role, not only on plant growth-promotion (PGP), antagonistic action against pathogens and insect pests and biofortification traits in many agriculturally important crops, but also on decomposition of organic materials and production of secondary metabolites (Glick, 2010). Plant growth-promoting properties of actinomycetes was reported on cereals (such as wheat (Sadeghi et al., 2012), rice (Gopalakrishnan et al., 2014) and sorghum (Gopalakrishnan et al., 2013)) as well as legumes (such as bean (Nassar et al., 2003), chickpea (Gopalakrishnan et al., 2015, 2016a), pigeonpea (Gopalakrishnan et al., 2016b) and pea (Tokala et al., 2002). Actinomycetes have also been demonstrated for biocontrol of soil-borne pathogens (Mahadevan and Crawford, 1997; Trejo-Estrada et al., 1998; Macagnan et al., 2008; Gopalakrishnan et al., 2011) and insect pests (Gopalakrishnan et al., 2016c; Sathya et al., 2016 a, b). However, not much information on the usefulness of actinomycetes on prebiotics is available. Therefore, in the present investigation, an effort has been made to characterize probiotic properties of actinomycete(s) from flour and batter samples of pearl millet cultivars.

MATERIALS AND METHODS

Reference bacteria used

Reference human pathogens used in this study, *Salmonella typhi* (ATCC 14028), *Staphylococcus aureus* (ATCC 6538) and *Escherichia coli* (ATCC 25922), were acquired from ATCC, USA. Reference plant pathogens used in this study, *Fusarium oxysporum* f. sp. *ciceri* (FOC), *Rhizoctonia bataticola* (RB-6, RB-24 and RB-115), *Sclerotium rolfsii*, *Botrytis cinerea*, *Macrophomina phaseolina*, *Fusarium proliferatum* -242 and *Fusarium andiyazi* were acquired from Plant Pathology unit at ICRISAT-Patancheru, India.

Collection and preparation of pearl millet grain samples

A total of 2 pearl millet grain varieties including dual purpose hybrid (DPH) and high Fe hybrid were grown at ICRISAT-Patancheru and used for this study. The grain samples were dried at 30 to 32°C for 72 h and milled in Cyclotech^{TM}- Mill. The flour were sieved through 0.2 mm sieve and mixed (5 g) in sterilized water (5 ml) and further incubated at 28°C for 12 h. At the end of incubation, the batter samples were used for isolating the actinomycetes.

Isolation of actinomycete(s)

Ten grams of flour/batter sample was separately suspended in 90 ml of sterilized physiological saline (0.85% of NaCl in distilled water) in a flask and placed on an orbital shaker (at 120 rpm) for 45 min. At the end of shaking, the samples were serially diluted (up to 10^6 dilutions) with physiological saline. Dilutions 10^4 to 10^6 were spread plated (0.1 ml) on actinomycetes isolation agar and incubated at 28°C for 72 h. The most prominent actinomycete colonies, were found abundantly in the AIA plate, isolated and maintained on AIA slants at 4°C for further studies.

Morphological and biochemical properties of the actinomycetes

The actinomycete isolates were streaked on AIA plate and incubated at 28°C for 72 h. At the end of incubation, the colonies were observed for its morphological traits including form, surface, texture, color, elevation and margin. Gram staining of the isolates was done as per the protocols of Pelczar et al. (2008). The isolates were further characterized for their biochemical properties including hydrogen sulphide, urease, catalase, oxidase, nitrate reduction, gelatin liquefaction, starch hydrolysis and IMViC (Indole, Methyl red, Voges Proskauer and Simmons citrate) tests as per the methods of Holt (1984). Utilization of carbohydrates including lactose and sucrose were determined as per Forouhandeh et al. (2010).

Antibiotic resistance pattern of the isolates was conducted by disc diffusion method. The resistance or susceptibility to antibiotics of actinomycete isolates to ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (10 µg), erythromycin (15 µg), kanamycin (30 µg), penicillin (10 µg), streptomycin (10 µg), tetracycline (30 µg) and vancomycin (10 µg) (HiMedia, Mumbai, India) were determined as per the guidelines of Clinical and Laboratory Standards Institute (Wilker, 2006). In brief, antibiotic discs were placed on the AIA plates immediately after swab with actively grown actinomycete isolates. The plates were incubated at 28°C for 72 h. At the end of incubation, zone of inhibition was measured.

Antimicrobial activity of the actinomycetes

The antimicrobial activity of the actinomycete isolates against human pathogens such as *E. coli*, *S. aureus* and *S. typhi*, was done by the ditch assay method (Aswathy et al., 2008) with slight modifications. In brief, the isolates were grown in Muller-Hinton broth at 28°C for 72 h. At the end of incubation, the culture filtrates were collected by centrifuging at 10,000 *g* for 20 min, concentrated on a rotary evaporator until one fifth of the original volume and filter-sterilized through 0.2 µm membrane filter. The pathogens grown separately on nutrient broth at 37°C for 24 h were amended with sterilized nutrient agar (2%) at 45°C and poured on Petri plates. Upon solidification, a ditch (6 mm) was cut in the Petri plate and filled with filter sterilized culture filtrate of actinomycete isolates (0.2 ml). The plates were initially placed at 4C for 1 h, for diffusion of metabolites present in the culture filtrate, and further incubated at 37°C for 18 h. At the end of incubation, zone of inhibition was measured.

Probiotic traits of the actinomycetes

The isolates were characterized for their probiotic traits including tolerance to acid (low pH), bile salt, phenol and NaCl. Acid tolerance was done, for identifying the actinomycetes which could tolerate simulated gut acidic conditions, as per the protocols of Liu et al. (2007) and Kunchala et al. (2016). Bile tolerance test was done as per the methods of Aswathy et al. (2008) and Kunchala et al. (2016) by checking the growth of actinomycetes in brain heart infusion (BHI) broth amended with various concentrations (0.3, 0.5 and 0.8%) of bile salt. NaCl tolerance was done as per the methods of Graciela and Maria (2001) and Kunchala et al. (2016) in Muller-Hinton broth (MHB) adjusted with different concentration of NaCl (3, 6, 9, and 12%). The phenol tolerance of the actinomycete isolates was assessed using the protocols of Teply (1984) and Kunchala et al. (2016) in MHB containing 0.2 and 0.4% of phenol.

Plant growth-promoting (PGP) and biocontrol traits of the actinomycetes

The actinomycete isolates were evaluated for their PGP and biocontrol traits including indole acetic acid (IAA), siderophore, lipase, cellulase, chitinase and hydrocyanic acid (HCN) production. Estimation of IAA and siderophore productions was done as per the protocols of Patten and Glick (2002) and Schwyn and Neilands (1987), respectively. The lipase and cellulase productions were estimated as per the standard protocols of Bhattacharya et al. (2009) and Hendricks et al. (1995), respectively. Chitinase production was estimated by amending agar plates with colloidal chitin suspension and mineral salts according to the protocols of Hsu and Lockwood (1975). HCN was qualitatively assessed by the method described by Lorck (1948). The rating scales for lipase, cellulase and chitinase were as follows: 0 = no halo zone; 1 = halo zone of 1-10 mm; 2 = halo zone of 11-20 mm; 3 = halo zone of 21-30 mm; 4 = halo zone of 31-40 mm; and 5 = 41-50 mm. The following rating scale was used for HCN production: 0 = no color change, 1 = light reddish brown, 2 = medium reddish brown, and 3 = dark reddish brown.

The actinomycete isolates were also screened for their antagonistic potential against selected plant pathogens of chickpea (including *R. bataticola* [three strains, viz., RB-6, RB-24 and RB-115], *S. rolfsii*, *B. cinerea* and *F. oxysporum* f. sp. *ciceri* [FOC]) and sorghum (*M. phaseolina*, *F. proliferatum*-242 and *F. andiyazi*) by dual culture assay (Gopalakrishnan et al., 2011) and the zone of inhibition measured.

Molecular identification of the actinomycetes

Pure cultures of probiotic potential actinomycete isolates were grown in starch casein broth until log phase and genomic DNA isolated as per the standardized protocols of Bazzicalupo and Fani (1995). The amplification of 16S rDNA gene was done using universal bacterial primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3') and 27F (5'-AGAGTTTGATCMTGGCTC AG-3') (Pandey et al., 2005). The PCR product was sequenced at Macrogen Inc., Seoul, South Korea. The sequences were compared with those from GenBank using the BLAST program (Alschul et al., 1990), aligned using the ClustalW software (Thompson et al., 1997) and phylogenetic trees inferred using the neighbor-joining method (Saitou and Nei, 1987). The sequences of potential probiotic actinomycete were submitted to NCBI and the GenBank accession number was obtained.

RESULTS

Isolation, morphological and biochemical characterization of the actinomycete

The most prominent isolate, PDPF-21 (which was found abundantly in the actinomycetes isolation agar (AIA); selective and specific media for isolation of actinomycete), was isolated from the fermented batter samples of pearl millet and maintained on AIA slants at 4°C for further studies. PDPF-21 was found to be circular in form, punctiform in size, rough in surface, dry in texture, white in color, umbonate in elevation and entire in margin in the morphological studies. It was found to be Gram positive in the Gram staining test. When characterized for biochemical traits, it was found positive for urease, catalase, Vogues Proskauer and sucrose utilization tests but negative for hydrogen sulfide, oxidase, nitrate reduction, gelatin liquefaction, starch hydrolysis, indole, methyl red, citrate utilization and lactose utilization tests (Table 1).

Antibiotic resistance pattern and antimicrobial activity of the actinomycete PDPF-21

The selected actinomycete PDPF-21 was found resistant to tetracycline (at 30 µg), streptomycin (at 10 µg), kanamycin (at 30 µg), chloramphenicol (at 30 µg), ciprofloxacin (at 10 µg), ampicillin (at 10 µg), penicillin (at 10 µg), erythromycin (at 15 µg) and vancomycin (at 10 µg). PDPF-21 was also found to have antagonistic properties against human pathogens including *E. coli*, *S. aureus* and *S. typhi*, as it significantly inhibited all three pathogens (more than 10 mm inhibition zone), of which the strongest antagonistic activity was noted against *E. coli* (Table 2).

Probiotic traits and molecular identification of the actinomycete PDPF-21

When PDPF-21 was tested for its probiotic properties, it

Table 1. Morphological and biochemical characterizations of the actinomycete isolate PDPF-21.

Morphological traits		Biochemical traits		Carbohydrate utilization traits	
Form	Circular	Hydrogen sulfide test	-	Lactose test	-
Size	Punctiform	Urease test	+	Lactose gas production test	-
Surface	Rough	Catalase test	+	Sucrose test	+
Texture	Dry	Oxidase test	-	Sucrose gas production test	=
Color	White	Nitrate reduction test	-		
Elevation	Umbonate	Gelatin liquefaction test	-		
Margin	Entire	Starch hydrolysis test	-		
Gram staining	+	Indole test	-	-	
		Methyl red test	-		
-		Vogues Proskauer test	+		
		Citrate utilization test	-		

Table 2. Antibiotic resistance pattern and antimicrobial activity of the actinomycete isolate PDPF-21.

Antibiotic resistance pattern (zone of inhibition in mm)		Antimicrobial activity (zone of inhibition in mm)	
Tetracycline (30 µg)	34	*Escherichia coli*	18
Streptomycin (10 µg)	30	*Staphylococcus aureus*	11
Kanamycin (30 µg)	31	*Salmonella typhi*	14
Chloramphenicol (30 µg)	31		
Ciprofloxacin (10 µg)	32		
Ampicillin (10 µg)	14	-	
Penicillin (10 µg)	17		
Erythromycin (15 µg)	22		
Vancomycin (10 µg)	29		

Table 3. Probiotic properties, identity and NCBI accession number of the actinomycete isolate PDPF-21.

Probiotic properties		Identification by 16S rDNA analysis	NCBI accession number
Acid tolerance (pH)	2	*Streptomyces* spp.	KP326565
Bile tolerance (%)	0.5		
Phenol tolerance (%)	0		
NaCl tolerance (%)	6		

was found to tolerate acidic pH (pH 2), bile (0.5%) and NaCl (6%) but not phenol (Table 3 and Figure 1). A neighbor-joining dendrogram was prepared using the sequences of PDPF-21 (1400 bp) and representative sequences from the databases. Phylogenetic analysis of sequences of the PDPF-21 matched with *Streptomyces* species (Table 3 and Figure 1).

Plant growth-promoting (PGP) and biocontrol properties of PDPF-21

The actinomycete PDPF-21 produced PGP and biocontrol traits including IAA, siderophore, lipase, cellulase, chitinase and HCN. In the dual culture assay, PDPF-21 inhibited plant pathogens of chickpea including three strains of *R. bataticola* (RB-6, RB-24 and RB-115), *S. rolfsii*, *B. cinerea* and FOC and in sorghum, *M. phaseolina*, *F. proliferatum*-242 and *F. andiyazi*-943 (Table 4).

DISCUSSION

Probiotic foods help the existing microflora to stabilize or repopulate the microflora in the colon, lost due to disease, antibiotics and/or chemotherapy. Pearl millet is one of the important staple food for millions of poor

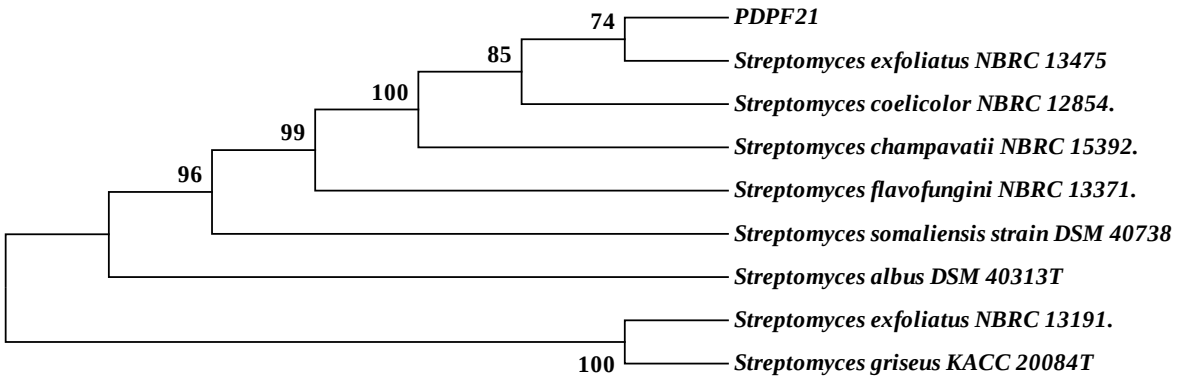

Figure 1. Phylogenetic relationship between PDPF-21 and representative species based on full length 16S rDNA sequences constructed using the neighbor-joining method.

Table 4. Plant growth-promoting (PGP) and biocontrol traits and antagonistic (against important pathogens) properties of the actinomycete isolate PDPF-21

PGP and biocontrol traits		Antagonistic properties (zone of inhibition in mm)	
Indole acetic acid (µg/ml)	0.06	*Rhizoctonia bataticola* -6	4
Siderophore (% units)	0.50	*Rhizoctonia bataticola* -24	4
Lipase (rating)	4	*Rhizoctonia bataticola* -115	1
Cellulase (rating)	2	*Macrophomina phaseolina*	4
Chitinase (rating)	3	*Sclerotium rolfsii*	3
Hydro cyanic acid (rating)	2	*Botrytis cinerea*	3
		Fusarium oxysporum f. sp. *ciceri*	4
-		*Fusarium proliferatum* -242	4
		Fusarium andiyazi -943	4

The rating scale for lipase, cellulase and chitinase were as follows: 0 = no halo zone, 1 = halo zone of 1-10 mm, 2 = halo zone of 11-20 mm, 3 = halo zone of 21-30 mm, 4 = halo zone of 31-40 mm, 5 = 41-50 mm. For hydro cyanic acid production, the following rating scale was used: 0 = no color change, 1 = light reddish brown, 2 = medium reddish brown, 3 = dark reddish brown.

people in Africa and Asia as it contains high carbohydrate energy and nutrition, thus making it useful component of dietary and nutritional balance in human food and animal feed. Foods from pearl millet are reported to be rich in phytochemicals (such as phytic acid and phytates) and generate vitamins, fatty acids and other vital nutrients that improve body's resistance against pathogens, lower cholesterol and reduce the risk of cancer (Coulibaly et al., 2011; El-Salam et al., 2012). In the present study, pearl millet flour and batter samples were explored for specifically isolating and characterizing probiotic actinomycetes.

It is widely accepted that one of the important criteria for isolating and/or selecting probiotic potential bacteria should be its ability to inhibit human pathogens and resistance to broad range of antibiotics. In the present investigation, the selected actinomycete PDPF-21 was found to inhibit all the tested pathogens, including *E. coli*, *S. aureus* and *S. typhi* and exhibited resistance to tetracycline, streptomycin, kanamycin, chloramphenicol, ciprofloxacin, ampicillin, penicillin, erythromycin and vancomycin in the antibiotics resistance pattern (Table 2). The main aim of using probiotic strains should be to beneficially affect the gut microbial composition and functionality. Oluwajoba et al. (2013) reported similar results on lactic acid bacteria (LAB). The same authors noted that LAB, isolated from millet grains and fermented products, inhibited human pathogens such as *S. aureus* 25923, *E. coli* 25922, *Pseudomonas aeruginosa* 27853 and *Enterococcus faecalis* 29212. Probiotic potential bacteria such as *Lactobacillus fermentum*, *Bifidobacterium* species and *Weissella confuse* were also found to help in preventing and treating acute diarrhea (Lei and Jakobsen, 2004). It is concluded that the selected actinomycete PDPF-21 has good antagonistic potential against important human pathogens and antibiotic resistance patterns.

Perhaps, the most important selection criteria for

isolating and identifying suitable probiotic microbe should be their ability to survive in acidic environment of the final fermented product and the adverse conditions of the gastrointestinal tract. Hence, in the present investigation, the actinomycete PDPF-21 was characterized for its probiotic properties including acid tolerance, bile tolerance, phenol tolerance and NaCl tolerance and found to tolerate acidic pH (pH 2), bile (up to 0.5%) and NaCl (up to 6%) but not phenol (Table 3). Therefore, it is concluded that the selected actinomycete has the desirable properties in order to qualify as a probiotic.

In order to determine the identity of the selected actinomycete, its 16S rDNA was sequenced and analyzed. A neighbor joining dendrogram generated using the sequence from the selected actinomycete (1400 bp) and representative sequences from the databases revealed that it belonged to *Streptomyces* spp. Pearl millet flour has been used for isolation of LAB including *Lactobacillus plantarum*, *Lactobacillus cellobiosus*, *Lactobacillus pentosus*, *Leuconostoc mesenteroids*, *Bacillus subtilis*, *Pedicoccus pentosaceus*, *Streptococcus lactis* and *Torulopsis glabrata* (Badau, 2006; Kamgar et al., 2013; Okoronkwo, 2014). Microbes such as *Lactobacillus*, *Bifidobacterium* and *Weissella* spp. are reported widely as probiotic microbes. Mridula and Sharma (2015) reported a non-dairy probiotic drink from the sprouted cereals, legumes and soy milk using LAB. *Lactobacillus acidophilus* was used to ferment a food mixture containing sorghum flour, whey powder and tomato pulp (Jood et al., 2012). However, to our knowledge, there are no reports till date on any actinomycete, *Streptomyces* spp., showing probiotic potential. Therefore, the selected actinomycete *Streptomyces* spp. can be exploited for development of functional foods. *Streptomyces* spp. have been widely reported and demonstrated under field conditions as plant growth-promoters and biocontrol agents in both cereals and legumes (Tokala et al., 2002; Nassar et al., 2003; Sadeghi et al., 2012; Gopalakrishnan et al., 2013, 2014, 2015, 2016a, b, c). In the present investigation, when the *Streptomyces* spp. PDPF-21 was evaluated for its PGP and biocontrol traits, it was found to produce IAA, siderophore, lipase, cellulase, chitinase and HCN and inhibit many plant pathogens of chickpea and sorghum (Table 4). Hence, it is concluded that the selected *Streptomyces* spp. PDPF-21 is having not only probiotic properties but also plant growth-promoting and biocontrol properties.

Conclusion

Pearl millet is not only a valuable source of prebiotics and bioactive compounds (such as resistant starch, total oligosaccharides, total dietary fiber and β-glucan), useful for development of functional foods, but also source of probiotic microbial cultures, which should be exploited for the production of new and innovative functional foods. The multiple beneficial effects of pearl millet can be used in association with probiotic microbes isolated from pearl-millet in designing novel cereal-based functional foods targeting different consumer segments having specific health requirements. The present work has identified a probiotic actinomycete strain from pearl millet that can be exploited for designing novel cereal based functional foods for addressing food and nutritional security for millions of malnourished people living in the poor countries of Africa and Asia. Probiotic foods obtained using a multiple microbial strains provide the opportunity to develop innovative food products with enhanced flavor, taste and texture based on consumer preference, unlike single strain based products that are usually sour and acidic in taste (Saarela et al., 2000). This study, thus, gives an opportunity to further explore the possibility of amending the selected actinomycetes with other known probiotic strains and/or exploring more probiotic actinomycetes towards development of functional foods.

CONFLICT OF INTERESTS

The authors have not declared any conflicts of interests.

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Potential of *Streptomyces* sp. and *Trichoderma* sp. as compost microbiota for coffee husk

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About 70 to 80% of the coffee berries husk end up as agricultural waste after processing. Its neglect or improper utility can cause environmental pollution. The present study investigated the efficiency of *Streptomyces, Trichoderma harzianum* and the combination of both as bio-degradable agents of coffee husk which is regarded as agricultural waste. Identification of their efficiency is necessary to stimulate further research on their probable utility in converting huge mass of coffee husk to organic manure for soil amendment and higher crop productivity. Coffee husk from the coffee processing unit of the Cocoa Research Institute of Nigeria (CRIN), Ibadan was the substrate for the isolates of Streptomyces, *Trichoderma* and Streptomyces/*Trichoderma*. Culturing was observed for 0, 15 and 30 days. The factorial in completely randomized design experiment had ten treatment combinations (control inclusive) of three replications. Significant ($P < 0.001$) variation existed among the three periodic days for all the generated biochemical products. The three organisms and the interaction between organisms and days differed significantly ($P \leq 0.05$) for moisture content, dry matter content, phosphorus content and the pH. The production trend of most of the biochemical as aided by *Streptomyces* was both linear and quadratic for the three days. Caffeine, tannin, phosphorus, potassium and dry matter content were significantly highest in the control. Wricke ecovalence was least for the production of moisture content, phosphorus, dry matter, tannin and pH in the treatment which combines the two organisms. For the production of biochemical from the incubated substrate, the nine treatments significantly differed from the control denoting that microorganisms are needed in organic matter decomposition process.

Key words: Agricultural waste, mineralization, compost, microorganisms, recycling.

INTRODUCTION

Careless assemblage and neglect of proper disposal of agricultural wastes can lead to environmental problems such as pollution, blockage of drainage and water ways and hence flooding. The challenge has also resulted in out-break of epidemics. Yahaya and Ibrahim (2012) included rice husk, groundnut shells and coffee husk

among the major agricultural wastes in Nigeria. Coffee husk is rich in lignocelluloses materials, which makes it an ideal substrate for microbial processes. Among the attempts for coffee husk utilization are: production of biogas, enzymes, mushroom and compost (Pandey et al., 2000; Neves et al., 2006; Dias et al., 2010).

The projected high coffee production for the future would only be sustainable if the policy programme for the production would incorporate the proper disposal, recycling and use of the resulting residues (Murthy and Naidu, 2012). Coffee husk and coffee pulp have been usually incubated for composting as the recycling of organic wastes in agriculture. Fan et al. (2003) remarked that coffee husk has high tannin and phenolic compound content which makes degradation of the material slow in nature; hence, its direct release into the environment could inhibit plant root growth and increase in greenhouse gas emissions through anaerobic decomposition.

Bidappa et al. (1998) asserted that the nutritional quality and environmental safety of compost with coffee husk base was improved when in mixture with animal manures and rock phosphate. A summary of the derivable importance of compost generated from coffee husk and other agricultural wastes according to Westerman and Bicudo (2005) are: reduction in wastes of natural resources, environmentally save recycling of nutrients, increase of soil organic matter and improvement of the physical, chemical and biological characteristics of soils.

Aerobic and anaerobic microorganisms produces extra cellular enzymes capable of degrading macromolecules like starch, cellulose, hemicelluloses, lignin and pectin of the plant cell wall (Priest, 1984). *Tricoderma harzianum* is a soil borne green-spored Ascomycetes. It is known as a successful colonizer of their habitats. It has potent degradative machinery for heterogenous substrate (Andre and Monica, 2010). Streptomyces are also soil borne and a member of the *Actinomycetales* bacteria order. Streptomyces remarkably plays an important role in the degradation of organic matter. Kizilkaya et al. (2015) identified the prominence of *Streptomyces* spp. for hazelnut husk degradation. Converting huge and disgusting heaps of coffee husk in coffee plantation to compost for soil amendment is a worthwhile essence. Since the degradation potential of organic compounds by microbes differs and the competence of *T. harzianum* and *Streptomyces* sp. have been ascertained in some previous research, it would be needful to understand their potential degradation ability for coffee husk. This is primary to developing strategy for coffee husk compost production for utilization. Assessment of their potential in sole and combination over time would provide information on the stability and dynamics of each for dried coffee husk degradation.

MATERIALS AND METHODS

Dried coffee husk samples were collected from the coffee processing unit, Cocoa Research Institute of Nigeria (CRIN), Ibadan, Nigeria. The collected husk was air dried to remove any moisture and then shred into small particles which were presented as substrate for the experiment. The the pure culture of *T. harzianum* and *Streptomyces* sp. were obtained from farm soils and maintained on potato dextrose agar (PDA) slant (Adeniyi et al., 2013) in the Pathology Laboratory of CRIN.

Fifty grams of the dried milled husk of was weighed into thirty 500 ml clean beaker, 100 ml of distilled water was added into each and vortex. Each beaker was covered with foil paper, sterilized at 121°C for 15 min and allowed to cool. The experiment was a factorial in completely randomized design consisting of three levels of organisms (*T. harzianum, Streptomyces* sp. and their combinations), three different periods (0, 15 and 30 days) for fermentation and a control. The experiment was replicated three times.

Five inoculum discs of a 5-day old pure culture of *T. harzianum* and *Streptomyces* sp. were inoculated into the sterilized coffee husk singly and in combinations and incubated for 15 and 30 days and a control (without either *T. harzianum* or *Streptomyces* sp.). The inoculated coffee husk in the flasks were gently agitated, labeled and incubated at room temperature (28 ± 5°C) for the specify periods. The inoculated flasks containing coffee husks were agitated occasionally for homogeneity, harvested in a sterile container after the specified period and dried aseptically.

Ten grammes of each sample were homogenized in 50 ml of distilled water. The resulting suspensions were cleanse and decanted and their pH determined with the pH meter. Following Oyewole (1990), the pH meter was standardized using a standard buffer of pH 4.0 and sterilized water. The mineral components were determined following the procedure of AOAC (2005). Triplicate sample of one gram each were weighed into porcelain crucible and the sample were ashed at 550°C for 5 - 6 h. After cooling to room temperature, the ash was dissolved in 1 ml of 0.5% HNO_3. The sample volume was made up to 100 ml and the level of mineral present was analyzed by atomic absorption spectrophotometer Buck 201 VGP. The tannin and caffeine content of the fermented coffee husk were determined according to the method of Pearson (1991).

Generated data were subjected to analysis of variance using PROC GLM in SAS (version 9.3, 2011). Trend analysis was performed on the eleven measured characters in R (R Team, 2010) to understand the pattern of response of each organism to the three days intervals. Six characters which showed significant organism by interval interaction were further investigated to understand their stability using Wricke Ecovalence Stability statistics (Wricke, 1962) which generated unvariate stability estimates for the three organisms for six traits. Mean performances of the treatments were plotted as histogram in Microsoft Excel (version 2010). In SAS (version 9.3, 2011), Gower genetic distance (Gower, 1971) was

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Table 1. Analysis of variance summary of the bio-degraded coffee husk.

Source of variation	DF	Mean square										
		MC	DMC	CafC	TanC	PTN	PN	PTC	PC	Phos	Pota	pH
Rep	2	0.21	0.15	142.46	0.17	0.50	0.21	17.72	1.29	0.02	0.04	0.02
Organisms	2	22.67***	18.23***	378.61*	0.16	1.15	0.05	49.22**	0.82	0.64***	0.06	0.11***
Days	2	352.53***	325.43***	2713.45***	4.79***	4.89***	16.27***	104.23***	31.67***	0.03*	0.64***	0.07***
Organisms*days	4	13.76***	15.41***	130.47	1.42***	0.12	0.17	18.92	1.33	0.18***	0.25**	0.08***
Error	17	0.53	0.56	64.30	0.08	0.39	0.14	7.91	1.23	0.008	0.034	0.004
CV (%)		1.20	1.92	4.69	4.58	10.15	11.41	14.52	12.64	1.28	1.22	0.92
Mean		66.64	39.09	170.95	6.12	6.22	3.29	19.38	8.77	7.09	15.18	7.13

MC– Moisture content, DMC–dry matter content, CafC– caffeine content, TanC– tannin content, PTN– percentage total nitrogen, PN– percentage nitrogen, PTC– percentage total carbon, PC– percentage carbon, Phos– phosphorus and Pota– potassium. *, ** and *** represented probability level of significance at 0.05, 0.01 and 0.001, respectively.

used to understand similarities among the ten treatments. The similarity matrix from procedure was used as the basic data for the principal component analysis in SAS (version 9.3, 2011). Scores from the first three principal component axes was used to generate a tri-dimensional plane diagram in SAS (version 9.3, 2011) for the diverse positional view of the ten treatments.

RESULTS

The two organisms and the combination of both significantly (P≤0.05) differed in the production of moisture, dry matter, caffeine content, total percentage of carbon, phosphorous and pH (Table 1). Moreover, the three different days of treatment application showed significance (P≤0.05) for the eleven measured parameters. Organism by day interaction was significant (P≤0.05) for moisture, dry matter, tannin, phosphorus, potassium and pH (Table 1). For the eleven parameters, coefficient of variation ranged between 1.20 (moisture content) and 14.52 (carbon total percentage) (Table 1).

Streptomyces significantly (P≤0.001) affected the trend of production of caffeine content, nitrogen total percentage (PTN), percentage nitrogen (PN), carbon total percentage (PTC), percentage carbon (PC) and potassium within the three days interval in both linear and positive quadratic fashion (Table 2). Further with Streptomyces, trend of production of tannin content was significant (P≤0.001) and linearly negative; however, the trend of phosphorus was significant (P≤0.001) and linearly positive. The influence of *Trichoderma* on dry matter content (DMC) and total nitrogen percentage (PTN) for the three intervals of days was significant (P≤0.001) and positively quadratic (Table 2). Furthermore in Table 2, the combination of the two organisms produced significant (P≤0.001) but negative trend on the total carbon percentage (PTC) and the pH across the three days interval.

The highest moisture content was observed in the culture containing both organisms at 15 days of fermentation; the least moisture content occurred in the control (Figure 1). However, caffeine, tannin and dry matter was significantly highest in the control. The least content of the three parameters occurred on the 15^{th} and 30^{th} days of fermentation with *Streptomyces* and *Trichoderma* in combination (Figure 1).

Figure 2 clearly showed how the control differed significantly from the other treatments for the five fermentation products. Except for the pH (for which every treatment including the control did not differ), the percentage nitrogen, carbon, phosphorus and potassium were significantly higher in the other treatments as compared to the control (Figure 2).

From Table 3, the combination of *Streptomyces* and *T. harzianum* displayed the highest stability for the production of moisture content (*Wi* = 3.68), dry matter (*Wi* = 3.43) and tannin (*Wi* = 0.28). Stability in the production of phosphorus (*Wi* = 0.02), potasium (*Wi* = 0.04) and pH (*Wi* = 0.01) was mostly supported by *T. harzianum* (Table 2). Enhancement of pH (*Wi* = 0.03) and potassium (*Wi* = 0.13) stability by *Streptomyces* was next to *T. harzianum*. The sole culture of each of the two organisms displayed highest dynamism in the production of moisture, dry matter and tannin content (Table 3).

The similarity of the ten treatments involved in this study is presented in Table 4. Similarity coefficient of Gower distance among the ten

Table 2. Trend analysis of microbes, their interactions and period of degradation of coffee husk.

Character	*Streptomyces*		*Trichoderma*		*Streptomyces*/*Trichoderma*	
	Linear	Quadratic	Linear	Quadratic	Linear	Quadratic
MC	ns	ns	ns	ns	ns	ns
DMC	ns	***	ns	***	ns	ns
CafC	***	***	ns	ns	ns	ns
TanC	***	ns	ns	ns	ns	ns
PTN	***	***	ns	***	ns	ns
PN	***	***	ns	ns	ns	ns
PTC	***	***	ns	ns	***	ns
PC	***	***	ns	ns	ns	ns
Phos	***	ns	ns	ns	ns	ns
Pota	***	***	ns	ns	ns	ns
PH	ns	ns	ns	ns	***	ns

MC – Moisture content, DMC– dry matter content, CafC– caffeine content, TanC– tannin content, PTN– percent total nitrogen, PN– percent nitrogen, PTC– percent total carbon, PC– percent carbon, Phos– phosphorus and Pota– potassium. *, ** and *** - Level of significance measured at 5, 1 and 0.1%, respectively.

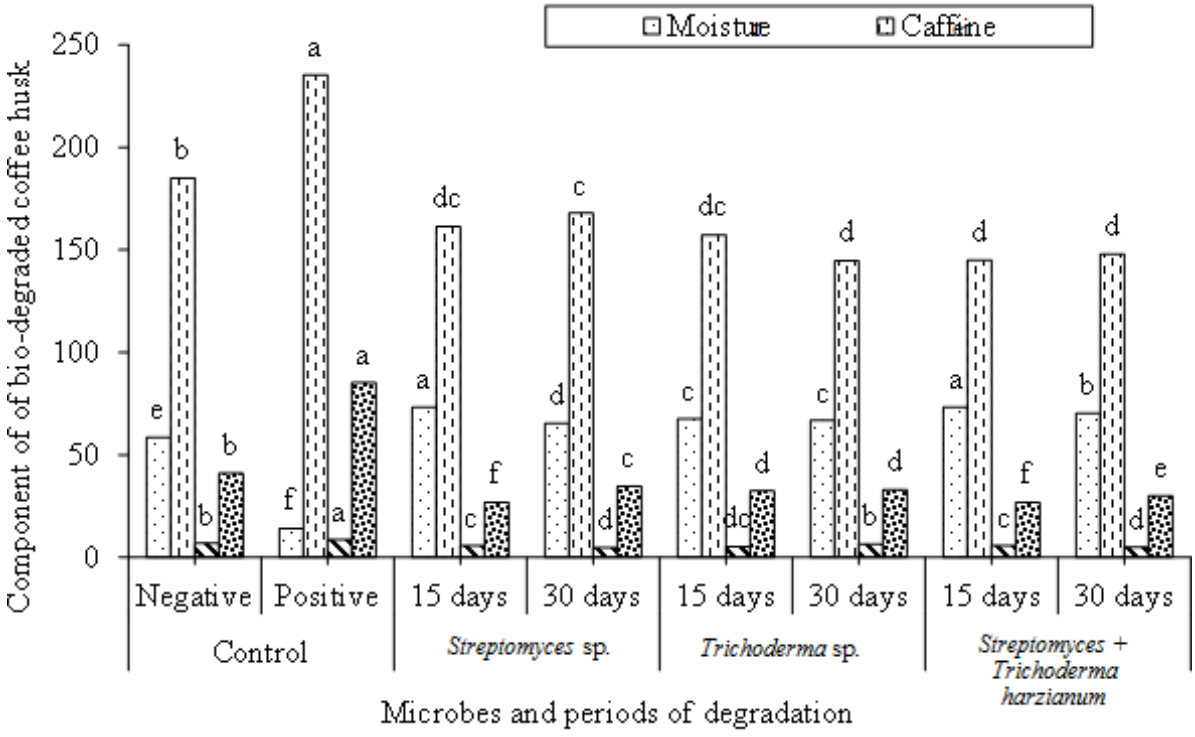

Figure 1. Effect of microbes on moisture and caffeine content in biodegraded coffee husk.

treatments ranged between 0.3216 (Control and S_15) and 0.9285 (T_15 and T_30) with a mean of 0.709. The most similar treatments in this study are T_15 and T_30; with quantitative similarity measure of 0.93 (Table 4). Within Table 3, other pairs of treatment with high similarities includes: T_15 and S_30 (0.92), S_0 and T_0 (0.90), ST_0 with T_0 (0.87) and ST_0 with S_0 (0.87). Three principal components (PC) axes explained a cumulative of 94% of the total variance among the ten treatments (Table 5). The highest (60%) contribution to the total variance occurred in PC1. The positive and higher (≥ 0.35) Eigenvector loadings of dry matter content

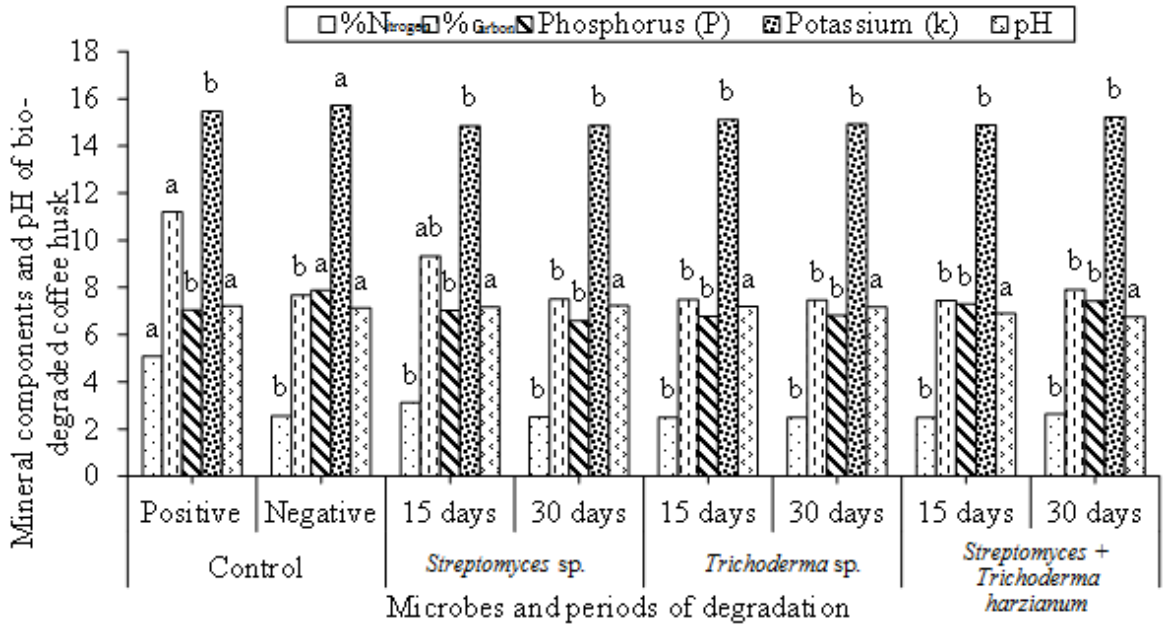

Figure 2. Mineral contents and pH of biodegraded coffee husks.

Table 3. Wricke Ecovalence analysis of contents of bio-degraded coffee husks.

Organism	MC	Phos	DMC	Pota	TanC	pH
Streptomyces sp. + *Trichoderma harzianum*	3.68	0.07	3.43	0.22	0.28	0.08
Streptomyces sp.	7.60	0.17	7.75	0.13	0.36	0.03
Trichoderma harzianum	7.07	0.02	9.30	0.04	1.25	0.01

MC– Moisture content, Phos– phosphorus, DMC–dry matter content, Pota– potassium, TanC– tannin content.

(DMC), caffeine content, tannin content, nitrogen total percentage (PTN) and carbon total percentage (PTC) were most contributory to the significance of PC1. The significance of percentage carbon was prominent (Eigenvector = 0.5829) in PC2 and that of phosphorus (Eigenvector = 0.4739) and potassium (Eigenvector = 0.3796) were higher and significant in PC3 (Table 5).

Two prominent clusters exist in the tri-dimensional plane generated by the scores of the first three principal component axes (Figure 3). The control uniquely separated from the other nine treatments. However, the nine treatments further separated into three different sub-clusters, each with three members (Figure 3). Treatments in sub-cluster I had the highest mean value for dry matter content, caffeine content, percentage total nitrogen, percentage nitrogen, percentage carbon and potassium. The highest mean for moisture, tannin content, percentage total content and phosphorus occurred in cluster II. Except for pH, cluster III had the lowest mean for most of the studied parameters.

DISCUSSION

The C/N proportion in the tested coffee husk provided an enabling environment for *Streptomyces, T. harzianum* and the combination of both as microbiota. The report of Biu (2014) updated a review and catalogued list of new and different cellulose-degrading microorganisms isolated from various natural habitats. The selection of the two organisms in the present study provided evidence that both were highly effective in facilitating coffee husk decomposition processes. Smits et al. (1996) reported on the effectiveness of *Trichoderma reesei* in the composting process of wheat bran. Kizilkaya et al. (2015) equally identified the prominence of *Streptomyces* in the decomposition of hazelnut husk.

Cellulose degradation according to Ryckeboer et al. (2003) and Sundberg et al. (2011) is a process completely controlled and carried out by microorganisms. From our result, the linear trend of the production of the biochemical products and the significant higher values of

Table 4. Gower genetic distances similarity expression.

Treatment	Control	ST_0	ST_15	ST_30	S_0	S_15	S_30	T_0	T_15
ST_0	0.3287								
ST_15	0.3280	0.5795							
ST_30	0.3327	0.5481	0.8669						
S_0	0.4318	0.8718	0.5515	0.5564					
S_15	0.3216	0.7200	0.8290	0.7549	0.6686				
S_30	0.3443	0.6485	0.7979	0.7458	0.5773	0.8409			
T_0	0.3489	0.8735	0.5207	0.5256	0.9003	0.6794	0.6474		
T_15	0.3744	0.6533	0.8327	0.8108	0.6073	0.8530	0.9211	0.6643	
T_30	0.3805	0.6648	0.8663	0.7753	0.6134	0.8573	0.8940	0.6535	0.9285

ST_0– *Streptomyces*/*Trichoderma* combination at zero day, ST_15- *Streptomyces*/*Trichoderma* combination at 15 days, ST_30- *Streptomyces*/*Trichoderma* combination at 30 days, S_0- *Streptomyces* at zero day, S_15- *Streptomyces* at 15 days, S_30- *Streptomyces* at 30 days, T_0- *Trichoderma* at zero day, T_15 *Trichoderma* at 15 days, T_30- *Trichoderma* at 30 days.

Table 5. Proportion of total variance of content of bio-degraded coffee husk by Eigenvalues and Eigenvectors matrix.

Variables	Eigen values	Variance proportion	Cumulative variance proportion
PC Axes			
PC1	6.6200	0.6018	0.6018
PC2	2.4743	0.2249	0.8268
PC3	1.2453	0.1132	0.9400
Eigenvector			
Traits	PC1	PC2	PC3
Moisture content	-0.3772	0.1174	0.0908
Dry matter content	0.3765	-0.1259	-0.0889
Caffeine content	0.3760	0.0721	-0.0786
Tannic content	0.3546	0.0551	0.0266
Percentage total nitrogen	0.3729	-0.1522	-0.0840
Percentage nitrogen	0.1000	0.5839	0.2493
Percentage total carbon	0.3697	-0.0535	-0.2384
Percentage carbon	0.0900	0.5829	0.2562
Phosphorus	0.2446	-0.2848	0.4739
Potassium	0.2904	0.1360	0.3796
pH	0.1037	0.3947	-0.6454

the treated above the control seem to reveal the importance and the effective role of the different microorganisms and their combinations in the composting process of coffee husk. Potential of the three organisms for coffee husk degradation were revealed and further substantiates in earlier report, that microorganisms are useful for the manufacture of natural compost from organic matter (Biu, 2014; Kizilkaya et al., 2015).

The significant increase of K and P observed in this experiment was in line with the work of Warman and Termeer (1996) and Chane (1999). The increase in K observed by the authors was due to the decomposition of racetrack manure, grass clippings and sewage sludge. However, the reduction in total N observed in the present study was consistent with the report of Mahimairaja et al. (1994) on losses and transformation of nitrogen during composting of poultry manure. High similarity existed among all the treatments at the beginning of the experiment because degrading process/activities of the organisms may have rarely started. *Streptomyces, Trichoderma* and their combination as treatment for 15 and 30 days displayed very high (≥ 85%) similarities. The work of Kaewchai et al. (2009) on *Trichoderma* spp. identified seemingly similarity in the culture activities for

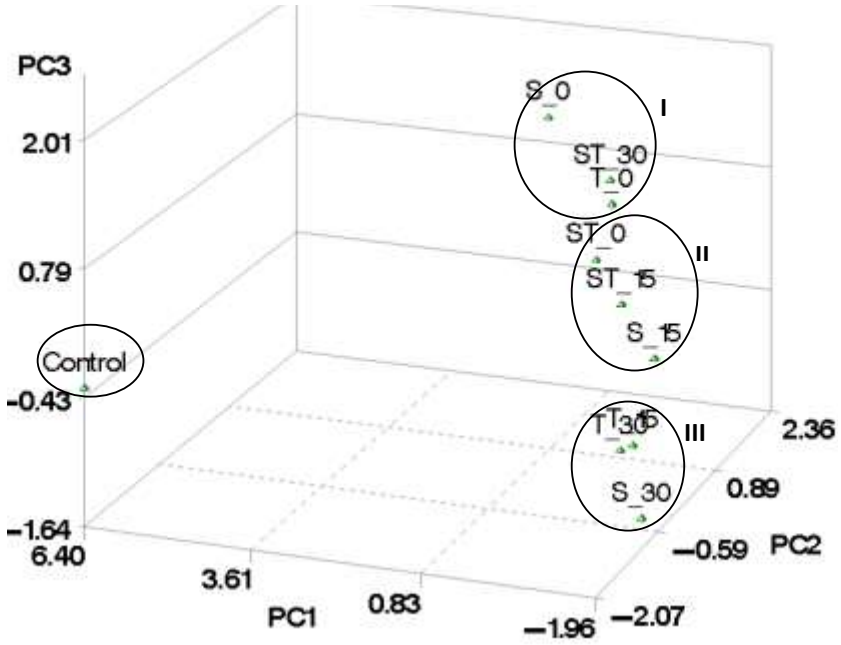

Figure 3. Diversity and similarities among the parameters.

days 15 and 30. It is suspected that degradation activities may have stopped at 15 days or probably earlier. Therefore, the choice of 30 days for effective degradation of coffee husk by these organisms and their combination in this experiment seems too long. Biu (2014) reported that 3 days was optimum for cellulolytic bacteria strains to degrade coffee exocarps.

Quadratic trend in the production of the proximate biochemical products is observed in this study. The prolongation of culture time affects the growth potential as well as cellulose biosynthesis of actinomycetes and the bacteria strains (Biu, 2014). Therefore, cellulolytic activities of the isolates in the medium reduced with time. For coffee exocarp, significant decline in biochemical production as facilitated by actinomycetes and bacteria strains resulted after three days of culture in the report of Biu (2014). A consideration of lower number of days (less than 15) of incubation may be a focus of another research on coffee husk degradation.

Natural system does not select for single species but mixtures in different proportion to enhance equilibrium in the degradation process (Kizilkaya et al., 2015). However, understanding individual potential of the different bio-degrading species is key to generating formula for identified useful species in combination for effective production of environmentally stable products to mimic the natural habitat. Individual degrading potentials of the two organisms in this study revealed dynamism in organic matter decomposition. Their combination revealed high stability and equilibrium in the production of simpler organic and inorganic molecules. The best proportional combination of the two organisms for optimum coffee husk degradation may be another revealing investigation.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Microbiological study of cases of early neonatal sepsis and evaluation of the role of C-reactive protein, interleukin-6 and interleukin-8 as diagnostic biomarkers of such cases

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Neonatal sepsis is responsible for significant morbidity and mortality. Its early diagnosis is very important but very difficult. This study aimed to evaluate the value of C reactive protein (CRP), interleukin 6 (IL-6) and interleukin 8 (IL-8) as early diagnostic biomarkers for early neonatal sepsis (ENOS). Forty neonates with prenatal risk for neonatal sepsis with their mother were taken in this study (group I). Ten healthy neonates and their healthy mothers were taken as the control group (group II) in Tanta University Hospital in the period of March to December, 2016. White blood count (WBC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), interleukin-6 (IL-6) and interleukin-8 (IL-8) were evaluated in cord blood of neonates and in mother's sera. Also, cultures were done for neonates to confirm neonatal sepsis. The results of this study showed that the gestational age was shorter in the study group than the control group. *Staphylococcus aureus* represent 55% of the organisms isolated from cases of early neonatal sepsis. WBC, ESR, CRP, IL-6 and IL-8 were significantly higher in the study group than the control group. In the study group, 50% had positive cultures, while there were only 10% in thr control group with P value of 0.001. The sensitivity, specificity, positive predictive value (PPV) and negative predictive values (NPV) for CRP were 88.6, 84.7, 86.9 and 90.4%, for IL-6, they were 92.4, 97.6, 90.4 and 86.6%, for IL-8, they were 90.8, 88.9, 92.4 and 91.7%, respectively. The best sensitivity and NPV was for IL-6. Cord blood CRP alone has little utility in EONS diagnosis. IL-6 and IL-8 has great superiority than CRP when combined with other hematological markers. IL-6 was better diagnostic biomarker for ENOS than IL-8 and CRP.

Key words: Early neonatal sepsis, C-reactive protein (CRP), IL-6, IL-8.

INTRODUCTION

Neonatal sepsis is defined as generalized bacterial infection of neonates and is considered one of the major causes of morbidity and mortality in the newborn, especially in developing countries. Surviving infants can

have significant neurologic sequelae as a consequence of central nervous system involvement, septic shock or hypoxemia (Betty and Inderpreet, 2005).

Early onset neonatal sepsis (EONS), referred to infection occurring during the first 72 h after birth, is generally caused by pathogens prevalent in the maternal genital tract, delivery room and/or operation theatre. Infection can be acquired by ascending colonization following the rupture of membranes, during fetal passage through an infected birth canal, or at the time of resuscitation procedures. EONS has an incidence of 1.5 to 3.5%/000 live births with mortality rates ranging from 5 to 10% in developed countries (Vergnano et al., 2005).

The prognosis and outcome of neonatal sepsis depend on early diagnosis and treatment with efficient antibiotic therapy. The accurate diagnosis of early neonatal sepsis remains a challenge for the neonatologist because early signs and symptoms are often minimal, variable, nonspecific and often difficult to differentiate from the physiologic changes that occur during transition to extra uterine life (Ng, 2004)

Blood cultures were considered the gold standard method of diagnosis of neonatal sepsis for years but it is time consuming and can give negative results because of antibiotic treatment of mothers during labour (Kumar et al., 2001). In addition to the blood culture, other tests were usually used for the diagnosis of neonatal sepsis including: white blood cell count (WBC) and erythrocyte sedimentation rate (ESR). Unfortunately, these tests do not have a high sensitivity and specificity in diagnosing neonatal sepsis. CRP has also been used for long time as a biomarker for infection as it is simple, fast and effective but its sensitivity and specificity became a matter of questions (Gonzalez et al., 2003)

Meanwhile, infants who are suspected to have neonatal sepsis have to start antibiotic treatment which could be unnecessary and even harmful with higher incidence of antibiotic resistance and longer hospitalization with increased health costs (Bindlish et al., 2015). Therefore, efforts have been made to improve diagnosis and to find the ideal rapid biomarker that provides high sensitivity and specificity for diagnosis of presence or absence of sepsis, severity of sepsis, type of microorganisms, and prognosis during treatment (Modi et al., 2009).

In recent years, the search for new diagnostic tests for ENOS has turned to cytokines as more early and effective indicators of acute infections. IL-6 and IL-8 are potent proinflammatory cytokine and are responsible for a strong inflammatory reaction, which if left uncontrolled, may lead to severe hypotension, multiple organ dysfunction and death (Ng et al., 2004).

The aim of our study was to evaluate the role of CRP, IL-6 and IL-8 as early diagnostic biomarkers for early onset neonatal sepsis.

METHODOLOGY

The study is a prospective one performed in obstetric department and neonatal intensive care unit (NICU) with cooperation of Medical Microbiology and Immunology Departments at Tanta University Hospital, Egypt during the period of March 2016 to December 2016. The study was approved by Tanta Ethical Committee and informed consent was obtained from the parents.

Fifty (50) neonates with gestational age above 28 weeks and their mothers were enrolled in the study. They were divided into two groups:

Group I (study group): 40 maternal and cord blood samples were taken at the same time with gestational age above 28 weeks from mothers with prenatal risk factors for infection. Eligible neonates had to fulfill one or more of prenatal risk factors for infection such as premature rupture of membranes (PROM), chorioamnionitis and/or maternal high fever.

Group II (control group): 10 maternal and cord blood samples were taken with the same gestational age above 28 weeks from mothers with no risk factors.

Maternal samples were taken during the 1st and/or 2nd stage of labor and cord samples were taken just after the fetal delivery and during resuscitation of the newborn by the help of obstetricians. All mothers were delivered vaginally.

CRP level was studied immediately by the latex immuno-nephelometric method (BNA analyser, Behring-Werke AG, Marburg, Germany). CRP levels ≤6 mg/L were considered normal. The blood samples were then centrifuged and serum samples were stored at -70°C. Both mothers' and cord blood serum were tested for IL-6 and IL-8 using a micro enzyme linked immunosorbent assay (ELISA) method. All of the laboratory analyses were carried out in the central laboratory of Tanta University Hospital.

Exclusion criteria

Stillbirth, delivery outside the hospital, inadequate blood sampling, parents refusal to participate, presence of any other systemic infections e.g. chest, renal, hepatic, instrumental deliveries, Caesarean section, and mother taking antibiotics are the exclusion criteria.

All mothers were evaluated by ultrasonography, and biophysical profiles by obstetricians. Gestational age was calculated by mother's last menstrual date, and/or ultrasonographic fetal measurements. PROM was defined as a prolonged rupture of membranes exceeding 24 h. Clinical findings of chorioamnionitis were evaluated, and then placental remains from mothers with clinical chorioamnionitis were evaluated for histological chorioamnionitis.

All neonates of group I were admitted to NICU and subsequent complications such as early neonatal sepsis, pneumonia, intraventricular hemorrhage (IVH), respiratory distress syndrome (RDS), necrotizing enterocolitis (NEC) and chronic lung disease were recorded. Diagnosis of early neonatal sepsis was based upon criteria according to Cernada et al. (2012). The Tollner scoring system was used to evaluate risk of early neonatal sepsis. Both mothers' and cord blood serum were tested for IL-6, IL-8, CRP, ESR and CBC.

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Table 1. Age of mothers and gestational age of both groups.

Parameter	Group I	Group II	P. value
Gestational age	30.2±5.63	36.21±7.52	0.024*
Age of mothers	28.63±8.63	31.14±6.15	0.528

Diagnosis of microbiological sepsis was based on clinical signs and confirmed with blood culture where blood samples were collected from neonates who were showing signs and symptoms of sepsis. Each sample was 0.5 ml of blood. Blood cultures bottles (Salix®) were incubated at 37°C for 24 h. Subcultures on blood agar and mannitol salt agar were done, and incubated at 37°C for another 24 h. Characteristic colonies were identified by Gram stain, and biochemical reactions according to standard bacteriological procedures (Campos et al., 2010). Clinical sepsis was defined as the presence of 3 or more of the following:

1) Temperature instability (rectal temperature >38 or <36°C);
2) Respiratory symptoms (respiratory distress, apnea or cyanosis);
3) Cardiovascular symptoms: hypotension (blood pressure <5th percentile for age), tachycardia (HR >180/min), bradycardia (HR < 100/min) or poor perfusion; (iv) neurological symptoms: clinical or electrical seizures, bulging fontanelle, hypotonia or lethargy;
4) Gastrointestinal symptoms: vomiting, poor feeding or feeding intolerance and/or abdominal distension, without identification of a bacterial pathogen from a sterile site.

Statistics

Statistical presentation and analysis of the present study was conducted, using the mean, standard deviation and Chi-square test by SPSS V.20. P<0.05 was considered as significant. Sensitivity, specificity, negative predictive value and positive predictive value of the studied biomarkers were calculated.

RESULTS

The results show that the gestational age among group I in patients with early neonatal sepsis was significantly lesser than the age of the control group (group II) (P-value:0.024). Regarding the age of the mother, there was no significant difference between the two groups (P-value:0.528) (Table 1).

Leukocyte count for both mothers and neonatal cord samples in group I was significantly higher than in the group II. Also, ESR 1st and 2nd hours for both mothers and neonatal cord samples in group I was significantly higher than in the group II (Table 2). There was significant difference between both groups as regard levels of CRP, IL 6, and IL 8 of mothers which were higher in groups I as compared to II (Table 3).

There was highly significant difference between both groups as regarded CRP, IL 6, and IL 8 of neonatal cord blood which were significantly higher in group I than group II (Table 4). Number of newborn entered neonatal intensive care unit with proven ENOS by blood culture was 20 patients (50%) in group I, and one patient (10%) in group II with p-value < 0.05. There were high significant difference between groups I and II regarding number of patient diagnosed with ENOS and high IL-6, IL-8 and CRP (Table 5). The sensitivity of CRP mother blood was 59%, specificity 85%, PPV 90.6% and NPV 74.6%, while sensitivity of CRP neonatal cord blood was 88.6%, specificity 84.7%, PPV 86.9% and NPV 90.4% (Table 6).

The sensitivity of IL-6 mother sample was 95.1%, specificity 91.8%, PPV 86.4% and NPV 92.1%. While sensitivity of IL-6 neonatal cord blood was 92.4%, specificity 97.6%, PPV 90.4% and NPV 86.6 % (Table 6).

The sensitivity of IL-8 mother sample was 91.2%, specificity 86.3%, PPV 85.9% and NPV 87.3%, while sensitivity of IL-8 neonatal cord blood was 90.8%, specificity, 88.9%, PPV 92.4% and NPV 91.7% (Table 6). Table 7 shows the results of blood culture among samples of cord blood of cases with early neonatal sepsis where *Staphylococcus aureus* was the main organism causing early neonatal sepsis in this study as it was isolated from 11 cases (55%), followed by *Klebsiella pneumoniae* (22.5%), *Acinatobacter baumanni* and *Pseudomonas auruginosa* isolated from 4 cases (10%) and *E. coli* isolated from only one case (2.5%). The best sensitivity for early neonatal sepsis was achieved by IL 6 (Table 6).

DISCUSSION

Early diagnosis of neonatal sepsis and appropriate management can be lifesaving and significantly decrease morbidity and mortality in newborns. However, early diagnosis of sepsis is very difficult because symptoms are minimal and non-specific. So, the search for an ideal rapid diagnostic test, which has maximum sensitivity and maximum negative predictive value, is very important for prognosis and to decrease unnecessary exposure to antibiotics (Ng et al., 2004). So, the authors measured CRP and cytokines (IL- 6 and IL-8) in high risk neonates to identify a reliable test for diagnosis of ENOS.

Presence of risk factors for neonatal sepsis usually results in preterm labour that was confirmed in the results by lower gestational age in the study group than control group. This is higher in comparison with the results of other authors (Modi et al., 2009). In this study, leukocytic count and ESR for both mothers and neonates in group I were significantly higher than in the control group, indicating the presence of infection. This showed agreement with other studies (Malik et al., 2003).

CRP is synthesized in the liver in response to IL 6, IL β and TNF-CRP. CRP is synthesized within 6 and 8 h of exposure to infection, peaks at 24 to 48 h, and then diminishes when the inflammation subsides (Kocabaş et al., 2007). This study reported increase in levels of CRP for both mothers and neonates in groups I as compared to group II with sensitivity, specificity, PPV and NPV of 88.6 and 84.7, 86.9 and 90.4%, respectively in neonates. These results are similar to that of other investigators

Table 2. Leukocytic count and ESR in both groups.

Parameter	Group I		Group II		P value
	Maternal samples	Neonatal cord samples	Maternal samples	Neonatal cord samples	
WBC	14.380±4.098 c/µl	15.180±6.992 c/µl	11.759±2.994 c/µl	10.898±3.556 c/µl	0.004*
ESR 1st h	105±30 ml/h	97±35 ml/h	50±15 ml/h	30±10 ml/h	0.002*
ESR 2nd h	122±40 ml/h	99±40 ml/h	40±20 ml/h	35±12 ml/h	0.008*

*h: Hour c/µl: cell/microliter.

Table 3. CRP, IL 6, IL 8 in serum of mothers in both groups.

Parameter	Group I	Group II	P value
CRP	17.63±14.16mg/l	12.25±6.71 mg/l	0.019*
IL 6	52.36±11.6 pg/ml	18.32±3.62pg/ml	0.001*
IL 8	395.2±85.6 pg/ml	311.2±42.6 pg/ml	0.019*

Table 4. CRP, IL 6, IL 8 in neonatal cord samples of both groups.

Parameter	Group I	Group II	P value
CRP	16.32±4.626 mg/l	13.9±2.93mg/l	0.011*
IL 6	65.35±8.25 pg/ml	35.4±8.52 pg/ml	0.001*
IL 8	385.4±74.1 pg/ml	291.4±63.8 pg/ml	0.019*

Table 5. Number of neonate admitted to NICU with EONS and that with high biomarkers.

Number of newborn entered ICU for ENOS	Group I	Group II	P value
Proven EONS by culture	20/40 (50%)	1/10 (10%)	0.001*
High CRP	18	2	0.004*
High IL-6	16	4	0.001*
High IL-8	15	1	0.001*

High CRP>6 mg/l high IL-6>9pg/ml high IL-8>33 pg/ml.

Table 6. Diagnostic value of blood cytokines in early-onset neonatal sepsis in both mother and neonate.

Parameter	CRP		IL-6		IL-8	
	Mothers	Neonates	Mothers	Neonates	Mothers	Neonates
Sensitivity	59	88.6	95.1	92.4	91.2	90.8
Specificity	85	84.7	91.8	97.6	86.3	88.9
PPV	90.6	86.9	86.4	90.4	85.9	92.4
NPV	74.6	90.4	92.1	86.6	87.3	91.7

(Reyes et al., 2003).

In contrast to the results, Dollner et al. (2002) found that CRP levels were low and undetectable in nearly all of the neonates, both in infectious and control groups. This can be explained by the fact that infection may be initiated relatively close to delivery, resulting in low levels of umbilical CRP concentration. Moreover, CRP responses could be undetectable several days after birth

Table 7. Results of blood culture among samples of cord blood in cases with early neonatal sepsis:

Isolated organism	No. of cases (%)
Staphylococcus aureus	11(55)
Klebsiella pneumonia	4 (22.5)
Acinatobacter baumanni	2 (10)
Pseudomonas aeruginosa	2(10)
E.coli	1(2.5)
Total	20(100)

in infected extremely premature neonates. Also, cord blood CRP may not increase in presence of umbilical vasculitis which often reflects severe chorioamnionitis with neonatal diseases, whereas many other inflammatory markers do.

It is known that CRP does not cross the placenta (Beceiro-Mosquera et al., 2009). Accordingly, the present study confirmed that serum concentrations of CRP and its production in the mother and fetus/newborn are independent of one another as lower levels of CRP were present in mothers' blood. However, the same stimulus may be operating concurrently in each. The major obstetric condition in which determination of maternal serum CRP concentrations might be clinically useful is chorioamnionitis (Baltimore, 2002).

Possible sources of heterogeneity of results in different studies had wide differences in postnatal age, single versus serial measurements, different cutoff level, different sample sizes and different measurement methods. Thus, the fact that there are no established CRP reference intervals in the neonatal period can explain the wide range of reported CRP sensitivities (47 to 100%) and specificities (6 to 97%) for detection of neonatal sepsis (Beceiro-Mosquera et al., 2009).

IL-6 is a pro-inflammatory cytokine that promotes the synthesis of CRP by the liver. IL-6 is produced by monocytes, endothelial cells and fibroblasts. IL-6 is released soon after the inflammatory stimulus takes place, peaks within 6 h post-insult, preceding the increase in CRP, and decreases 48 h thereafter. Several studies that have used IL-6 as a biomarker for established neonatal sepsis have reported contradictory results (Beceiro-Mosquera et al., 2009).

It was found that IL-6 levels in group I was significantly higher than group II in both neonatal cord blood and maternal blood. IL-6 levels in maternal blood was valuable in detecting early fetal infection with a sensitivity, specificity, NPV, PPV of 95.1, 91.8, 92.1 and 86.4%, respectively and was comparable to the results of other investigators (Tasci et al., 2006). Also, the finding of higher IL-6 concentrations in cord sera of babies compared with the corresponding maternal samples argues against the dependency of neonatal serum IL-6 concentrations on maternal IL-6 concentrations.

This study reports higher values of IL-6 in newborns with culture-proven sepsis compared with IL-8 and similar to studies. IL 6 was found to have the highest sensitivity and NPV as a predictor of EONS as compared to CRP and IL-8 like the result of other investigators (Martin et al., 2012).

The different results of these studies may be partially explained by timing at which sampling was performed in the course of the disease as serum IL-6 values inversely correlate with timing of sample collection (Cernada et al., 2012). The samples were obtained immediately after birth before the patients featured any clinical sign of sepsis. This could explain why we found low values of CRP and high ones of IL-6 as compared to other publications.

IL-8 is a pro-inflammatory cytokine predominantly produced by monocytes, macrophages and endothelial cells. It has a role in release, activation and chemotaxis of neutrophils and rises early in the course of neonatal bacterial infections. IL-8 is considered to be an accurate early and late marker for neonatal sepsis in many studies (Dima et al., 2012).

In this study, it was found that IL-8 was significantly higher in group I than group II. This agreed with the results of other investigators (Dollner et al., 2002; Fukuda et al., 2012) who reported that a significant initial elevation of IL-8 in cord blood collected immediately at birth was observed in infected preterm neonates. unlike the results of Fukuda et al. (2012) who studied cord levels of IL-6 and IL-8 and showed no increase in preterm with high risk for infection as compared to a control healthy group.

The sensitivity, specificity, PPV, NPV for IL-8 in neonatal cord blood were 90.8, 88.9, 92.4 and 91.7% and this was in agreement with the results of Fan and Yu (2012) who reported the sensitivity of cord blood IL-8 91%, specificity 93%, PPV 91% and NPV 97%.

Many investigators reported that IL-6 and IL-8 were produced rapidly in ENOS and peaked on day zero and they fell into their normal baseline within 24 h (*Kocabaş* et al., 2007*).* This makes them very important early predictors of ENOS but in the same time, could not be used alone for diagnosis as we could not be sure at which stage of infection blood samples were taken for IL-6 and IL-8. In contrast, CRP peak concentration occurs later so it is highly specific to confirm infection and the presence of high CRP in the presence of normal IL-6 and IL-8 suggest infection was there for 24-48 h (Ng et al., 2003). However, low sensitivity and NPV values for CRP in this study made this test alone not sufficient in the early diagnosis of neonatal sepsis. However, the relatively low cost of the rapid automated CRP test makes it an important test to screen for rather than to diagnose early neonatal sepsis. Combination of CRP with IL-6 or IL-8 improves its diagnostic value.

In this study, 50% of neonates in group I entered NICU with sepsis that was confirmed by positive blood culture and 10% of neonates in group II proved to have sepsis

also. There was statistically significant difference between number of newborns in NICU with EONS in both groups. This shows agreement with Jan et al. (2013) who reported incidence of neonatal sepsis (54%) among high risk newborn.

The results showed that 50% of cases suspected to be neonatal sepsis were positive for blood culture where *S. aureus* was the main organism causing early neonatal sepsis in the study as it was isolated from 11 cases (55%), followed by *K. pneumoniae* (22.5%), *A. baumanni* and *P. auruginosa* isolated from 4 cases (10%) and *E. coli* isolated from only one case (2.5%).

The result of Shrestha et al. (2013) showed that out of 120 neonates suspected of having neonatal sepsis, 30.8% (37/120) were blood culture positive (prevalence = 30.8%). The most common causative agents of neonatal sepsis was *S. aureus* (56.8%; 21/37) followed by *K. pneumoniae* (21.7%; 8/37) and *P. aeruginosa* (13.4%; 5/37) and others. In another study by Vrishali et al. (2015), *K. pneumoniae* was the predominant pathogen (35.4%) among the Gram-negative pathogens and *S. aureus* (22.9%) was the predominant Gram-positive.

In conclusion, IL-6 and IL-8 levels in cord blood and mothers sera were superior markers for ENOS than CRP and did not show a significant difference in sensitivity and specificity and IL-6 had a better marker as compared to CRP and IL-8 with the highest sensitivity and PPV. Also, it is concluded that the use of multiple markers as CRP, IL-6 and Il-8 improves the diagnostic accuracy of ENOS than using each marker alone.

CONFLICT OF INTERESTS

Authors have declared that no competing interests exist.

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Microbiological quality and safety of some-street-vended foods in Jimma Town, Southwestern Ethiopia

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Street food vending has been benefiting both consumers, who are the in low socio economic status, as well as vendors, by creating job opportunities. However, street foods are perceived to be a major public health risk due to contamination. The objective of this study was to evaluate the microbiological quality and safety of street-vended foods in Jimma town in Ethiopia. The study involved collection of socio-economic data using structured questionnaire and laboratory analysis for microbial quality and safety. A total of 160 ready-to-eat street foods (40 each of firfir (mixture of majority of cabbage, watt, macaroni and injera), bread, injera (Ethiopian traditional food) and sambussa) samples were collected from Merkato, Kochi and Agip vending sites in Jimma. Result of the study shows that 85.5% of vendors were women, 54.5% had primary education, 90.9% did not use special apparel for their job as street food vendors, 80.9% handled food with bare hands, 49.1% used well water for cleaning of utensils, and 40% wore no hair covering. The mean microbial counts ($CFUg^{-1}$) of food samples were dominated by aerobic mesophilic bacteria (5.0 ± 0.5), aerobic bacterial spore (4.0 ±0.4), lactic acid bacteria (4.0 ±0.4), Enterobacteriaceae (3.9 ± 0.6), staphylococci (3.7 ± 0.6), coliform (2.6 ± 0.4), yeasts (3.8 ± 0.5) and moulds (2.6 ± 0.4). Of the total 1697 isolates characterized, the most predominant were Bacillus spp. (41.96%) followed by *Staphylococcus* spp. (24.28%). Out of the food samples, 29.38% were positive for *S. aureus* and 13.13% samples were positive for Salmonella. *Staphylococcus aureus isolates* were resistant to maximum of six antibiotics (8.51%) but Salmonella had showed resistance to four antibiotics (14.29%). Generally, the microbial quality of street-vended food in Jimma town was poor and calls for special attention.

Key words: Foodborne Pathogens, Street-vended Foods, Vendors.

INTRODUCTION

Street-vended foods are cooked ready-to-eat solid foods and beverages prepared and sold by vendors, especially, on streets and other public places (FAO, 1990). According to WHO (1996), street-vended foods enable urban and rural poor consumers obtain a source of readily available, inexpensive, convenient and often nutritious food. In the developing countries, street-vended food is a source of income for a vast number of vendors (Muzaffar et al., 2009).

Moy et al. (1997) reported that in contrast to the potential benefits of street-vended foods, concerns over their safety and quality have been raised. Vendors lack

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appreciation of basic food safety issues. Unhygienic conditions and practices of vendors and environment are likely to lead to cross-contamination of cooked foods (Yasin et al., 2012).

Microbiological studies on street-vended foods in different countries have suggested the presence of high bacteria counts and a high incidence of food-borne bacterial pathogens (Rane, 2011). For instance, in the Malaysian state, 14 people died because of eating rice noodles bought from street vendors (Dawson and Canet, 1991). In Cuba, 14 people died because of eating foods sold by a private vendor (Barro et al., 2006). In Senegal, over 200 cases of food poisoning were traced to street foods (Bryan et al., 1992). In Egypt, pathogenic microorganisms were reported from street-vended foods (El-Sherbeeny et al., 1985). In Ethiopia, particularly Addis Ababa, Muleta and Ashenafi (2001a) reported, the presence of *Salmonella* and *Shigella* from street- vended foods.

In Nigeria, pathogens isolated from street-vended food showed high resistance to different antibiotics (Oladipo and Adejumobi, 2010). *S. aureus* strains isolated from street-vended food in Cotonou, Benin, were resistant to a wide range of antibiotics. According to Crump and Mintz (2010) the multiple drug resistant (MDR) strains of *Salmonella* are encountered frequently and their rates of occurrence have increased considerably in the recent years.

The present study evaluate the microbial safety and quality of street-vended foods like *injera*, *sambussa*, *firfir* (mixture of majority of cabbage, *watt*, macaroni and injera) and bread (prepared locally at home) from Markato, Agip and Kochi vending sites of Jimma town.

Injera and *wot* are two very important traditional foods in Ethiopia. While *injera is* thin, pancake-like, sour, leavened bread, which can be made of either teff, corn, sorghum, barley or a mixture of two or three of these. W*ot* is a traditional Ethiopian stew. Sambussa is a thin dough shell stuffed with lentils and spice. Homemade bread is mainly made of wheat and corn. Injera, firfir and bread are largely produced and consumed by millions urban and rural areas Ethiopians. In Jimma town, these food items are sold in hotels, markets and streets.

MATERIALS AND METHODS

Description of the study site

The study was conducted in Jimma town, located at 353 km southwest of Addis Ababa, the capital city of Ethiopia. The town's geographical coordinates are $7^{o}41'$N latitude and $36^{o}50'$E longitude. The study area has an average altitude of 1780 m above sea level (Alemu et al., 2011).

Study design and population

A cross sectional study design was used. The sample size was calculated by using Cochran (1977) formula. The total population of the street venders at three sites of this study was 274 irrespective of vendors' age and sex. There were 102 street food vendors from Merkato, 96 from Kochi, and 76 from Agip, all of them locations in Jimma.

Sampling technique

A systematic random sampling technique was used to select representative street food vendors in the study area. Systematic sampling is a statistical method involving the selection of elements from an ordered sampling frame.

Socio-demographic data collection

Data about general sanitation were collected from the street vendors using a structured questionnaire.

Sample collection

A total of 160 ready-to -eat food samples comprising 40 samples each of firfir, bread, injera and sambussa were collected from the three different vending sites of Jimma town, namely, Merkato, Kochi and Agip between the months of November 2011 to March 2012. Food samples were purchased from vendors between 5 to 6 pm during the period. Food samples were collected from vendors using vendors' own serving utensils and placing into sterilized aluminum plates. All food samples were transported to Postgraduate Research Laboratory, Department of Biology, College of Natural Sciences, Jimma University The food samples were kept in the refrigerator at 4□C until microbial analysis was conducted. Microbial analysis was conducted with in one to three hours after sample collection.

Microbiological methods

Sample preparation

Approximately 250 g of each food sample was collected from street vendors to be analyzed for microbiological quality and safety. Accordingly, 25 g of each well-mixed food sample was taken and mixed with 225 ml buffered peptone water (BPW) (oxiod), homogenized in a flask for five minutes using A shaker at 160 rpm. After homogenization, 1 ml of each food sample was aseptically transferred into 9 ml of BPW, and mixed thoroughly by using vortex mixer. The homogenates were serially diluted from 10^{-1} to 10^{-6} and a volume of 0.1 ml aliquot of appropriate dilution was spread-plated on pre-solidified plates and incubated at appropriate temperature and time for enumeration. The colonies were counted from plate containing microbial colonies between 30 and 300. The counted colonies were expressed in colony forming units per gram ($CFUg^{-1}$) and later converted to log $CFUg^{-1}$.

Microbial enumeration

Enterobacteriaceae count: From the serial dilutions, 0.1 ml of the aliquot was spread-plated on MacConkey agar (Oxoid) and incubated at 32°C for 18 - 24 h after which, pink to red purple colonies were counted as member of the family Enterobacteriaceae (Spencer et al., 2007).

Coliform count: From the serial dilutions, 0.1 ml of the aliquot was spread plated on pre-solidified surfaces of Violet Red Bile Agar (VRBA) (Oxoid) plates. Then the plates were incubated at 32°C

for 18 - 24 h. After this, purplish red colonies surrounded by reddish zone of precipitated bile were counted as coliforms (Weil et al., 2006).

Aerobic Bacterial Spore Count: For aerobic bacterial spore counts, 10 ml of the serial dilutions were heated in a water bath kept 80°C for 10 min and then cooled rapidly in tap water. From appropriate dilution, 0.1 ml aliquot was spread-plated on pre-dried surface of plate count agar and incubated at 32°C for 72 h (Acco et al., 2003).

Staphylococci count

From the serial dilutions, 0.1 ml of the aliquot was spread plated onto Mannitol Salt Agar (MSA) (Oxoid) and incubated at 32 °C for 48 h (Acco et al., 2003).

Lactic acid bacteria count

From the serial dilutions, 0.1 ml of the aliquot was spread plated on de Mann Rogosa Sharpe (MRS) agar media (Oxoid) and incubated at 37°C for 48 h under anaerobic condition using anaerobic Jar (Patra et al., 2011).

Yeasts and moulds counts: From the serial dilutions, 0.1 ml aliquot was spread-plated on pre-solidified surfaces of Potato Dextrose Agar (Oxoid) supplemented with 0.1 g chloramphenicol and incubated at 25°C for 5-7 days (Spencer et al., 2007). Smooth (non-hairy) colonies without extension at periphery were counted as yeasts whereas hairy colonies with extension at periphery were counted as moulds.

Microbial characterization

After enumeration of aerobic mesophilic bacteria, 10 to 15 colonies with distinct morphological differences such as color, size and shape were randomly picked from countable plates and aseptically transferred in to a tube containing 5 ml nutrient broth (Oxoid). The inoculated cultures were incubated at 32^{o}C for 24 h. Cultures were purified by repeated plating and preserved on slants at 4^{o}C for a month. Finally, the obtained organisms were characterized to genus and family levels. The characterizations of isolates were done based on John (2012) bacterial classification manual.

To determine the morphology and biochemical characteristics of the bacterial isolates, bacterial cells were Gram-stained (Gram, 1884). Motility test was conducted according to Shields and Cathcart (2012). Endospore test was done according to Schaeffer and Fulton (1933) method. Presence of lipopolysaccharide that is Gram positivity or negativity was determined according to Gregerson (1978). Oxidation-reduction properties were determined according to Hugh and Leifson (1953). Catalase test was conducted according to MacFaddin (1980). Cytochrome test was conducted using the method outlined by Kovacs (1956).

Isolation of *Salmonella* spp.

For the detection of *Salmonella* spp., 25 g of food samples were mixed with 225 ml of BPW and incubated at 37^{o}C for 24 h. Then 1 ml pre-enrichment broth culture was added to 10 ml of selenite cysteine broth (Oxoid) and again incubated at 37°C for 24 h. Thereafter, a loopful of suspension from a tube was streaked onto Xylose Lysine Deoxycholate Agar (XLD) (Oxoid). The presumptive *Salmonella* colonies (black colony surrounded by red color) were picked off, transferred to 5 ml nutrient broth (Oxoid), incubated at 37°C for 24 h, then streaked onto Nutrient Agar (Oxoid) for purity, and incubated at 37°C for 24 h (Arvanitidou et al., 1998). The presumptive *Salmonella* spp. were characterized by standard biochemical tests. The biochemical testes were done according to the procedure of Johnson and Case (2007).

Isolation of *Staphylococcus aureus*

After counting staphylococci, golden yellow colonies on MSA plates were aseptically picked and transferred into 5 ml nutrient broth and incubated at 37°C for 24 h for further purification. Then, a loopful of culture from the nutrient broth was streaked on nutrient agar supplemented with 0.75% NaCl and again incubated at 37°C for 24 h. Finally, the distinct colonies were characterized using the established microbiological methods (Acco et al., 2003). Gram-positive cocci with clustered arrangement under the microscope were subjected to preliminary biochemical tests: oxidase, catalase and coagulase tests (Cheesbrough, 2006).

Antimicrobial Susceptibility testing for some pathogens

Antimicrobial susceptibility testing was investigated on Mueller Hinton Agar (Oxoid) plates following the standard disk diffusion techniques. The antibiotic discs were placed on the medium by using forceps and incubated at 35°C for 18 h and the zone of inhibition was measured manually with a transparent ruler. The results of the antimicrobial susceptibility were interpreted based on the guidance of National Committee for Clinical Laboratory Standards (NCCLS, 2007).

The following standard discs (Oxoid) and their potency (μgml^{-1}) were used depending up on the antibacterial spectrum, toxicity, effectiveness and availability (Vlkova et al., 2006): ampicillin (10), chloramphenicol (30), ciprofloxacin (5), gentamycin (10), kanamycin (30), naldixic acid (30), norflaxacin (10), streptomycin (10) and tetracycline (30) were used for *Salmonella* spp. Chloramphenicol (30), ciprofloxacin (5), clindamycin (2), erythromycin (15), gentamycin (10), kanamycin (30), penicillin G (10), streptomycin (10) and tetracycline (30) were used for *Staphylococcus aureus.* The reference strains were *Salmonella typhimurium* (ATCC13311*)* and *S. aureus* (ATCC25923).

Data analysis

The Percentage of Coefficient of variation (% CV) was calculated to verify if there was significant variation in microbial counts within the food samples analyzed. The data obtained from the respondents were analyzed using SPSS software version 16.0. Mean values of food samples from different sites were compared using one way ANOVA and the significance of differences were considered at 95% confidence interval ($P < 0.05$).

RESULTS

Socio-demographic characteristics of the street vendors

Table 1, which presents the socio-demographic characteristics of street vendors shows that 39.1% of street vendors' belonged to the age group between 31-40 years and the majority (85.5%) of the vendors were

Table 1. Socio-demographic characteristics of street vendors in Jimma town, southwestern Ethiopia 2011/2012.

Parameter	Number of respondents (N=110)	Percentage
Age		
< 20	10	9.1
21-30	26	23.6
31-40	43	39.1
41-50	19	17.3
> 51	12	10.9
Sex		
Male	16	14.5
Female	94	85.5
Academic Status		
Illiterate	34	30.9
Primary education	60	54.5
Secondary education	8	7.3
Experience in vending food (years)		
< 5	29	26.4
6-10	15	13.6
11-15	22	20.0
16-20	40	36.4
>20	4	3.6

females. Educationally, 54.5% had primary education. Among the vendors 36.4% had an experience of 16-20 years, 26.4% < 5 years and 20% had 11-15 years' experience in street food vending.

General hygiene of street vendors and the vended foods

In Table 2, majority (64.5%) of the street vendors used tap water for preparation of food while 27.3% used well water. On the other hand, 49.1% of street vendors used well water for cleaning utensils. In addition, 43.6% of the vendors cleaned the utensils using hand and water only.

Table 3 shows that, 80.9% of street food vendors handled food with their bare hands. Again, 80.91% of the vendors worked in dusty environment and 70.9% in the vicinity of litter. Training- wise, 92.7% of the vendors did not get training on food hygiene, although 7.3% had exposure on personal hygiene. A significant number (66.4%) of the vendors had no information about food and water-borne diseases whereas 33.6% had information about foodborne and waterborne diseases such as diarrhea and giardia.

Microbial counts

From Table 4, the mean count of aerobic mesophilic bacteria (AMB) was the highest (6.6 log $CFUg^{-1}$) in firfir followed by bread (5.1 log $CFUg^{-1}$) whereas the lowest was in sambussa (4.0 log $CFUg^{-1}$). The mean count of Enterobacteriaceae was the highest in firfir (4.9 log $CFUg^{-1}$) whereas it was lower in bread (4.1 log $CFUg^{-1}$) and injera (4.0 log $CFUg^{-1}$). Likewise, the mean counts (log $CFUg^{-1}$) of yeast, coliform and mould were the highest in firfir (5.5, 4.0 and 3.7, respectively) and the mean count of lactic acid bacteria (LAB) was low in sambussa (3.2 log $CFUg^{-1}$). Furthermore, the mean counts (log $CFUg^{-1}$) of staphylococci were the highest (4.8) in firfir followed by bread (4.1). However, it was relatively lower in injera (3.1) and sambussa (2.7). Generally, the mean counts of all bacteria and fungi (yeast and moulds) in all food samples were above detectable level whereas the mean counts of mould and coliform in sambussa were below detectable level.

The percentage coefficient of variation (% CV) ranged from 6.8 to 22.7%. Accordingly, the highest (22.7%) was observed for yeasts count in sambussa and the lowest (6.8%) for AMB count in injera sample. The highest mean counts of AMB were recorded in firfir from Merkato site (6.8 log $CFUg^{-1}$) and bread from Kochi site (5.1 log $CFUg^{-1}$). Accordingly, the maximum mean counts (log $CFUg^{-1}$) of AMB, yeasts, staphylococci, Enterobacteriaceae, coliform, and moulds were observed in firfir, which accounted 7.3, 6.2, 5.6, 5.5, 4.8 and 4.6, respectively.

There was statistically significant difference ($p < 0.05$) among the mean counts of AMB, Enterobacteriaceae, coliform, ABS, staphylococci, LAB, yeasts and moulds in all food samples between the groups. However, there was no significant difference ($p > 0.05$) of the mean counts in all microbes among the three sites.

Table 2. Source of water and utensils handling of street vendors in Jimma town, southwestern Ethiopia 2011/2012.

Parameter	Number of respondents (N=110)	Percentage
Source of water for preparation of food		
Tap	71	64.5
Well	30	27.3
Spring	7	6.4
Source of water for cleaning utensils		
Tap	49	44.5
Well	54	49.1
River	7	6.4
Clean the utensils		
By hand using water only	48	43.6
With warm water and soap	14	12.7
With cold water and soap	28	25.5
Frequency of changing the vending utensils		
Daily	16	14.6
Weekly	25	22.7
Monthly	11	10.0
Yearly	4	3.6
Not changed	54	49.1

Table 3. Food-handling practices, vendors personal hygiene and awareness on food and water-borne disease in Jimma town, southwestern Ethiopia 2011/2012.

Parameter	Number of respondents (N=110)	Percentage
Apparel for vending		
Yes	10	9.1
No	100	90.9
Handling food with bare hands		
Yes	89	80.9
No	21	19.1
Vendors covered their hair		
Yes	66	60.0
No	44	40.0
Vending site neatness		
No litter	7	6.4
Some litter	25	22.7
Much litter	78	70.9
Dusty vending site		
Yes	89	80.91
No	21	19.09
Undergone training about of vending food		
Yes	8	7.3
No	102	92.7
Informed about food and water-borne disease		
Yes	37	33.6
No	73	66.4

Microbial analysis of street-vended foods

From Table 5 out of total 160 food samples analyzed, 457 bacterial strains were isolated from firfir, 440 from bread and 400 from each of injera and sambussa. Totaling 1697 bacterial isolates. Generally, among the

Table 4. Mean microbial counts (log $CFUg^{-1}$) in Jimma town, southwestern Ethiopia 2011/2012.

Food type	Microbial mean counts (log $CFUg^{-1}$ ± SD)															
	Number	AMB	% CV	Entero	% CV	Coli	% CV	ABS	% CV	Staph	% CV	LAB	% CV	Yeast	% CV	Mould	% CV
Firfir	40	6.6 ± 0.6	9.1	4.9±0.5	10.2	4.0±0.5	12.5	4.3±0.4	9.3	4.8±0.7	14.6	4.3±0.5	11.6	5.5±0.5	9.1	3.7±0.6	16.2
Bread	40	5.1 ± 0.5	9.8	4.1±0.6	14.6	2.5±0.5	20.0	4.0±0.4	10.0	4.1±0.6	14.6	4.2±0.3	7.1	4.0±0.5	12.5	2.6±0.3	11.5
Injera	40	4.4 ± 0.3	6.8	4.0±0.6	15.0	2.2±0.3	13.6	3.8±0.5	13.2	3.1±0.6	19.4	4.1±0.5	12.2	3.4±0.4	11.8	2.4±0.2	8.3
Sambussa	40	4.0 ± 0.4	10.0	2.7±0.5	18.5	1.7±0.3	17.6	3.8±0.3	7.9	2.7±0.4	14.8	3.2±0.4	12.5	2.2±0.5	22.7	1.8±0.4	22.2
Total		5.0 ±0.5	10.0	3.9 ±0.6	15.4	2.6±0.4	15.4	4.0±0.4	10.0	3.7±0.6	16.2	4.0±0.4	10.0	3.8±0.5	13.2	2.6 ±0.4	15.4

AMB = Aerobic Mesophilic Bacteria; Entero = Enterobacteriaceae; ABS = Aerobic Bacterial Spore; Staph =Staphylococci; LAB =Lactic Acid Bacteria, Coli = Coliform.

Table 5. Frequency distribution of dominant bacteria in some street-vendedfood, Jimma town, southwestern Ethiopia 2011/2012.

Food Type	Number of isolates	*Bacillus* spp.	*Staphylococcus* spp.	*Micrococcus* spp.	Enterobacteriaceae	*Pseudomonas* spp.	*Acinetobacter* spp.	*Alcaligens* spp.	*Aeromonas* spp.
	Number	Frequency (%)	Frequency (%)	Frequency (%)	Frequency (%)	Frequency (%)	Frequency (%)	Frequency (%)	Frequency (%)
Firfir	457	157 (34.35)	143 (31.29)	85 (18.6)	42 (9.19)	12 (2.63)	9 (1.97)	6 (1.31)	3 (0.66)
Bread	440	227(51.59)	67 (15.23)	80 (18.18)	27 (6.14)	18 (4.09)	11 (2.5)	10 (2.27)	-
Injera	400	148 (37)	104 (26)	59 (14.75)	65 (16.25)	15 (3.75)	-	6 (1.5)	3 (0.75)
Sambussa	400	180 (45)	98 (24.5)	76 (19)	17 (4.25)	13 (3.25)	4 (1)	5 (1.25)	7 (1.75)
Total	1697	712 (41.96)	412 (24.28)	300 (17.68)	151 (8.90)	53 (3.12)	26 (1.53)	30 (1.77)	13 (0.77)

total isolates, the predominant bacterial group was *Bacillus* spp. (41.96%) followed by *Staphylococcus* spp. (24.28%), *Micrococcus* spp. (17.68%) and Enterobacteriaceae (8.9%).

Prevalence of *S. aureus* and *Salmonella* spp.

In Table 6, the overall 29.38% samples were positive for *S. aureus*. However, the frequency distribution varied among the food samples. the highest (57.5%) being found in firfir and the lowest being found in sambussa (12.5%) . With regard to sites, the prevalence of *S.aureus* was comparably higher in firfir from Merkato, Agip and Kochi sites. No *S.aureus* was detected in sambussa sample. from Agip site.

For overall, 13.13% samples were positive for *Salmonella* isolates. As a result, the prevalence of *Salmonella* spp. was higher (27.5%) in firfir and bread (12.5%) and low in injera (7.5%) and sambussa (5.0%). In terms of site, the prevalence of *Salmonella* spp. was highest in firfir from Merkato (33.3%) and Agip (27.27%). However, *Salmonella* spp. were not isolated from injera at Merkato site and sambussa from Agip and Merkato sites.

Antimicrobial Susceptibility patterns of *S. aureus* and *Salmonella* spp.

From Table 7, out of a total 47 isolates of S. *aureus,* the majority were susceptible to chloramphenicol and gentamycin (95.74% each) followed by ciprofloxacin (93.62%), streptomycin (85.11%) and kanamycin (76.59%). However, the isolates were highly resistant to penicillin G (100%) followed by clindamycin (80.85%) and tetracycline (63.83%).

From Table 8, all the 21 isolates of *Salmonella* spp. were susceptible (100%) to ciprofloxacin, gentamycin and norflaxacin followed by chloramphenicol (95.24%), and kanamycin (85.71%) (Table 10). However, the highest frequency of resistance to ampicillin (95.24%) was observed followed by naldixic acid (76.19%) and streptomycin (47.62%).

From Table 9, the multi-drug resistance (MDR) patterns of *S.aureus* showed that, 38.30% of the

Table 6. Prevalence of *S. aureus* and *Salmonella* spp. from street-vended food in Jimma town, southwestern Ethiopia 2011/2012.

Sample	Sample size (160)	Number *S. aureus* positive samples (%)	Number *Salmonella* spp. positive samples (%)
		Frequency (%)	
Firfir	40	23 (57.5)	11 (27.5)
Bread	40	13 (32.5)	5 (12.5)
Injera	40	6 (15.0)	3 (7.5)
Sambussa	40	5 (12.5)	2 (5.0)
Total	160	47 (29.38)	21 (13.13)

Table 7. Antimicrobial susceptibility patterns of *S. aureus* isolated from street-vended food in Jimma town, southwestern Ethiopia 2011/2012.

Antimicrobial Agents	Disc concentration (µg/ml)	Resistance	Intermediate	Sensitive
		Frequency (%)	Frequency (%)	Frequency (%)
Chloramphenicol (C)	30	1(2.13)	1 (2.13)	45 (95.74)
Ciprofloxacin (CIP)	5	-	3 (6.38)	44 (93.62)
Clindamycin (DA)	2	35 (74.47)	3 (6.38)	9 (19.15)
Erythromycin (E)	15	5 (10.64)	21 (44.68)	21 (44.68)
Gentamycin (CN)	10	1(2.13)	1 (2.13)	45 (95.74)
Kanamycin (K)	30	2 (4.26)	9 (19.15)	36 (76.59)
Penicillin G (P)	10	47 (100)	-	-
Streptomycin (S)	10	-	7 (14.89)	40 (85.11)
Tetracycline (TE)	30	6 (12.77)	24 (51.06)	17 (36.17)

Table 8. Antimicrobial susceptibility patterns of *Salmonella* spp. Jimma town, southwestern Ethiopia 2011/2012.

Antimicrobial Agents	Disc concentration (µg/ml)	Resistance	Intermediate	Sensitive
		Frequency (%)	Frequency (%)	Frequency (%)
Ampicillin (AMP)	10	20 (95.24)	-	1 (4.76)
Chloramphenicol (C)	30	-	1 (4.76)	20 (95.24)
Ciprofloxacin (CIP)	5	-	-	21 (100)
Gentamycin (CN)	10	-	-	21 (100)
Kanamycin (K)	30	-	3 (14.29)	18 (85.71)
Naldixic Acid (NA)	30	12 (57.14)	4 (19.05)	5 (23.81)
Norflaxacin (NOR)	10	-	-	21(100)
Streptomycin (S)	10	5 (23.81)	5 (23.81)	11 (52.38)
Tetracycline (TE)	30	8 (38.10)	-	13 (61.90)

isolates were resistant to 3 antibiotics (mainly DA/ P/ E and DA/ P/TE combinations) followed by 23.40% to 4 antibiotics and 14.89% to 5 antibiotics (Table 11). The highest MDR in *S.aureus* (17.02%) was for four antibiotics (mainly DA/P /E/TE). The maximum number of antibiotics resisted by *S.aureus* was six antibiotics with total proportion of 8.51%. Generally, MDR to three and four antibiotics dominated the resistance pattern (Table 9).

In Table 10, the MDR profile of *Salmonella* spp. showed the highest resistance (33.33%) of the isolates towards three antibiotics followed by two and four antibiotics (14.29% each). In case of *Salmonella* spp., the maximum number of antibiotics resisted was four antibiotics. However, the highest MDR (14.29%) was observed for NA/AMP/S (resistance to naldixic acid, ampicillin and streptomycin). Generally, MDR to three antibiotics dominated the resistance pattern

Table 9. MDR patterns of *S. aureus* isolated from street-vended food in Jimma town, southwestern Ethiopia 2011/2012.

Number of antimicrobial resistance	Antimicrobial resistance patterns	Number of isolates (%)	Total (%)
Two	DA/ P	3(6.38)	4(8.51)
	P/TE	1(2.13)	
Three	P/E/S	2(4.26)	18(38.30)
	P/E/CIP	1(2.13)	
	P/K/TE	1(2.13)	
	DA/ P/K	2(4.26)	
	DA/ P/ E	6(12.77)	
	DA/ P/TE	6(12.77)	
Four	DA/P/K/S	1(2.13)	11(23.40)
	DA/P/K/TE	2(4.26)	
	DA/P /E/TE	8(17.02)	
Five	DA/P/E/S/TE	1(2.13)	7(14.89)
	DA/P/E/K/TE	4(8.51)	
	DA/P/E/C/TE	1(2.13)	
	DA/P/E/CN/TE	1(2.13)	
Six	DA/P/E/S/TE/C	1(2.13)	4(8.51)
	DA/P/E/K/S/TE	1(2.13)	
	DA/P/E/CIP/S/TE	1(2.13)	
	DA/P/E/K/CIP/TE	1(2.13)	

DA=Clindamycin; P=Penicillin, TE=Tetracycline; E=Erythromycin; C=Chloramphenicol; CN= Gentamycin; K=kanamycin, NA= Naldixic Acid; S= Streptomycin; CIP= Ciprofloxacin.

Table 10. MDR of *Salmonella* spp. isolated from street-vended food in Jimma town, southwestern Ethiopia 2011/2012.

Number of antimicrobial resistance	Antimicrobial resistance patterns	Number of isolates (%)	Total (%)
Two	AMP /S	1(4.76)	3 (14.29)
	TE/AMP	1(4.76)	
	NA /AMP	1(4.76)	
Three	TE/AMP/S	2 (9.52)	7 (33.33)
	TE/AMP/K	2 (9.52)	
	NA/AMP/S	3 (14.29)	
Four	NA/AMP/S/K	1(4.76)	3 (14.29)
	NA/TE/AMP/S	1(4.76)	
	NA/TE/AMP/C	1(4.76)	

AMP, Ampicillin; TE, Tetracycline; C, Chloramphenicol; K, kanamycin; NA, Naldixic Acid; S, Streptomycin.

(33.33%)

DISCUSSION

The socio-demographic characteristics of street vendors in the present study showed that the majority of street vendors were females. Similarly, Mensah et al. (2002) in their study in Accra, Ghana, found that 100% of vendors were females. In addition, Chukuezi (2010) in Nigeria also reported that majority (66.67%) of the street vendors were females. In the current study, 49.1% of vendors

used well water for cleaning utensils and 27.3% of them used well water for cooking. Ali et al. (2011) reported that well waters used for drinking and washing in Jimma town were not of acceptable range both in physico - chemical property and bacteriological safety. Water is a critical raw material in many street vending food operations. One of the most critical problems in street food vending is the supply of water of acceptable quality and insufficient quantities for drinking, washing, cleaning and other operations.

The food handling practices of the street vendors in the present study showed that, 90.9% served their customers without having a special apparel and 80.9% handled food with their bare hands. These numbers were higher than what was reported by Chukuezi (2010) from Owerri, Nigeria, where 42.86% had not used special apparel for vending food and 47.62% handled food with bare hands. Insanitary handlings of street foods by some of the vendors lead cross contamination (Dawson and Canet, 1991). The vendors can be carriers of pathogens like *E.coli*, *Salmonella* and *S. aureus* and eventually can transfer these foodborne pathogens to the consumers. The hands of the food handlers are the most important vehicle for the transfer of organisms from faeces, nose, and skin to the food (WHO, 1989).

In the present study, the majority of vendors had no education or training about the hygiene of vending food and many had no information about food and waterborne diseases. Education or training is critical for street vendors at all ages to prevent cross contamination and mishandling of foods at home and vending site. Hence, training and sharing of information to the vendors are critical at all levels of preparation (Collins, 1997). FAO (1998) also suggested that the food handlers should have the necessary knowledge and skills to handle food hygienically

The mean total counts of AMB (5.0 $CFUg^{-1}$) observed in the present study is in agreement with the findings of Bryan et al. (1997), who reported between 3 to 9 log $CFUg^{-1}$ from meatballs of street-vended foods from Zambia. In general, there is no standard set for the permissible level of microbes for street-vended food being served in Ethiopia. However, Gilbert et al. (2000) set the recommended guideline of street-vended food in London. According to this guideline, all food samples in the present study belonged to Level 1, which mean all food samples were fully cooked. Specifically the mean counts of AMB in all food samples (firfir, bread, injera and sambussa) in the present study were 4 log $CFUg^{-1}$ and above. Hence, they belonged to unsatisfactory level (≥ 4 log $CFUg^{-1}$). This could be as a result of from vendors' personal hygiene and dust, and litter at the vending sites. Most processed foods are regarded as harmful when they have large populations of aerobic mesophilic microorganisms, even if the organisms are not known to be pathogens (Sudershan et al., 2009).

The mean count of Enterobacteriaceae in the present study was 3.9 log $CFUg^{-1}$. This agrees with the findings of Mustafa and Abdulla (2011), who reported the counts between 2.3 to 4.4 log $CFUg^{-1}$ in Sudanese street-vended traditional foods. According to the guideline, the mean counts ($CFUg^{-1}$) of Enterobacteriaceae in firfir (4.9), injera (4.1) and bread (4.0) showed unsatisfactory level (≥ 4 log $CFUg^{-1}$), whereas sambussa (2.7) belonged to the group with acceptable level (2 to < 4 log $CFUg^{-1}$). Enterobacteriaceae and the high number AMB clearly suggest that the poor hygiene could be a source of foodborne disease (Motarjemi et al., 1993).

The mean count of coliform in the present study is between 1.7 to 4.0 $CFUg^{-1}$. The presence of coliforms in the present study could be due to fecal contamination of food or water after preparation. Heat-processed foods usually have no vegetative microbial contaminants immediately after cooking. However, food could be contaminated later. Most probably, the source of contamination for food was the water used in washing utensils or directly from hands or bodies of vendors. Of course, once introduced into the food samples and the foods left at ambient temperature for a while, the contaminating coliform would multiply to higher counts (Tomkins, 1981). The fecal coliforms have been used as an indicator of the sanitary conditions. Since the indicator is a typical component of the fecal microbiota, its detection indicate the potential occurrence of other microorganisms which could be even more pathogenic to human and both domestic and wild animals (Souza et al., 2005).

The mean aerobic bacterial spore (ABS) count (4.0 log $CFUg^{-1}$) of the present study is higher compared to report by Mosupye and Van Holy (1999) where the counts ranged between 1.2 to 2.0 log $CFUg^{-1}$ in ready to eat food samples from Johannesburg, South Africa. According to the guideline, mean counts of ABS in firfir (4.3 log $CFUg^{-1}$) and bread (4.0 log $CFUg^{-1}$) were of unsatisfactory level (>4 log $CFUg^{-1}$) whereas injera and sambussa (3.8 log $CFUg^{-1}$each) were in acceptable level (3 to < 4 log $CFUg^{-1}$). The higher counts in the present study could be due to the contamination of food by the heat resistant spores, which had survived cooking temperature because of temperature / time inadequacy during preparation of food.

The mean counts of staphylococci in the present study were 3.7 log $CFUg^{-1}$. This is in agreement with the microbiological studies made on street-vendedfoods in India (Mohapatra et al., 2002), where counts were greater than 3 log $CFUg^{-1}$. The higher levels of staphylococci in the present study could be due to unhygienic handling of food items by the vendors and vendors' personal hygiene. The interview results showed 80.9% of the vendors handled food with their bare hands and 90.9% did not use special cloth while processing and vending the food samples. According to Mensah et al. (2002), the uses of a fork or spoon to serve food reduced the level of contamination, while the use of bare hands resulted in an increase of contamination. Staphylococci exist in air,

dust, sewage, water and food or on food equipment and environmental surfaces. *S.aureus* can be found in the nose (50%), on hands (5-30%), in hair, eyes and throat of healthy persons (Hammad, 2004).

In the present study, the mean count of LAB was 4.0 log $CFUg^{-1}$. Incontrast to the present study, Omemu and Omeike (2010) reported the higher count ranging between 4.5 to 9.2 log $CFUg^{-1}$ in cooked ogi used as weaning food from Nigeria. The high count of LAB in food has significant impact in lowering the counts of pathogens. The growth of many bacteria is inhibited or decreased in the presence of lactic acid bacteria (Shirazinejad et al., 2010).

The mean counts (log $CFUg^{-1}$) of moulds and yeasts in the present study were 2.6 and 3.8, respectively. The presence of high count of yeasts and moulds in the present study could be due to litters in the environment. Yeasts do not produce adverse toxins to humans. However, some moulds produce toxic substances that can cause disease or illness when eaten by humans. It is difficult to prevent moulds from growing on foods but their levels can be decreased by maintaining hygienic conditions during food processing and storage. Spores of mould can be carried by wind, and hence can easily enter into food sample (Stratford, 2006).

The predominant microfloral of street-vended foods in the present study was generally *Bacillus* spp. (41.96%) followed by *Staphylococcus* spp. (24.28%), and *Micrococcus* spp. (17.68%). The current study showed higher percentage of isolates than Muleta and Ashenafi (2001b) report where the isolates from street-vended food were dominated by *Bacillus* spp. (29.1%), followed by *Staphylococcus* spp. (22.8%) and *Micrococcus* spp. (15.4%) in Addis Ababa, Ethiopia. The predominance of *Bacillus* spp. among isolates on aerobic plate count plates was possibly due to the presence of spores in the raw materials. The heat-resistant spores may have survived cooking while vegetative bacteria were eliminated (Mosupye and von Holy, 1999). High number of *Bacillus* spp. could cause food poisoning result foodborne disease. *Micrococcus* spp. and *Staphylococcus* spp. were among the dominant isolates possibly due to much litter and dust in vending sites, handling of food with bare hands and the vendors serving utensils stayed for a long period before replacement. They are common environmental bacteria that could be introduced into the food after cooking through cross-contamination (Cardinale et al., 2005).

The prevalence of *S. aureus* and *Salmonella* spp. in the present study were 29.38 and 13.13% respectively. The higher prevalence of *Salmonella* spp. and *S. aureus* in the present study could be due to handling of food with bare hand, washing of the utensils using well water, litter and dust at vending site. The presence of a large number of *S. aureus* in a food may indicate poor handling or sanitation and lead to cross contamination from vendors to food (Sina et al., 2011). Ready-to-eat foods should be free of *Salmonella* as consumption of food containing this pathogen may result in foodborne illness. The presence of this organism indicates poor food preparation and handling practices such as inadequate cooking (Tunung et al., 2007).

The antibiotic resistance patterns of the isolates revealed low number of resistance (4.26%) of *S.aureus* to each of chloramphenicol and gentamycin. Similarly, Alina et al. (2011) reported lower number of S*. aureus* isolates resistant to chloramphenicol (0.5%) and gentamycin (1.5%). In the current study, all S*. aureus* isolates were resistant to Penicillin G. This was in agreement with Sina et al. (2011) who reported that, 100% of the isolates were resistant to Penicillin G. The resistance of *S. aureus* to penicillin G could be due to the production of penicillinase enzyme (a type of ß-lactamase) that hydrolyzed the beta-lactam ring of penicillin (Lowy, 2003).

The prevalence of antimicrobial resistance among foodborne pathogens has increased during recent decades (Chui et al., 2002), possibly as a result of selection pressure created by the use of antimicrobials in food-producing animals (Bywater, 2004). The coexistence of resistance genes with mobile elements such as plasmids and transposons facilitate the rapid spread of antibiotic resistant genes among bacteria (Sunde, 2005).

The study in Sarab, Iran by Akbarmehr (2012), showed that *Salmonella* spp. were highly susceptible to chloramphenicol (100%) followed by ciprofloxacin and gentamycin (91.89% each). However, isolates of *Salmonella* spp. exhibited resistance to streptomycin and tetracycline (29.72% each) and ampicillin (13.51%). In the present study, high number of *Salmonella* spp. was susceptible to ciprofloxacin and gentamycin (100% each) and lower number to chloramphenicol (95.24%). In this study we found, lower resistance to streptomycin (23.81%) while higher resistance was observed in tetracycline (38.1%) and ampicillin (95.24%). Antibiotics such as ampicillin and sulphamethoxazole are the first line antibiotics used for the treatment of salmonellosis. However, *Salmonella* strains which are resistant to these first-line antibiotics have recently emerged worldwide, and is causing great concern. With that increase, the risk to public health has also increased. It is particularly serious in low-resource countries where bacterial infections remain among the major causes of death (Bartoloni et al., 2005).

Conclusions

The overall microbial quality of street-vended foods assessed in the current study was poor as compared to the guidelines set by other regulatory bodies. This could be due to the poor personal hygienic practice of street vendors such as handling food with bare hand, washing the utensils using well water, availability of dust and litters

at vending site and lack of training for vendors lead to the higher level of microbial load though cross contamination. The most predominant microbial groups were *Bacillus* spp., *Staphylococci* spp., *Micrococcus* spp. and Enterobacteriaceae. Thus, the presence these microorganisms could be a possible prediction for the presence of pathogens. The presence of high number of pathogenic bacteria such as *Salmonella* spp. and *S.aureus* could cause foodborne diseases like diarrhea, typhoid fever and food poisoning. *S.aureus* isolates were susceptible to chloramphenicol, gentamycin and ciprofloxacin but resistant to penicillin G. On the other hand, *Salmonella* spp. was susceptible to ciprofloxacin, gentamycin, norflaxacin and chloramphenicol. However, it was resistant to ampicillin and naldixic acid. The consumption of un hygienically prepared and contaminated street-vended foods could lead to the dissemination of drug resistant bacteria such as *Salmonella* and *S. aureus.*

RECOMMENDATIONS

Vendors should wash hands before and after preparation of food, cleaning the vending site with the collaboration of the municipal of Jimma town, washing the utensils with soap and warm water and serve the consumers using fork or spoon. The concerned bodies like the municipal of Jimma town, health official of Jimma town and other voluntary NGO's should give attention to improve the quality of street vending food by providing training to the vendors to keep their personal hygiene and clean the vending sites. Moreover, vendors should adequately heat vended food. The guideline for Ethiopia street vending food should be set. The unusually high microbial load of firfir and bread samples calls for regular inspection for safety of street-vended foods. As significant numbers of population in Jimma town is earning income from street vending business, government should give attention and provide the necessary infrastructure to the vendors in order to improve the safety of street-vended foods. Currently, different antibiotics are available in a market among these and it is better to use chloramphenicol, gentamycin and ciprofloxacin for foodborne disease caused by *S. aureus* and ciprofloxacin, gentamycin, norflaxacin and chloramphenicol for foodborne disease caused by *Salmonella* spp. It is necessary to enhance food hygiene practices to reduce or eliminate the risk from resistance to antibiotic and pathogenic bacteria originating from food. Identification of the isolates could not show the exact identity. Thus, molecular approach can overcome this drawback.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Isolation of gut associated bacteria from mangrove crabs collected from different mangrove regions of Tamil Nadu, South east coast of India

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Mangrove crabs are mostly herbivorous which consume more amount of leaf litter of various mangrove species and also plays most important role in leaf degradation. Several studies reported that crab harbor bacteria from the environment through water and food. Bacterial species of the gut can influence the health and robustness of the host. The present study aims to isolate and enumerate the bacterial count from the gut of crabs collected from different mangrove environments. The results shows that maximum bacterial load was recorded in *Sesarma brockii* crabs gut collected from Pichavaram mangroves and minimum was observed in *Metopograpsus maculatus* crabs collected from Uppanar estuarine mangrove. In the same way, maximum bacterial load was observed in both water and sediment samples of Pichavaram. Bacteria belonging to the genera *Bacillus*, *Pseudomonas* and *Aeromonas* were found at higher levels in all the different mangrove regions. In conclusion, crabs in the various mangrove environments carry a particular bacterial flora, which reflects their environment. The Pichavaram mangrove ecosystem is endowed with a high bacterial load due to the continuous shedding of foliage into the water and subsequent decomposition than other mangrove environments. SEM results confirm that crowded populations of bacteria were attached to the gut region of the mangrove crabs.

Key words: Mangrove crabs, gut microflora, isolation, characterization, sediments.

INTRODUCTION

Several species of mangrove crabs are recognized as herbivorous and can consume a good quantity of leaf litter of different mangrove species. These crabs play a key role in the process of leaf degradation and making mangrove leaves more rapidly available to meiofauna (Ravichandran et al., 2007b). Crab receives bacteria in the gut from their aquatic environment through water and food that are populated with bacteria. Being rich in nutrient, the environment of the crab gut confers favourable conditions for the microorganisms.

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The intestine microbial diversity has been studied in a wide range of aquatic animals (Liu et al., 2011).

The importance of gut bacteria in the nutrition and well being of their hosts has been established for homoeothermic species, such as birds and mammals. It is clear that bacterial species of the gut can influence the health and development of the host. Extreme examples of the influence of the gut flora embrace the negative effects of pathogenic organisms and in contrast, the total reliance that ruminants have on their gut flora for the assimilation of organic carbon from the environment (Kennedy et al., 1991). An understanding of the host intestinal bacterial floral interactions is of much significance for the development of a healthy cultivation environment and also to optimize the possible species growth in aqua culture (Oxley et al., 2002). Recent studies suggest that the predominant rod-shaped bacteria in the hindgut are tightly attached to the epithelium surface by an unusual pili-like structure (Chen et al., 2015). Several studies reported on gut flora of marine crabs which includes *Callinectes sapidus* (Huq et al., 1986), *Macrocheira kaempferi* (Ueda et al., 1989), *Eriocheir sinensis* (Li et al., 2007), *Scylla serrata, Scylla tranquebarica, Portunus pelagicus, Portunus sanguinolentus, Charybdis helleri* (Ravichandran and Kannupandi, 2005; Rameshkumar et al., 2009) and reports also includes on pathogenic bacteria isolated from gut of *Portunus pelagicus* (Talpur et al., 2011). Despite these reports, there is still lack of similar information on crab species which inhabit mangrove environment. Hence, the present study was designed to isolate and enumerate the total bacterial population from fifteen different mangrove crabs species which were collected from various stations mangrove environments and also examined the gut associated bacteria using scanning electron microscope (SEM).

MATERIALS AND METHODS

Sample collection

From five different mangrove regions including Muthupet, Pazhayar, Pichavaram, Vellar and Uppanar, crabs were collected during the period of November 2013 to October 2014. Fifteen crab species including *Heteropanope indica*, *Macrophthalmus depressus*, *Metapograpsus maculatus*, *Metapograpsus messor, Nanosesarma batavicum, Nanosesarma minutum, Neoepisesarma mederi, Neoepisesarma tetragonum, Pseudograpsus intermedius, Sesarma andersoni, Sesarma bidens, Sesarma brockii, Sesarma plicatum, Uca annulipes* and *Uca triangularis* were collected and each species contain 25 individuals taken for the present study. Sterile forceps were used to pick the crabs and crab species were collected immediately and transferred to sterile polythene bags. All the samples were transported immediately to the laboratory and subjected to several analysis. Water and sediment sample were collected from all the five stations using sterile bottles and sterile polythene bags (approximately, 100 ml of water and 100 g of sediment from each site) using sterile spatula.

Segregation of the gut

Prior to segregation of the gut of each crab, species were bathed in 10% formalin for 30 s. Again, the crabs were washed with tap water for 5 min and finally with sterile de-mineralized water in order to remove surface micro flora. Sterilized dissecting materials were used for this study. Crab specimens were dissected and the whole gut was removed (Talpur et al., 2011).

Enumeration of total bacterial load

Gut sample

To avoid individual variations of the gut microflora (Spanggaard et al., 2000), the gut of 15 species of crabs were pooled by species and homogenized in 10 ml of a sterilized nine-salt solution (NSS) (Olsson et al., 1992). Gut homogenates and water sample were diluted in NSS up to ten fold times and appropriate dilutions were spread on the surface of Zobell marine agar (ZMA) plates (Hi-media, Mumbai) in triplicates. The plates were incubated at 28°C for 24 – 48 h. The microbial load was counted and mentioned as the number of colony forming units (CFU).

Water and sediment sample

From the collected water sample, 1 ml of water sample was pipetted out using a sterile pipette into a 9 ml blank and shaken well. From this, 1 ml was pipetted out and added to the 9 ml blank, likewise the serial dilutions were made upto sixth dilutions and used as inoculum. From the sediment sample, 1 g of sediment from each station was transferred aseptically to a 99 ml blank. The contents were homogenized for 10 min. From this, 1 ml was transferred aseptically to a 9 ml blank and mixed thoroughly. Likewise, serial dilutions were made and used as inoculum. Appropriate dilutions were spread on the surface of ZMA plates (Hi-media, Mumbai) in triplicate. The plates were incubated at 28°C for 24 - 48 h. The microbial load was counted and it was recorded as the number of CFU.

Characterisation of isolated bacteria

The isolates were characterised by Gram staining and biochemical test includes motility, oxidase activity, catalase activity, oxidation/fermentation, glucose acid, glucose gas, pigment production and citrate utilization. A series of secondary tests were used (indole, methyl red,Voges-Proskauer, citrate utilization, triple sugar iron,urease, lactose fermentation nitrate reduction, catalase, oxidase and starch hydrolysis tests), when required, to complete the genus level confirmation of isolates. 0% sodium chloride media tests were also included.

Scanning electron microscopic studies

Immediately after dissection, gut of the crabs were fixed in 3% gluteraldehyde in 0.1 M sodium cacodylate, pH 7.4, for 3 h. Samples were washed three times in sodium cacodylate buffer post fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate for 1 h, and washed another three times before dehydrating in a series of ethanol dilutions. Before examining, samples were mounted on aluminium stubs, coated with gold palladium by critical point and dried using carbon dioxide as the transitional fluid, and examined in a JEOL JSM-840 scanning electron microscope (SEM).

Table 1. Number of isolated bacterial genus from the mangrove crabs.

Mangrove crab	*Acinetobacter*	*Bacillus*	*Enterobacter*	*Vibrio*	*Alcaligenes*	*Photobacterium*	*Pseudomonas*	*Aeromonas*	*Flavobacterium*	*Staphylo coccus*	Total
Heteropanope indica	3	6	2	2	5	2	2	2	0	3	27
M. messor	2	5	2	4	2	2	4	5	1	3	30
Macrophthalmus depressus	4	5	3	3	4	2	1	1	0	2	25
Metapograpsus maculatus	1	7	5	3	5	6	2	2	2	1	34
N. batavicum	2	6	2	3	2	1	4	3	1	3	27
N. mederi	4	9	1	5	2	4	6	7	0	1	39
Nanosesarma minutum	2	5	2	2	2	5	4	1	2	0	25
Neoepisesarma tetragonum	2	6	1	2	3	2	4	5	0	0	25
Pseudograpsus intermedius	4	8	3	3	5	2	4	6	4	2	41
S. bidens	1	9	2	5	1	3	4	4	1	0	30
S. brockii	6	14	5	2	5	5	9	8	4	0	58
S. plicatum	3	9	3	4	4	5	8	8	2	0	46
Sesarma andersoni	2	10	3	2	3	4	5	1	0	2	32
U. triangularis	2	6	5	5	3	2	4	3	2	7	39
Uca annulipes	4	8	3	3	6	4	4	6	1	6	45
Total	42	113	42	48	52	49	65	62	20	30	523

RESULTS

Total bacterial population

Gut sample

The numbers of cultivable bacterial cells present in crab gut were estimated after isolation and growth on ZMA plates incubated at room temperature at 28°C. The total heterotrophic bacterial load ranged between 0.7 ± 0.49 x10^6 and 8.9 ± 0.13 x10^6 CFU/g of crabs gut sample and it was found to be the maximum (8.9 ± 0.13 x10^6 CFU/g) in *S. brockii* gut collected from Pichavaram mangrove region and minimum (0.7 ±0.49 x10^6) in *M. maculatus* collected in the mangrove region of Uppanar. Between the crab species, the highest bacterial load was noticed in *S. brockii* in all the five stations of Tamil Nadu (Table 1) and between the stations crabs collected from Pichavaram showing the maximum bacterial populations followed by Vellar, Pazhiyar, Muthupet and Uppanar (Figure 1).

Water and sediment sample

Total bacterial load of water from five mangrove regions were enumerated. Among the five stations, a high bacterial load was observed in the sample collected at Pichavaram mangrove region (7.2±0.37x10^6) and minimum of 4.2 ±0.51x10^6 was observed in Uppanar estuarine mangrove (Figure 2). Similarly, in sediment samples, maximum bacterial load was observed in Pichavaram mangrove region (9.4±0.51 x10^6) followed by Vellar (6.5±0.29x10^6), Pazhiyar (6.3 ±0.49x10^6), Muthupet (6.0 ±0.51 x10^6) and Uppanar estuary (5.3 ±0.81 x10^6), respectively (Figure 1). Two-way ANOVA showed significance at 0.05% level between all the stations (Table 1).

Characterisation of isolated bacteria

A total of 523 bacterial strains were isolated from gut of fifteen mangrove crab species collected from five different stations and classified into ten taxonomic groups including highest number of followed by *Acinetobacter* (42), *Aeromonas* (62), *Alcaligenes* (52), *Bacillus* (113) *Enterobacter* (42), *Flavobacterium* (20) *Photobacterium* (49), *Pseudomonas* (65), *Staphylococcus* (30) and *Vibrio* (48), (Table 1). All the isolated bacteria were tested for their biochemical characters which showed 89% of the isolates were Gram negative bacilli and 6% were Gram negative cocci, 4%

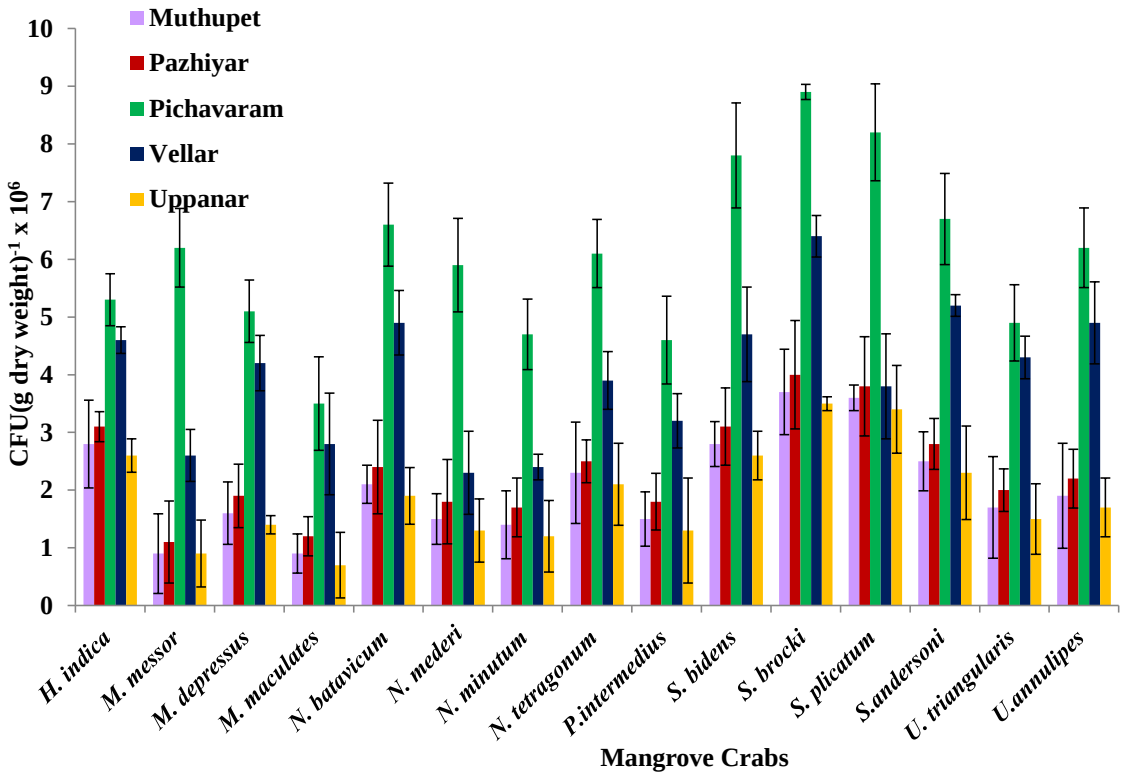

Figure 1. Total bacterial load in the gut of various mangrove crabs from five various stations.

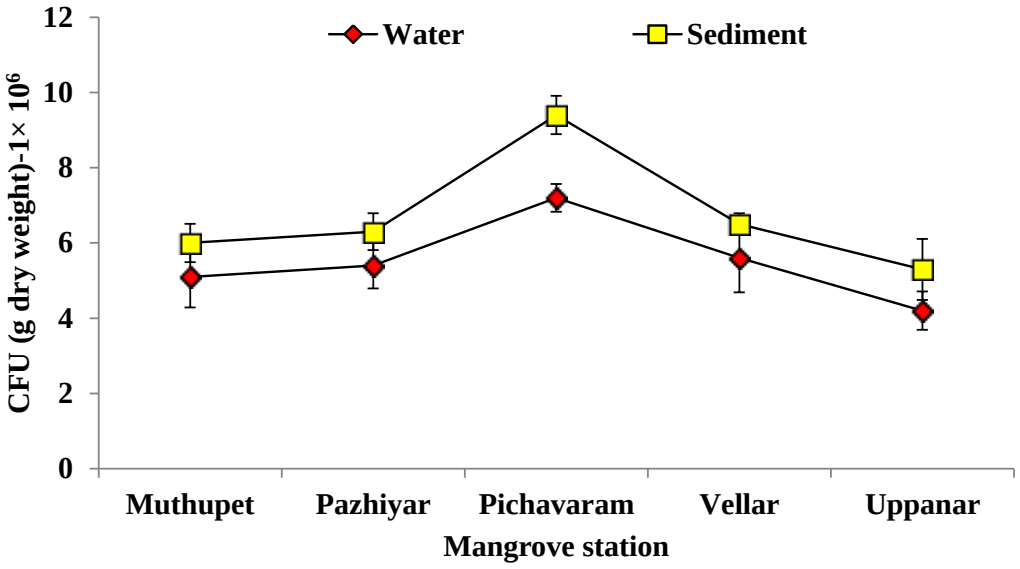

Figure 2. Total bacterial load from five mangrove environments.

were Gram positive bacilli and the remaining 1% was Gram positive cocci. The bacterial isolate showed 40% utilized the citrate and 20% strains were positive in indole and also in H_2S production test. Methyl red test and lactose fermentation showed positive on 50% of the isolates. 30% of the isolates showed positive results on

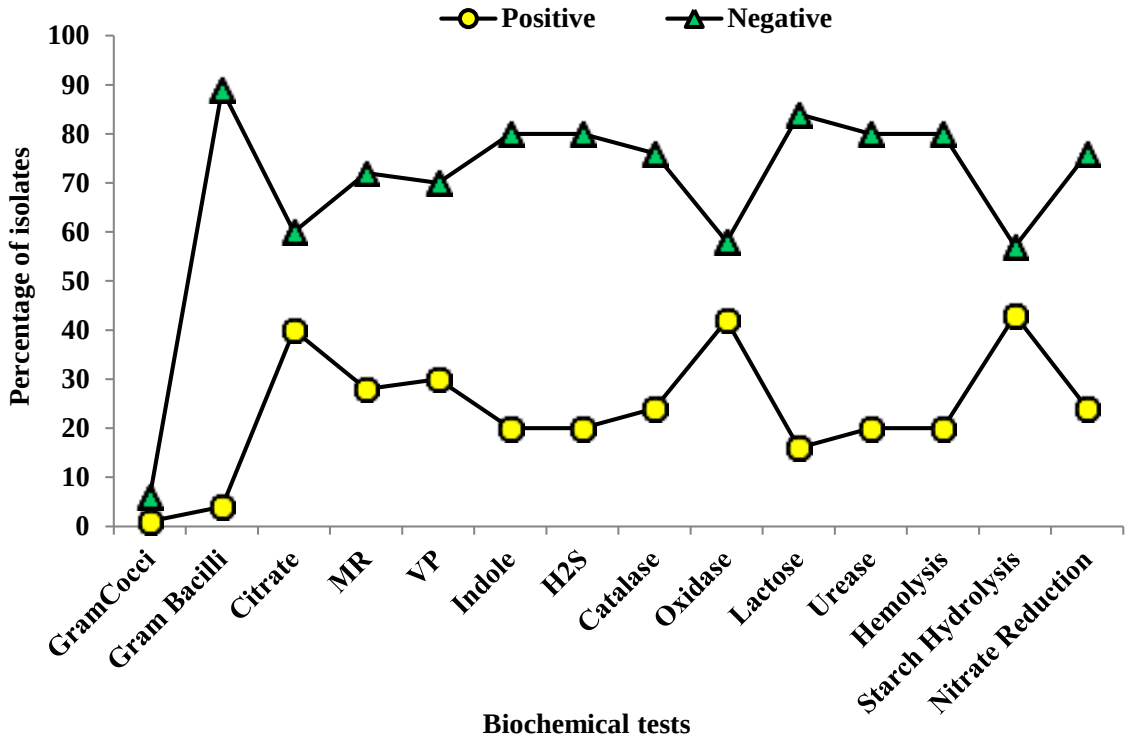

Figure 3. Biochemical characteristic of bacteria isolated from various mangrove crabs.

VP test and 20% of the isolated strains showed positive results on urease test. With regards to catalase character, about 24% of strains showed the positive results and 42, 43 and 24% of the strains showed positive activity in oxidase, starch hydrolysis and nitrate reduction analysis, respectively (Figure 3).

Scanning electron microscopic studies

Dense populations of apparent bacteria attached to gut regions of all the fifteen crabs were observed by SEM (Figure 4A to O). SEM study showed that common bacterial morphologies are rods and cocci form. Bacteria neither colonize all surfaces nor all projections in the pyloric stomach, but appeared restricted to chitinous pyloric finger like projections, where they colonized from base to tip with approximately uniform density. Rod shaped bacterial cells were attached to the fingerlets, images of semi-thin sections revealed coccoid bacteria in the gut of inspected crabs. At least two distinct bacterial morphologies colonized the hindgut lining, each consistently observed within a specific region. Curved, rod-shaped bacteria were associated with the anterior hindgut region. Dense aggregations of rod-shaped bacteria colonized the posterior region. hindgut region. Dense aggregations of rod-shaped bacteria colonized the posterior region.

DISCUSSION

Bacteria are continually ingested with food or water. For this reason, transient microorganisms probably have a more constant and important interaction with fish gastrointestinal ecosystems as compared to terrestrial animals (Cahill, 1990). The influence of the gut flora on the host is clearly of great interest in aquaculture, particularly where poor productivity and stock losses are widespread (Ravichandran and Kannupandi, 2005). Within aquatic and other marine animals, the colonization of the digestive system by microorganisms is influenced by a number of both host-related and non host-related factors (Harris, 1993). In the gut of another freshwater culture animal, the Chinese mitten crab, investigation illustrated that Proteobacteria and Bacteroidetes might be the dominant population (Li et al., 2007).

The purpose of this study was to isolate and enumerate the bacteria flora found in the gut of fifteen mangrove crab species from the five different mangrove regions. During the study of mud crabs, lactic acid bacteria, namely *Weissella fabaria* and *Streptococcus mutans* was also identified (Li et al., 2012). These results were to promote more systematic additional knowledge on the natural intestinal bacterial communities present in crabs and enhance the understanding of the effects of aquaculture operations. Furthermore, the data will promote the development of most favourable probiotic

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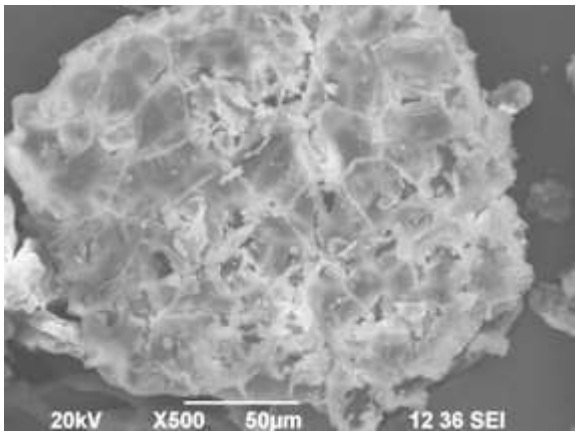
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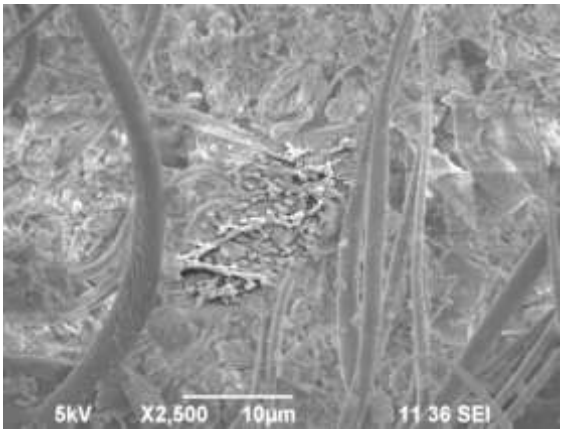
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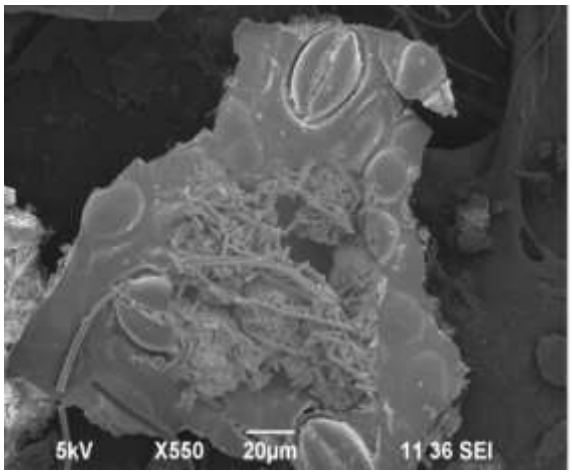
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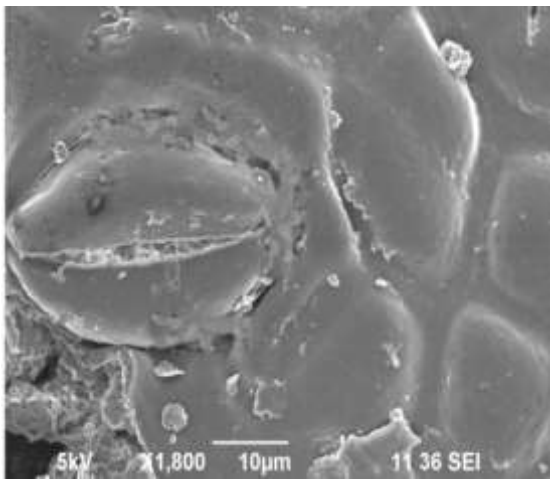
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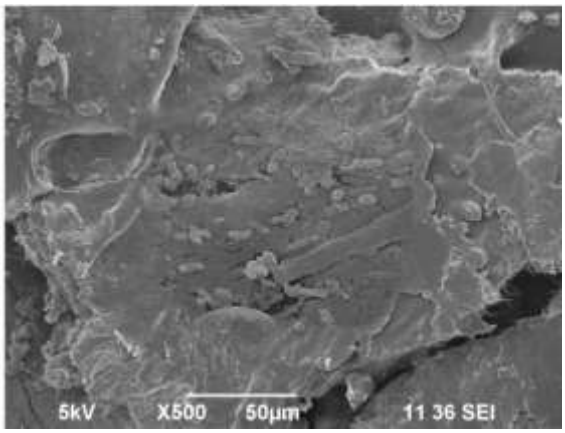
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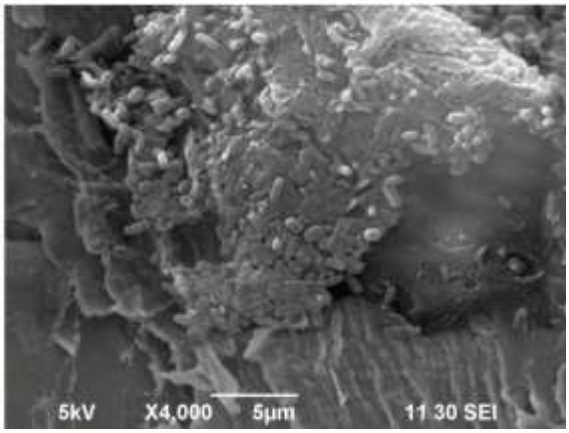
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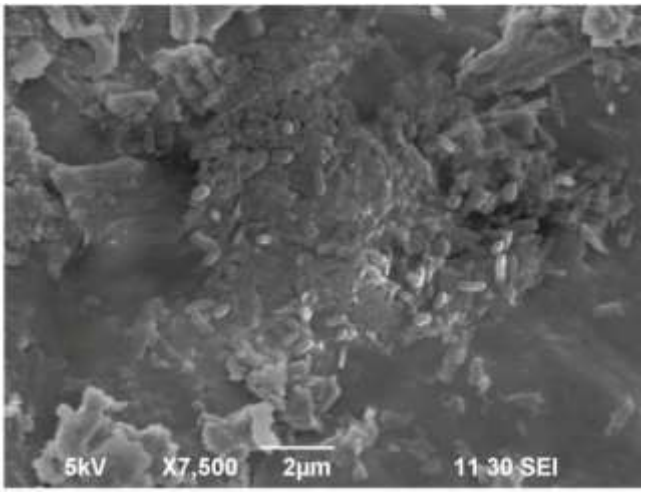
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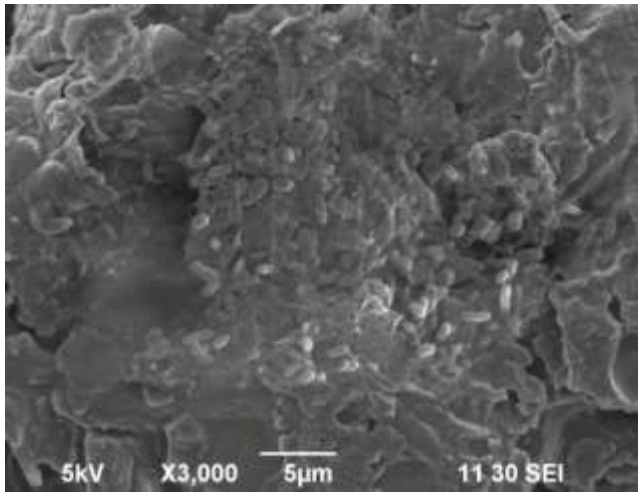
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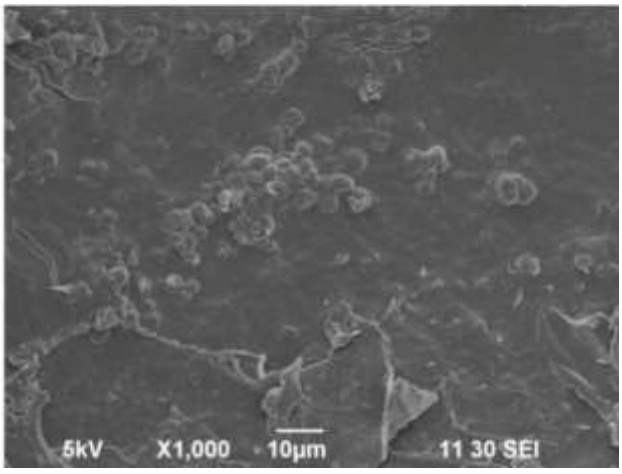
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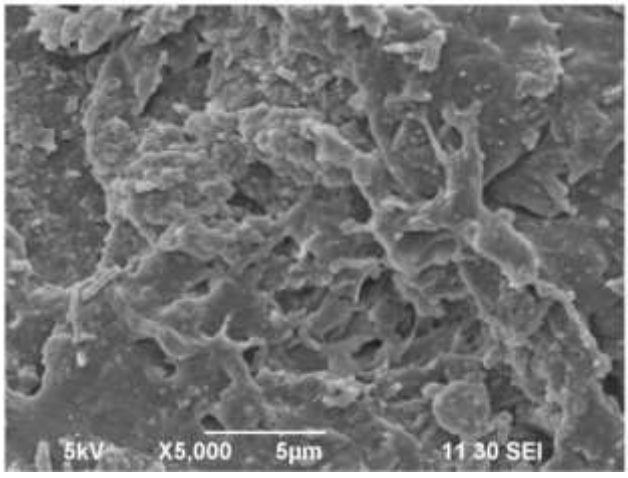
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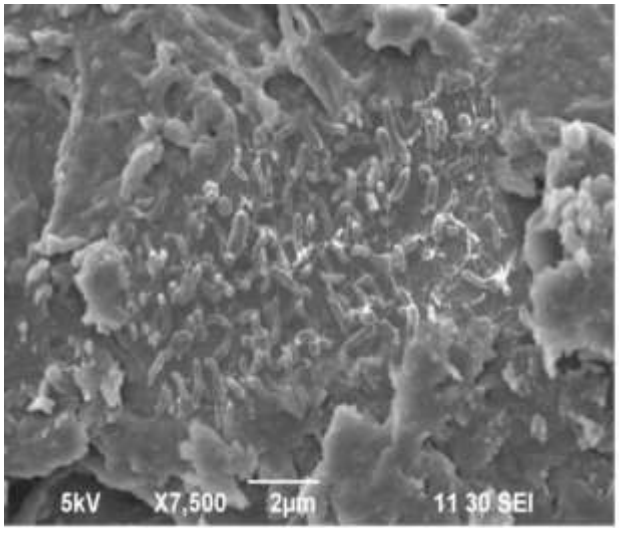
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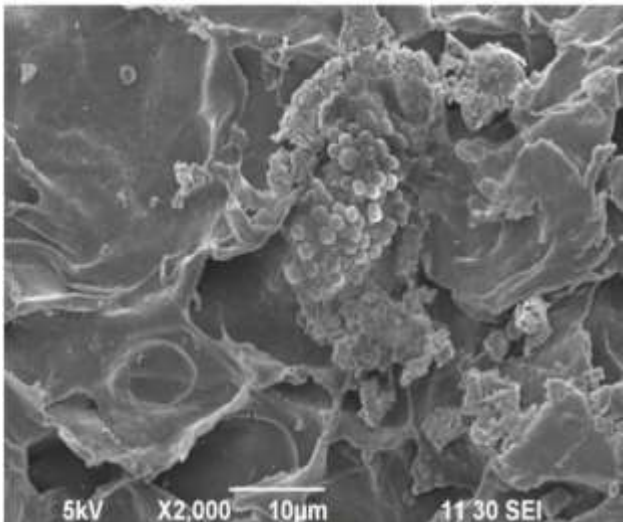
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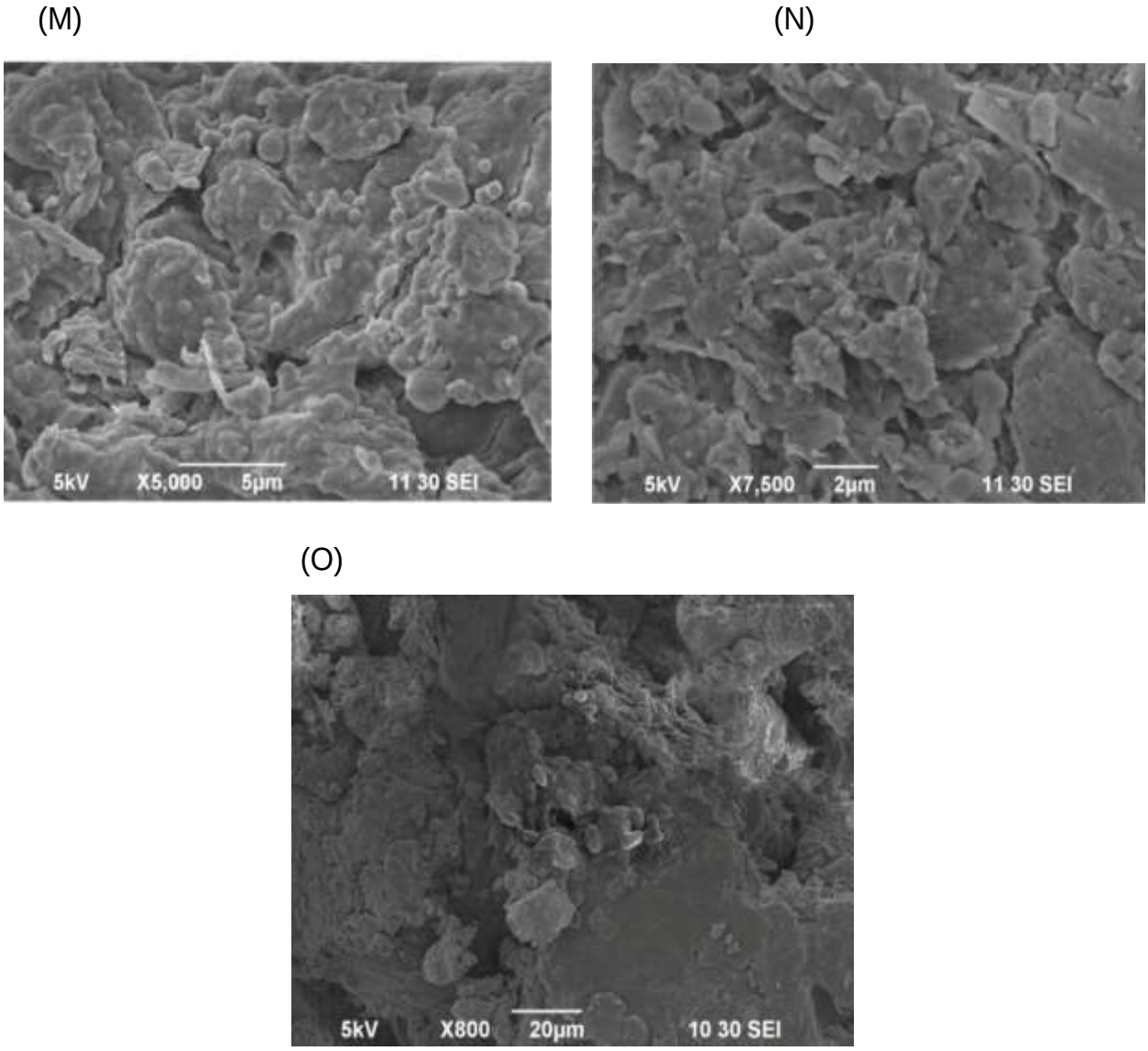

Figure 4. Scanning electron microscopic images of gut associated bacteria of various mangrove crabs. A) *Heteropanope indica*; B) *Macrophthalmus depressus*; C) *Metapograpsus maculatus*; D) *Metapograpsus messor*; E) *Nanosesarma batavicum*; F) *Nansesarma minutum*; G) *Neoepisesarma mederi*; H) *Neoepisesarma tetragonum*; I) *Pseudograpsus intermedius*; J) *Sesarma andersoni*; K) *Sesarma bidens*; L) *Sesarma brockii*; M) *Sesarma plicatum*; N) *Uca annulipes* and O) *Uca triangularis*.

products to increase feeding efficiencies by getting better intestinal microbial balance of crabs.

The total bacterial load ranged between 0.7 ±0.49 $x10^6$ and 8.9 ± 0.13 $x10^6$ CFU/g of crabs gut sample and it was found to be maximum (8.9 ± 0.13 $x10^6$ CFU/g) in gut of *S. brocki* collected from Pichavaram mangrove region. The present investigations also support the findings of some of earlier studies. *S. brockii* were found to be the dominant species in the Pichavaram mangrove (Ravichandran et al., 2007a). Sesarmid crabs prefer thedecaying mangrove leave for their most favourable nutritive status. Senescent mangrove leaves generally have high initial C : N ratios up to 100 which decrease in decomposing leaves. The food materials which have C : N ratios lower than 17 are considered nutritious to marine invertebrates (Russel-Hunter, 1970). The feeding activities of crabs speed up the rate of decomposition of leaf litter and may facilitate the release of nutrients to mangrove system (Lee, 1997). The mud crab, *Scylla serrata*, has a higher bacterial count in its gut than other

crabs (*Charybdis cruciata, Podophthalmus vigil* and *Portunus pelagicus* and *Portunus sanguinolentus*) and the luminescent bacterial floras were predominant in the hind gut and on the cuticular membranes of all the crabs (Venkateswaran et al., 1981).

The mangrove sediment harbours larger bacterial population than the water column in all the five mangrove regions. This is attributed to nutrient accumulation, precipitation of inorganic compounds and settlement of dead organic matter in the sediments (Ravikumar, 1995). Among the five stations, maximum bacterial load was noticed in the gut of crabs, sediment and water samples collected from Pichavaram. It may be due to more litter fall as compared to the other mangrove regions. Common genera in the mangrove environments were *Vibrio, Bacillus, Micrococcus, Pseudomonas, Aeromonas, Flavobacterium*, etc. (Sathiyamurthy et al., 1990). Similarly, in the present study, the *Bacillus* was the common and predominant genus which was present in all the examined crabs in all the stations. Several genera, *Bacteroides*, *Acinetobacter*, *Flavobacterium*, *Chryseobacterium* and *Porphyrobacter*, were identified in crab guts, and some species belonging to these genera ordinarily are related to some disease. For example, some Bacteroides species are opportunistic pathogens owing to their association with a variety of soft tissue and other infections (Liu et al., 2003). In shrimp, the intestinal microbial diversity investigated by molecular-dependent methods have revealed that the predominant bacterial population in the intestine of Chinese shrimp, *Fenneropenaeus chinensis* were *Proteobacteria* and *Vibrio* sp. (Liu et al*.,* 2011). In this present study, genus *Flavobacterium* was isolated rarely in the gut of the examined crabs. Other bacterial genus, *Pseudomonas*, *Aeromonas*, *Alcaligenes*, *Photobacterium*, *Vibrio*, *Enterobacter* and *Staphylococcus* were also present in the crabs gut.

Distinct morphologies of attached bacteria are consistently associated with different crab species gut, including the pyloric fingerlets and along the anterior and posterior hindgut. There were similar reports showing that bacteria resembling that observed in this research have been reported in other marsh fiddler crabs and detritivorous thalassinid prawns (Harris et al., 1991; Harris, 1992; Pinn et al., 1999). This findings suggest that the intestinal bacterial communities in the guts of mangrove crabs still require further study. The enrichment of nutrition in the gut of mangrove crabs, and therefore, the higher inter subject variation, total diversity and abundance of the intestinal bacteria in mangrove crabs were likely the effect of these feeding methods (Li et al., 2007). Such variations could lead to developmental efficiencies and differences in digestive mechanism among the crab populations. Generally, the tropical mangrove ecosystem is endowed with a high bacterial load due to animal waste contaminations, human wastes, the continuous shedding of foliage into the water and subsequent decomposition. Bacterial populations were high in water and sediment in the mangrove environment. Bacteria belonging to the genera *Bacillus*, *Pseudomonas* and *Aeromonas* were found at higher levels in all the different mangrove regions.

In conclusion, crabs in the various mangrove environments carry a particular bacterial flora, which reflects their environment. Moreover, some genera were identified as unique in some mangrove crabs. Additional studies are required for the use of gut associated bacteria in different aspects (that is, to degrade organic and inorganic industrial pollutants).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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