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Full Length Research Paper

Occurrence of *Vibrio parahaemolyticus* in oysters (*Crassostrea gigas*) and mussels (*Perna perna*) of the seacoast of Santa Catarina, Brazil

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This research aimed to identify and quantify *Vibrio parahaemolyticus* in fresh oysters (*Crassostrea gigas*), mussels (*Perna perna*) and seawater from different regions of cultivation of bivalve shellfishes in the seacoast of Santa Catarina, Brazil. Samples were collected between October 2012 and December 2013 and 130 oysters samples (*Crassostrea gigas*), 215 mussels samples (*Perna perna*) and 222 seawater were collected. The occurrence of *V. parahaemolyticus* in oysters and mussels was low, 10.76 and 11.62% of the samples tested. Higher incidences of *V. parahaemolyticus* were observed in seawater (18%). The density of *V. parahaemolyticus* in summer (December to March) was significantly greater than those in the other 3 seasons (P < 0.01). The occurrence of pathogenic *V. parahaemolyticus* in oyster, mussels and seawater was very low (<10%). It is recommend that control measures should be considered, including the establishment of an intensive and continuous monitoring of potentially pathogenic *V. parahaemolyticus* from all oyster-growing areas, the environmental parameters, and the assessment of the region-specific human health risk due to consumption of oyster.

Key words: Oyster, *Crassostrea gigas, Vibrio parahaemolyticus,* bivalve molluscs.

INTRODUCTION

Seafood is recognized as a nutritious food choice, and is liked by increasing numbers of consumers worldwide (Hellberg et al., 2012). For the last two decades, there has been a fourfold growth in commercial aquaculture worldwide (Cabello, 2006). In Brazil, the production of bivalve shellfishes occurs mainly in the state of Santa Catarina, in the southern region of Brazil, due to the excellent geographical conditions of this area for the cultivation of marine organisms, such as the presence of a large number of bays, which facilitates the establishment of marine farms (Coelho et al., 2003; Corrêa et al., 2007).

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Despite the increase, the main obstacles in the consumption of seafood are its high perishability and risk to health due to contamination by pathogens (Reyhana and Kutty, 2014). In addition to the indicators of faecal contamination, which are used to assess the microbiological quality of bivalve molluscs in Brazil, different species of the *Vibrio* genus occur naturally in marine, coastal and estuary environments, where some species such as *Vibrio parahaemolyticus, Vibrio vulnificus* and *Vibrio cholerae* are potentially pathogenic for men, and may be present in fishes and raw shellfishes or partially subjected to cooking (Thompson et al., 2004). The possibility of seafood consumers to be infected by pathogenic vibrios by eating oysters depends on the microbiological quality of the marine habitat, as well as on the practices of handling and processing of these shellfish (Vieira et al., 2011). The occurrence of these bacteria is not related to the counts of *Escherichia coli* or thermotolernat coliforms, therefore the specific constant monitoring is required (Pereira, 2002; Oliver, 2006; Suffredini et al., 2014).

Infections caused by *Vibrio parahaemolyticus* have been reported in several countries in Asia (Chiou et al., 2000; Chowdhury et al., 2013; Kubota et al., 2008; Ma et al., 2014; Okuda et al., 1997; Tuyet et al., 2002; Vuddhakul et al., 2006), United States (Haendiges et al., 2014; Sims et al., 2011), in Europe only a few outbreaks or sporadic cases were reported in the last decade as a consequence of the consumption of local or imported seafood (Martinez-Urtaza et al., 2005; Ottaviani et al., 2008, 2010b, 2012; Quilici et al., 2005; Sala et al., 2009), and some South American countries like Chile (Fuenzalida et al., 2006; Cabello et al., 2007; Harth et al., 2009), Peru (Gil et al., 2007; Martinez-Urtaza et al., 2008) and Brazil (Leal et al., 2008) have also reported outbreaks.

Pathogenic strains of *Vibrio parahaemolyticus* can be differentiated from non-pathogenic strains with its ability to produce thermostable hemolysin (TDH), whose production is called the Kanagawa phenomenon. The pathogenicity of *Vibrio parahaemolyticus* is associated with the presence of the *tdh* and *trh* gene in oysters (Nishibuchi and Kaper, 1995).

The concentration of *V. parahaemolyticus*, in oysters and mussels is directly related to water temperature, with a higher concentration being present when the bivalve molluscs are in warm water. Because of this, these microorganisms are rarely isolated when the water temperature is below 15°C (Pruzzo et al., 2005; Su and Liu, 2007). In Brazil, the temperature of sea waters is above 20°C in most of the year, favouring the occurrence of these microorganisms in the different stations.

This research aimed to identify and quantify *V. parahaemolyticus* in fresh oysters (*Crassostrea gigas*) and mussels from different regions of cultivation of bivalve shellfishes in the seacoast of Santa Catarina, Brazil.

MATERIALS AND METHODS

Collection and preparation of the samples

Between October, 2012 and December, 2013, 130 oysters samples (*Crassostrea gigas*), 215 mussels samples (*Perna perna*) and 222 seawater samples were collected directly from three geographical regions in Santa Catarina where there is shellfish farming in Brazil (Figure 1).

Each oysters and mussels sample consisted of 12 units. The oysters and mussels were transported to the laboratory in an isothermal box with packaged potable ice, and analyzed within 6 h of sampling.

The oysters and mussels were scrubbed under tap water to remove debris, allowed to dry, disinfected with 70% ethanol, and opened aseptically using a sterilized knife. The flesh and intervalve liquid were aseptically transferred to sterile bags and were homogenized for 1 min, forming the pool of 12 units.

Isolation and enumeration of *Vibrio parahaemolyticus* in oyster and mussels samples

Enumeration of *V. parahaemolyticus* was performed using most probable number (MPN) technique (Kaysner and DePaola, 2004). Approximately 25 g of the homogenate was added to 225 ml of phosphate buffered saline (PBS). Serial 10-fold dilutions were prepared up to 1:106 and three aliquots of each dilution were inoculated into alkaline peptone water tubes and incubated overnight at 37°C. After incubation, a loopful from the top 1 cm, approximately, of each broth tube with turbid growth was streaked onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar plates (Oxoid, UK) and incubated at 37°C for 24 h. Five to ten typical colonies from each plate were selected and isolated for identification. Sucrose-negative (blue-green on TCBS agar) colonies were submitted to confirmation as Gram-negative and oxidase-positive. Further biochemical differentiation for identification and confirmation of isolated items were performed using the API 20E system (bioMérieux, France). Total populations of *V. parahaemolyticus* in oysters and mussels were determined by converting numbers of APW tubes that were positive for *V. parahaemolyticus* to MPN g^{-1} using the MPN table. All strains of *V. parahaemolyticus* were confirmed genotypically through the detection of the *tlh* gene by multiplex qPCR.

Multiplex PCR for the detection of rox, tdh and trh genes

The extraction of bacterial DNA was made in QiaCube equipment (Qiagen) using the DNeasy Blood and Tissue kit (Qiagen) with specific protocol for the equipment. Real time multiplex PCR was performed using the kit *V. parahaemolyticus* multiplex kit (Biotecon). The target genes were the *Rox* to confirm the species, and *tdh* and *trh* genes of pathogenicity. The protocol used was indicated in the kit manual.

Statistical analysis

Results of microbiological tests were transformed into log values and were assumed to be normally distributed; statistical analyses were performed in the Statistica 7.0® software (Stat-Soft, Inc., USA). To facilitate statistical analyses of quantitative data obtained by most probable number for counts *V. parahaemolyticus* when levels were below the limit of detection, there was substitution for 2 MPN g^{-1} and test of significance of the observed differences in *V.*

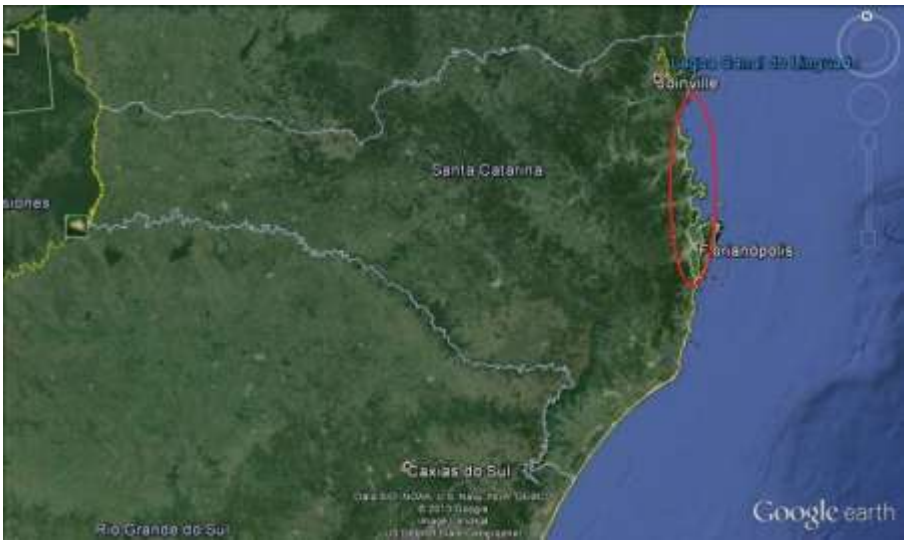

Figure 1. The coastal region which is the cultivation area of bivalve molluscs in the Santa Catarina.

Table 1. Occurrence of *Vibrio parahaemolyticus* in oyster, mussels and seawater samples.

Sample	Season	Number of samples tested	Number of positive samples (%)	Level of *V. parahaemolyticus* (MPN/g)		
				< 100	10^2-10^3	10^3-10^4
Oyster	Summer	50	10 (20%)	-	6	4
Oyster	Autumn	25	1 (4%)	1	-	-
Oyster	Winter	25	-	-	-	-
Oyster	Spring	30	3 (10%)	2	1	-
Mussel	Summer	120	21 (17.5%)	4	8	9
Mussel	Autumn	25	1(4%)	1	-	-
Mussel	Winter	25	-	-	-	-
Mussel	Spring	45	3 (6.6%)	2	1	-
Seawater	Summer	120	37 (30.8%)	10	18	9
Seawater	Autumn	30	9(30%)	9	-	-
Seawater	Winter	30	2(6.6%)	2	-	-
Seawater	Spring	42	12 (28.5)	10	2	-

parahaemolyticus levels, environmental parameters in oysters and mussels across the 22 samplings sites was conducted using a one-way analysis of variance (ANOVA), an alpha level of 0.05 was considered using the minimum level for statistical significance.

RESULTS AND DISCUSSION

The occurrence of *V. parahaemolyticus* in oysters and mussels was low, 10.76% and 11.62% of the samples tested (Table 1). Higher incidences of *V. parahaemolyticus* were observed in seawater (18%).

The densities of *V. parahaemolyticus* in oyster, mussels and seawater samples are listed in Table 1. They were higher in the summer months, especially in February and March. The density of *V. parahaemolyticus* in summer (December to March) was significantly greater than those in the other 3 seasons ($P < 0.01$). The occurrence of pathogenic *V. parahaemolyticus* in oyster, mussels and seawater was very low (<10%). Only 4 of 130 oysters, 5 of 215 and 5 of 220 seawater samples contained detectable levels of pathogenic strains. These results indicated that most *V. parahaemolyticus* in the environment were nonpathogenic to humans. Although, the levels of *V. parahaemolyticus* in oysters reported in this study were much lower, postharvest processing conditions and storage temperatures could allow contaminated *V. parahaemolyticus* to multiply to a higher level in market oysters. Studies have shown that the

populations of *V. parahaemolyticus* in unrefrigerated oysters could increase rapidly to reach 50-fold to 790-fold its original level within 24 h after harvest if oysters were exposed to an elevated temperature (Gooch et al., 2002).

Epidemiological data from CDC on association with *V. parahaemolyticus* gastroenteritis with tdh-carrying strains in the period 2001–2004 and US risk assessment studies on oysters (FDA, 2005), support the assumption that *V. parahaemolyticus* risk is proportional to exposure to different levels of pathogenic *V. parahaemolyticus* (WHO, 2011). According to some studies, pathogenic *V. parahaemolyticus* levels may be reliably estimated from total *V. parahaemolyticus* levels (Miwa et al., 2003; Nordstrom et al., 2007). On the other side, other studies showed that the ratio between total and pathogenic *V. parahaemolyticus* in the environment may be quite variable over time, as in the case of the monitoring performed in Alaskan waters, where percentage of potentially pathogenic strains in two consecutive summers (2004 and 2005) changed from 74 to 30% (WHO, 2011). Such variability, together with the limited number of quantitative data on *V. parahaemolyticus* levels in the environment and in shellfish harvested in regions as Europe (Cantet et al., 2013), Asia (Deepanjali et al., 2005), South America (Garcia et al., 2009), which are occasionally involved in outbreaks, underline the need for analytical assays which allow the enumeration of both total and potentially pathogenic (tdh and/or trh positive) *V. parahaemolyticus* strains. Trouble variables for the presence of *V. parahaemolyticus* in seafood have been shown in studies by many researchers around the world, using conventional bacteriological methods. The results found in this study are in agreement with the results reported by Nordstrom et al. (2007), a study conducted in Alaska (USA), Cabello et al. (2007) in Chile, Gil et al. (2006) in Peru and Quintoil et al. (2007), India.

Higher incidence of *V. parahaemolyticus*, however, was found using conventional methods of wild mullet in Italy (Serracca et al., 2011), cockles in Indonesia (Zulkifli et al., 2009), various seafood in India (Chakraborty et al., 2008) and in the USA mussels (Lu et al., 2006). Furthermore, the lower incidence of 8% (Hassan et al., 2012), were reported in the Netherlands seafood. Ramos et al. (2014) found an incidence of *V. parahaemolyticus,* 30.0% in samples of oysters and 33.3% in water samples from cultivation sites in Bahia Sul in Florianopolis, in the study region of this work.

Several factors are involved in the distribution and survival of microorganisms in estuarine ecosystems such as biotic and abiotic parameters of water, such as temperature, salinity, pH and turbidity (Ristori et al., 2007; Strom and Paranjpye, 2000). The concentration of *V. parahaemolyticus* in seawater increases with increasing temperature and is correlated with the seasonal increase in the occurrence of sporadic cases of infections in months with higher temperature (Hlady and Klontz, 1996).

The presence of *V. parahaemolyticus* seems to be constant where the sea water temperature is >10°C, unlike what occurs in Europe, where isolation of this pathogen decreases during the winter months (Baker-Austin et al., 2013). Hence, *V. parahaemolyticus* can be considered ubiquitous in the marine environment. The World Health Organization (WHO, 2011) listed the optimum temperature for *V. parahaemolyticus* growth as 37°C, with a wide growth range of 5-43°C. Several studies have shown a positive correlation between contamination of raw shellfish by *V. parahaemolyticus* and water temperature with higher frequencies being detected during warmer months in spring and summer seasons than in winter (DePaola et al., 2003; Parveen et al., 2008; Johnson et al., 2012; Ceccarelli et al., 2013).

The data provided in this study on contamination levels of total and potentially pathogenic *V. parahaemolyticus* and seasonal distribution, will help in defining appropriate monitoring programs and post-harvest policies for this hazard. The acquisition of further quantitative information on *V. parahaemolyticus* distribution in production areas and marketed products (exposure assessment), together with studies on the effectiveness of post-harvest treatments, will help in the definition of codes of practice for vibrios in shellfish and improve the safety of products.

Conclusion

In conclusion, these results demonstrate greater seasonal variations in total and pathogenic *V. parahaemolyticus* densities in oysters. Hence, there may be more uncertainty in the use of densities of total *V. parahaemolyticus* organisms as alternative for risk predictions as was previously recognized. These findings can provide a reference for the comprehensive management and control of the harvesting areas. Therefore, it is recommended that control measures should be considered, including the establishment of an intensive and continuous monitoring of potentially pathogenic *V. parahaemolyticus* from all oyster-growing areas, the environmental parameters, and the assessment of the region-specific human health risk due to consumption of oyster. Thus, more research is needed to assess differences in virulence among various toxigenic strains and to assess and manage the risk of illness due to human exposure to oysters harvested in contaminated environments under the light of the climate change.

Conflict of interests

Authors have not declared any conflict of interests.

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Multidrug-resistant bacteria isolated from patients hospitalized in Intensive Care Unit in University Hospital of Constantine, Algeria (2011 - 2015)

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The incidence of infections caused by multidrug resistant bacteria is increasing worldwide. The frequent misuse of antibiotic drug has greatly contributed to worldwide dissemination of antibiotics resistance. Multi-drug resistance in Gram-negative and Gram-positive bacteria causes a wide range of infections, particularly in the Intensive Care Unit (ICU) settings leading to an increased impact on morbidity, mortality and costs. This study was undertaken to determine the prevalence of multidrug resistant of bacterial isolates in patients admitted in ICU of university hospital of Constantine. We analyzed a 5-year period, from 2011 to 2015. Over five years period, 7472 clinical samples were collected in the Clinical Microbiology Laboratory of Benbadis University Hospital in Constantine. Identification of the isolates was performed by API automated systems (bioMérieux, Marcy l'Etoile, France) and automate microscan walkaway 96 (Siemens). Antibiotic resistance was determined by the Clinical and Laboratory Standards Institute (CLSI) disk diffusion test on Mueller-Hinton Agar. Multidrug-resistant isolates included in this study were methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, Enterobactericeae that produce extended-spectrum beta lactamases and/or carbapenemases, multidrug-resistant *Acinetobacter baumannii* and multidrug-resistant *Pseudomonas aeruginosa*. A total of 3528 isolates were collected from various specimens such as blood (47.05%).The *Staphylococcus sp*, *Klebsiella sp*, *Acinetobacter sp*, *P. aeruginosa* and *E. coli* are the most common isolates recovered from clinical specimens in ICU (26.3, 18.7, 14.3, 11.9 and 9.2% respectively). MRSA strains constituted over 65% of all *S. aureus* isolates and 30.3% of *E. faecium* were found to be vancomycin resistant. Extended spectrum β-lactmase producers were expressed in 53.2% and 50.6 from *K. pneumoniae* and *E. coli*. Carbapenem resistance among *K. pneumoniae* improved slightly from 2.89 to 4.21%. *A. baumannii* isolates exhibited extremely high levels of resistance to all antibiotics except colistin (100% sensitive). In addition, 80.4% of *A. baumannii* isolates were found to be resistant to imipenem. Imipenem resistant *P. aeruginosa* isolates showed 36.4%.

Key words: Multi-drug resistant bacteria, Intensive care unit, Gram negative bacteria, Gram positive bacteria.

INTRODUCTION

The increase and spread of multidrug resistant (MDR bacteria have become a major concern worldwide. The hospital acquired infections caused by MDR infections caused by MDR bacteria have led not only to

an increase in mortality, morbidity, and cost of treatment, but also continue to endanger the life of patients (Martin and Yost, 2011; Delle Rose et al., 2015).

MDR bacteria can cause a wide range of infections, including bacteremia, pneumonia, urinary tract infection, peritonitis etc., which can lead to substantial morbidity and mortality, particularly in the ICU settings (Chen et al., 2016).

The risk of acquiring MDR bacteria in the ICU is increased by severity of illness, length of stay, use of intravascular devices, exposure of ICU patients to invasive therapeutic procedures like endotracheal intubation, the intensity of exposure to infected patients and the frequent misuse of antibiotic drug (Khan et al., 2014; Royer et al. 2015; Wroblewska et al., 2006).

A review of emerging MDR bacterial pathogens in ICU includes methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant Enterococci (VRE) and multi-drug resistant Gram-negatives (Nayak et al., 2014; Thompson and FRCP, 2004; Russotto et al., 2015; Trubiano and Padiglione, 2015).

Infection and colonization with MRSA may be more frequent in the ICU than in general wards (Fridkin and Gaynes, 1999) and its resistance is conferred by the acquisition of one of several staphylococcal cassette chromosome mec elements that carry a gene (mecA) that encodes a penicillin-binding protein (PBP2a) with low affinity for β-lactam antibiotics (Katayama et al., 2000). Vancomycin-resistant enterococci (VRE) have emerged as a nosocomial pathogen especially in ICUs worldwide (Austin et al., 1999). VRE are among the major health care-associated MDR organisms causing a serious problem in choosing an appropriate therapy (Schouten et al., 2008).

Infections caused by Gram-negative bacteria have features that are of particular concern. Specifically, the rate of infections related to MDR gram-negative bacteria (MDR-GNB) in ICU is increasing. These organisms are highly efficient at up-regulating or acquiring genes that code for mechanisms of antibiotic drug resistance, especially in the presence of antibiotic selection pressure (Russotto et al., 2015).

The emergence of resistance in Enterobacteriaceae is considered an alarming health threat. During this last decade, a growing number of *K. pneumonia*e and *E. coli* have developed resistance against third-generation cephalosporin, due to extended-spectrum β-lactamases (ESBLs) (Pitout and Laupland, 2008). Carbapenems are the first line treatment of ESBLs producing bacteria, and the emergence of carbapenem-resistant isolates conferred by New Delhi Metallo-β-lactamase-1 (NDM-1) leaves limited therapeutic options.

Non - fermentative Gram-negative bacilli (NF-GNB) mainly *A. baumannii* and *P. aeruginosa*, have emerged worldwide and the resistance of these organisms to antibiotics, particularly to carbapenems, has posed important therapeutic challenges. Currently, carbapenems are considered the antimicrobials of choice for treatment of serious infections caused by *A. baumannii* and *P. aeruginosa* but their efficacy is increasingly compromised by resistance as reported worldwide (Al Jarousha et al., 2009). This resistance has been attributed to the production of carbapenem-hydrolysing-lactamase enzymes of Ambler molecular class D (oxacillinases) and B (metallo- lactamases) (Woodford et al., 2011).

Despite its importance, a few studies on the MDR bacteria isolated from patients hospitalized in ICU were investigated in Algeria.

The aim of the present study was to determine the prevalence of MDR Gram positive and Gram negative organisms isolated from patients hospitalized in ICU of a university hospital in Constantine, Algeria.

MATERIALS AND METHODS

Isolation site

This study was undertaken in the Laboratory of Clinical Microbiology at the University Hospital of Constantine, Algeria, over five years period 2011- 2015.

Clinical samples for microbiological culture comprised blood, urine, pus, body fluid or aspirates (joint fluid, pleural fluid, ascitic fluid and cérébrospinal fluid), other diverse material (such as catheter, sonde and swab) and others like sputum. Isolates were collected from 7472 patients suspected of bacterial infection from a single ICU patients. Only one isolate per patient infection episode was included in this study.

Microbiological cultures and identification

All samples collected were aseptically inoculated on the various media and incubated at 37°C for 24 h. Cultures were processed using standard microbiological methods. Identification of the isolates was performed by API automated systems (bioMérieux, Marcy l'Etoile, France) and confirmed on the basis of the results of automate Microscan Walkaway 96 (Siemens).

Susceptibility testing

Antibiotic resistance was determined by the Clinical and Laboratory Standards Institute (CLSI) disk diffusion test on Mueller-Hinton Agar, using calibrated inoculum of the isolates based on McFarland standard, with the following antibiotics according to the bacterial species: piperacillin, trimethoprim—sulfamethoxazole, cefotaxime, gentamicin, amikacin, ceftazidime, ciprofloxacin, pefloxacin, imipenem, amoxicillin/clavulanic acid, ticarcillin, cefazolin, colistin and vancomycin. The results were interpreted according to the

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Table 1. Number and rate of positive culture from all samples.

Year	Total of samples	Positive isolates	Contamined isolates	Negative isolates
2011	1641	765 (46.61)	86 (5.24)	790 (48.14)
2012	1711	799 (46.69)	83 (4.85)	829 (50.89)
2013	1629	751 (46.10)	78 (4.78)	800 (49.10)
2014	1367	696 (50.91)	85 (6.21)	586 (42.86)
2015	1124	517 (45.99)	94 (8.36)	513 (45.64)
Total	7472	3528 (47.21)	426 (5.70)	3518 (47.08)

Table 2. Nature of the analyzed samples.

Sample	2011	2012	2013	2014	2015	Total (%)
Blood	319	326	384	388	243	1660 (47.05)
Tracheal	133	183	138	100	36	590 (16.72)
Urine	91	112	76	138	97	514 (14.56)
Sonde	118	81	83	10	77	369 (10.45)
Catheter	57	32	31	24	36	180 (5.10)
Pus	22	42	19	14	15	112 (3.17)
Body fluid	16	16	20	21	10	83 (2.35)
Others	9	7	0	1	3	20 (0.56)
Total	765	799	751	696	517	3528 (100)

guidelines of Clinical and Laboratory Standards Institute 2007 (CLSI). The antimicrobial susceptibility results for the six most frequently isolated organisms were analyzed in the present study. MDR isolates included in the analyses were MRSA, vancomycin-resistant enterococci (VRE), Enterobactericeae (*K. pneumoniae* and *E. coli*) that produce extended-spectrum beta lactamases and/or carbapenemases (ESBLs and CREs, respectively), Imipenem- resistant *A. baumannii* and *P. aeruginosa*. ESBL-producing strains of Gram-negative rods were detected with a double disk method using amoxicillin/clavulanic acid, ceftazidime, cefotaxime and ceftriaxone disks.

Resistance of NFGNB to imipenem was verified by Etest (AB Biodisk). MIC of vancomycin was also determined by using E-test method for enterococci.

RESULTS

Among the 7472 samples collected, 3528 were positive (47.21% of total samples), 426 were contaminated and represented 5.70% of total samples and 47.08% of all samples were negative (Table 1).

The bacterial isolates were obtained from 8 different specimens with the following percentage representations: blood (47.05%), tracheal (16.72%), urine (14.56%), sonde (10.45%), catheter (5.10%), pus (3.17 %), different body fluids (2.35%), and others (0.56%) (Table 2). In which, 2398 (67.97%) were Gram negative bacteria and 1130 (32.02%) were Gram positive (Figure 1). The commonest organism isolated from all samples was *Staphylococci* sp. 931 (26.38), *Klebsiella* sp. 661 (18.73) followed by *Acinetobacter* sp. 506 (14.34), *Pseudomonas* sp. 422 (11.96), *E. coli* 326 (9.24), *Enterobacter* sp. 227 (6.43) and *Enterococcus* sp. 117 (3.31). Others organisms were isolated but in small proportions: *Proteus* sp. 83 (2.34), *Streptococcus* sp. 82 (2.32), *Serratia* sp. 81 (2.29), *Shigella* sp. 32 (0.9), *Providencia* sp. 24 (0.68), *Haemophylus influenzae* 15 (0.42), *Morganella* sp. 9 (0.25), *Stenotrophomonas* sp. 6 (0.17), *Salmonella* sp. 4 (0.11) *and Citrobacter* sp. 2 (0.06) (Table 3).

Among Gram-positive isolates, strains of *Staphylococcus* sp. amounted to 82.38% (931/1130) and strains of *Staphylococcus aureus* constituted 37.8% (352/931). Whilst isolates of coagulase-negative staphylococci (CNS) constituted 62.19% (579/931). Of all strains of *Staphylococcus sp*, 80.98% (754/931) were methicillin-resistant. The proportion of methicillin-resistant increased from 70.68 in 2011 to 87.33 in 2014.

MRSA comprised 65.9% of all *S. aureus* isolates (232/352) and methicillin-resistant CNS (MRCNS) constituted 90.15% (522/579). The proportion of MRSA decreased from 76.54% in 2012 to 66.66% in 2015. 82.14% of CNS were methicillin resistant in 2011 and the rate of methicillin resistance raised 93.65% in 2014. In MRSA, there was no resistance to vancomycin.

Among Gram-positive bacterial strains, *enterococci* comprised 10.35% (117/1130). Out of 117 isolates, 84 (71.79%) were *Enterococcus faecalis* and 33 (28.20%) were *Enterococcus faecium*. No other enterococcal species were isolated during the study period.

No vancomycin-resistant *Enterococci* were observed during the first two years of our study, 2 isolates (14.28%), 6 (21.42) and 2 (11.11) of *enterococci* were found to be resistant to vancomycine, in 2013, 2014 and 2015 respectively. Ten out of 33 isolates of *E. faecium* (30.3%) were resistant to vancomycin , 2 strains (40%) in 2013, 6 strains (75%) in 2014 and 2 strains (28.57%) in 2015. However, no strains of vancomycin-resistant *E.faecalis* were detected in the study (Table 4).

Klebsiella sp. ranked second overall among ICU patients (18.73%) and was the most frequently isolated Gram-negative organism (27.56%). *K. pneumoniae* was most common specie 608/661 (91.98). For *K. pneumoniae*, the highest resistance rate was for ticarcillin, cefazolin and amoxicillin/clavulinat (100, 85.29 and 79.82%, respectively). All strains of *K.*

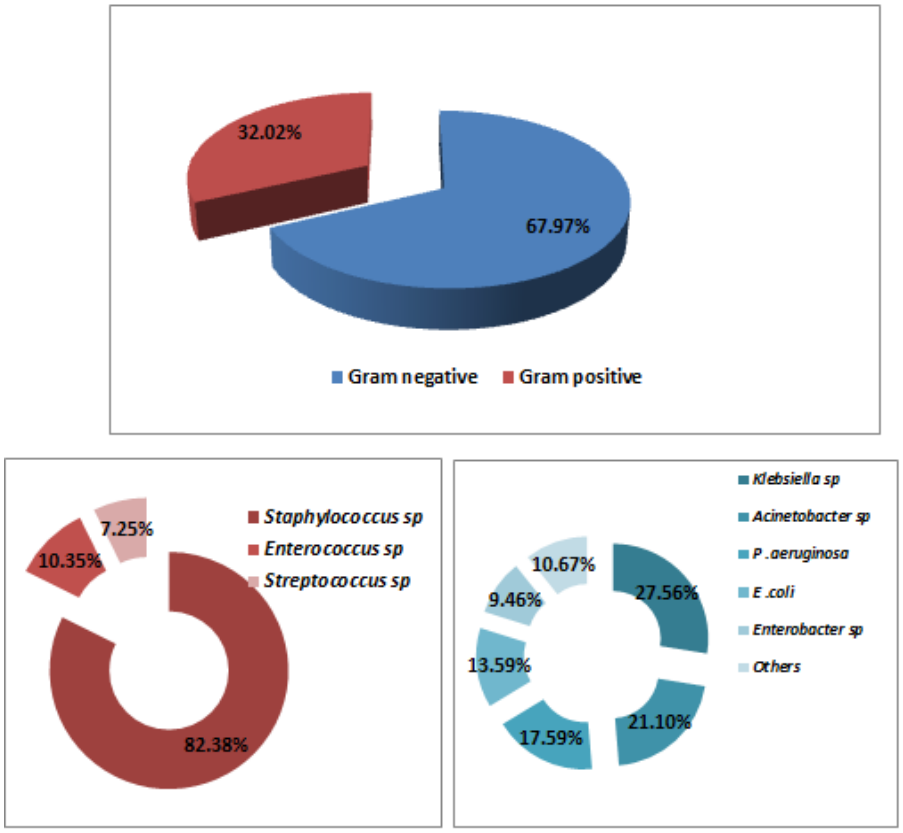

Figure 1. Prevalence of Gram positive and Gram negative isolates.

Table 3. Prevalence of clinical isolates.

Organisms	2011	2012	2013	2014	2015	Total(%)
Staphylococcus sp.	174	218	203	150	186	931(26.38)
Klebsiella sp.	124	143	154	140	100	661(18.73)
Acinetobacter sp.	112	96	119	107	72	506(14.34)
Pseudomonas aeruginosa	138	110	80	64	30	422(11.96)
Escherichia coli	60	65	70	84	47	326(9.24)
Enterobacter sp.	61	61	47	38	20	227(6.43)
Enterococcus sp.	31	26	14	28	18	117(3.31)
Proteus sp.	30	13	17	12	11	83(2.34)
Streptococcus sp.	18	22	12	14	16	82(2.32)
Serratia sp.	12	23	22	15	9	81(2.29)
Shigella sp.	0	0	0	30	2	32(0.90)
Providencia sp.	0	11	5	6	2	24(0.68)
Haemophilus influenzae	4	8	3	0	0	15(0.42)
Morganella sp.	0	1	4	3	1	9(0.25)
Stenotrophomonas sp.	0	0	0	5	1	6(0.17)
Salmonella sp.	0	1	1	0	2	4(0.11)
Citrobacter sp.	1	1	0	0	0	2(0.05)

pneumoniae were sensitive to colistin (Table 5).

ESBL production was detected in 53.28% of strains of *K.pneumoniae*. The proportion of ESBL increased from 52.06% in 2011 to 63.15% in 2015 (Table 6). Carbapenem resistance among *K. pneumoniae* improved slightly in 2013, 2014 and 2015 from 2.89 to 4.21%. *E.*

Table 4. Prevalence of multidrug resistant staphylococci and enterococci stratified by year.

Sample	Resistant isolate no./Total isolate no. (%)/ year					
	2011	2012	2013	2014	2015	Mean
Methicillin-resistant *Staphylococcus* sp.	123/174(70.68)	179/218(82.11)	172/203(84.72)	131/150(87.33)	149/186 (80.1)	754/931(80.98)
Methicillin-resistant *Staphylococcus aureus*	100/146 (68.49)	62/81 (76.54)	29/59 (49.15)	13/24 (54.16)	28/42 (66.66)	232/352 (65.9)
Methicillin-resistant *Staphylococcus* coagulase negative	23/28 (82.14)	117/137 (85.4)	143/144 (99.3)	118/126 (93.65)	121/144 (84)	522/579 (90.15)
Vancomycin resistant enterococci	0/31 (0)	0/26 (0)	2/14 (14.28)	6/28 (21.42)	2/18 (11.11)	10/117 (8.54)
VR *E. faecalis*	0/25 (0)	0/19 (0)	0/9 (0)	0/20 (0)	0/11 (0)	0/ 84 (0)
VR *E. faecium*	0/6 (0)	0/7 (0)	2/5 (40)	6/8 (75)	2/7 (28.57)	10/33 (30.3)

coli isolates from ICU patients exhibited elevated extended-spectrum β-lactamase (ESBL)-phenotype rates (50.61%) but the proportion of ESBL decreased from 61.66% in 2011 to 42.55% in 2015. the highest resistance rate for *E.coli* was for ticarcillin (82.65%), amoxicillin/clavulinat (64.57%) and cefazolin (63.11%). No strains showed resistance to imipenem and colistin.

NFGNB comprised 26.3% (928/3528) of all isolates. Among Gram negative isolates, strains of *Acinetobacter* sp. and *P.aeruginosa* comprised 21.1% (506/2398) and 17.59% (422/2398) of isolates respectively. Among strains of *Acinetobacter sp*, 93.87% (475/506) were *A.baumanii*.

In the present study, imipenem resistant was detected in 80.42% of *A. baumanii*, and 36.84% of *P. aeruginosa*. We recorded an increase of imipenem-resistant strains of *A. baumannii* during the study period from 60.39% in 2011 to 94.2% in 2014. The proportion of imipenem resitance increased slightly from 33.33% in 2011 to 50% in 2015 for *P.aeruginosa* (Table 6).

A. baumanii showed very high resistance rates to most antimicrobial agents. The highest resistance rates for *A.baumanii* were for piperacillin (97.54%), ticarcillin (97.52%) and ceftazidime (95.82%), while *P. aeruginosa* exhibited high susceptibility to most antimicrobial agents tested and the highest resistance rates was for pefloxacin (49.8%), piperacillin (37.82%) and ceftazidime (30.80%). All NFGNB isolates were sensitive to colistin.

DISCUSSION

Most isolates were recovered from the blood specimens (47.05%). This finding corroborated the results reported by other investigator in India (Jitendra et al., 2012). While in the ICU of Kingdom of Saudi Arabia (KSA), most isolates were recovered from the respiratory specimens (38.8%), followd by the blood specimens (33%) (Saeed et al., 2010).

Staphylococci constituted the group of bacteria most commonly isolated from ICU patients (Johnson et al., 2003). The commonest organism isolated from all samples was *Staphylococci* (26.38%) in our study. The most common GP cocci in this study were SCN, followed by *S.aureus*, whereas in the study of Fridkin et al. (1999), the most common GP cocci were *S. aureus*, followed by SCN.

In the present study, MRSA strains constituted over 65% of all *S. aureus* isolates. MRCNS strains represented 90.15% of CNS strains. These are high percentages from MRSA and MRCNS compared with data from other reports (Khan et al., 2014; Wang et al., 2011).

MRSA, first described in the 1960, is now endemic in many hospitals, infection and colonization with MRSA may be more frequent in the ICUs than in general wards (Thompson and FRCP, 2004).

Antibiotic susceptibility profile of MRSA showed that these strains exhibited the highest sensitivity to vancomycin (100%) In our study, *Enterococci* are important pathogens of patients hospitalized in the ICU, particularly in view of the increasing frequency of resistance to vancomycin. In this study, strains of *enterococci* comprised 3.31% of all bacterial isolates and 10.35% of Gram-positive isolates, this observation agreed with the finding of Johnson et al. (2003). Among *enterococci* isolates, 71.79% were *E. faecalis* and 25.46% were *E. faecium*. In the study of Sood et al. (2008), *E. faecalis* has been the predominant enterococcal species accounting for 80-85% of clinical isolates, followed by *E. faecium* which accounts for about 10-15% of clinical isolates.

In the present study, 10 (8.54%) isolates were found to be vancomycin resistant, 10 out of 33 isolates (30.3%) of *E. faecium* but no strains of vancomycin-resistant *E. faecalis* were detected. It has also been found in various studies that *E. faecium* accounts for far fewer clinical enterococcal isolates than *E. faecalis*, but it is far more resistant to glycopeptides.

In a study conducted by Saeed et al. (2010), less than 7.1% of *E. faecalis* were found to be resistant to vancomycin, whereas 40.1% of the *E. faecium* isolates were resistant to vancomycin. Zhanel et al. (2003) found that most of the VRE were *E. faecium* (88%), while only 12% were of *E. faecalis*. The Gram-negative organisms most frequently isolated in our study were *Klebsiella*

Table 5. Antimicrobial resistance of major four isolates Gram negative, stratified by year.

Organism/antimicrobial agent	Resistance rate (%) / Year					
	2011	2012	2013	2014	2015	Mean
Acinetobacter baumanii	**n=101**	**n=95**	**n=108**	**n=102**	**n=69**	
Ticarcillin	100	95.78	98.14	98.03	95.65	97.52
Piperacillin	98.01	97.89	98.14	98.03	95.65	97.54
Ceftazidime	95.04	98.89	94.44	95.09	95.65	95.82
Amikacin	72.27	57.89	70.37	66.66	86.95	70.82
Gentamycin	73.26	92.63	84.25	91.17	95.65	87.39
Ciprofloxacin	85.14	87.36	88.88	98.03	81.15	88.11
Pefloxacin	89.10	91.57	92.59	91.17	81.15	89.11
Trimethoprim-sulfamethoxazole	93.06	89.47	92.59	96.07	95.65	93.36
Colistin	0	0	0	0	0	0
Pseudomonas aeruginosa	**n=138**	**n=110**	**n=80**	**n=64**	**n=30**	
Ticarcillin	28.98	36.84	25	26.56	36.66	30.80
Piperacillin	16	24.6	27.5	84.37	36.66	37.82
Ceftazidime	23.91	11.81	18.75	78.12	20	30.51
Amikacin	4.34	3	3.75	79.68	23.33	22.82
Gentamycin	18	27.1	22.5	64.06	20	30.33
Ciprofloxacin	8	25.4	15	50	50	29.68
Pefloxacin	30	44	60	65	50	49.8
Trimethoprim-sulfamethoxazole	72.46	94.54	90	90.62	83.33	86.19
Colistin	0	0	0	0	0	0
Klebsiella pneumoniae	**n=121**	**n=125**	**n=138**	**n=129**	**n=95**	
Ticarcillin	100	100	100	100	100	100
Amoxicillin/clvulinat	66.94	88	86.95	78.29	78.94	79.82
Cefazolin	84.29	86.4	90.57	88.37	76.84	85.29
Imipenem	0	0	2.89	4.65	4.21	2.35
Amikacin	23.14	6.4	25.36	29.45	21.05	21.08
Gentamycin	74.38	81.6	61.59	66.66	54.73	67.79
Ciprofloxacin	41.32	38.4	21.73	48.06	33.68	36.63
Trimethoprim-sulfamethoxazole	67.5	82.64	74.63	68.21	56.84	69.96
Colistin	0	0	0	0	0	0
Escherichia coli	**n=60**	**n=65**	**n=70**	**n=84**	**n=47**	
Ticarcillin	95	70.76	94.28	75	78.72	82.65
Amoxicillin/clavulinat	41.66	92.30	68.51	52.38	68.08	64.57
Cefazolin	46.66	100	50	67.85	51.06	63.11
Imipenem	0	0	0	0	0	0
Amikacin	6.66	13.84	2.85	44.04	8.51	15.18
Gentamycin	38.33	66.15	18.57	21.42	34.04	35.70
Ciprofloxacin	43.33	21.53	80	47.61	46.8	47.85
Trimethoprim-sulfamethoxazole	66.66	62.5	52.30	55.95	53.19	58.12
Colistin	0	0	0	0	0	0

sp. (27.56%), *Acinetobacter* sp. (21.1%), *P. aeruginosa* (17.59%), and *E. coli* (13.59%). Similarly, these pathogens were the most frequently isolated Gram-negative organisms from ICU patients in Lybia (Zorgani et al., 2015). Whilst, the most common Gram-negative organisms in order of frequency from ICU patients were *Klebsiella* sp., *E. coli*, and *P. aeruginosa* in the USA and *E. coli*, *P. aeruginosa* and *Klebsiella* sp. In Europe (Sader

Table 6. Prevalence of multidrug resistant of major four isolates Gram negative, stratified by year.

Isolate	Resistant isolate no./Total isolate no. (%)/ Year					
	2011	2012	2013	2014	2015	Mean
ESBL-producing *K. pneumoniae*	63/121 (52.06)	57/125(45.6)	62/138 (44.92)	82/129 (63.56)	60/95 (63.15)	324/608 (53.28)
Carbapenemase-producing *K. pneumonia*	0/121 (0)	0/125 (0)	4/138 (2.89)	6/129 (4.65)	4/95 (4.21)	14/608 (2.3)
ESBL-producing *E. coli*	37/60 (61.66)	38/65(58.46)	30/70 (42.85)	40/84 (47.61)	20 /47 (42.55)	165/326 (50.61)
Imipenem- resistant *A.baumannii*	61/101 (60.39)	73/95 (76.84)	90/108 (83.33)	93/102 (91.17)	65/69 (94.2)	382/475 (80.42)
Imipenem–resistant *P. aeruginosa*	46/138 (33.33)	35/110 (31.18)	30/80 (37.5)	25/64 (40.62)	32/64 (50)	168/456 (36.84)

et al., 2014).

Over the past decade there has been a dramatic increase in MDR-Gram-negative rods, particularly among isolates recovered from ICU patients. In our study, ESBL production was detected in 53.28 and 50.61% of strains of *K. pneumoniae* and *E. coli* respectively. These data are similar to the proportion of 56.2 and 43.6% of ESBL-producing isolates amongst *E. coli* and *K. pneumoniae* isolated in clinical samples in China (Wang et al., 2008) but it is far higher than the same proportions reported in France (8 and 13%, respectively) (Arnaud et al., 2013). Whereas it is a less percentage compared with others reports in the literature. An Algerian's report of the resistance of bacteria to antibiotics (2012) have reported 76.73% of ESBL-producing isolates amongst *K. pneumoniae* in 2012. Saeed et al. (2010) found that ESBL producing *E. coli* were approximately 100% among the tested isolates, which were more common than that seen in *K. pneumoniae* (92%).

Carbapenem-resistant *K. pneumoniae* has emerged during recent years in several intensive care unit (Debby et al., 2012). Carbapenem resistance among *K. pneumoniae* improved slightly in 2013 and 2015 from 2.89% to 4.21% in the present study. Increasing rates of carbapenem resistance among Enterobacteriaceae have been reported by other investigators in various European countries (Mouloudi et al., 2010; Nordmann et al., 2011; Walsh, 2010). Rates of 2.3% and 38% of carbapenem resistance among *K. pneumoniae* have been reported (Sękowska et al., 2014; Chaudhary and Payasi (2013). KPC-producing strains appear to have the widest distribution, but a rising number of OXA-48–producing strains has been reported (Glasner et al., 2013).

In African countries, KPC producers were first identified by Brink et al. in 2012, in South Africa. In Algeria, a two recent studies described the first report of KPC-3-producing *K. pneumonia* (Bakour et al., 2015) and First outbreak of OXA-48-positive carbapenem-resistant *K. pneumoniae* isolates in ICU (Cuzon et al., 2015)**.** Strains of *P. aeruginosa* constituted 11.96% of all isolates ranking fourth in this study. *P. aeruginosa* was commonly isolated in ICU patients, ranking first in KSA (21.41%), second in Europe (18.2%) and China (15.6%), third in the USA (17.2%) and lybia (20%) (Saeed et al., 2010; Zorgani et al., 2015; Sader et al., 2014; Tan et al., 2014). It constituted 17.59% of Gram-negative strains in our study. This data is almost similar to the 24% reported from ICUs in Europe (Hanberger et al., 1999).

In the present study, the percentage of imipenem-resistant strains of *P. aeruginosa* was 36.84%. The proportion of imipenem resitance increased from 33.33% in 2011 to 50% in 2015. This is high compared with reports in the literature ranging from 2 to 20% (Glupczynski et al., 2001; Hsueh et al., 2001).

Strains of *Acinetobacter* sp. constituted 14.34% of all isolates and 21.10% of GN organisms. This is a high percentage compared with data from USA ICUs (Sader et al., 2014), but it is similar to others reports (Saeed et al., 2010; Tan et al., 2014). In present study, *A. baumanii* showed very high resistance rates to most antimicrobial agents and an important decrease in the susceptibility was observed for imipenem during the five years study period (60.39% in 2011 to 94.2% in 2015). This is very high compared with reports in the literature ranging from 9.6% to 23.8% (Wroblewska et al., 2006). While, among the four hundred and fifty four isolates of *A.baumani* isolated in India's ICU, 81.71% isolates were found to be carbapenemase producing (Chaudhary and Payasi, 2013).

The global spread of carbapenem-resistant *P. aeruginosa* and *A. baumannii* is of great concern (Hanberger et al., 1999). The significantly low susceptibility of ICU-acquired strains to carbapenems may be related to the increasing use of carbapenems in ICU. The most active compound against these organisms was colistin.

Conclusion

This study showed that *Staphylococcus sp*, *Klebsiella sp*, *Acinetobacter sp*, *P. aeruginosa* and *E. coli* were the most common isolates recovered from clinical specimens in the ICU of Constantine. Blood specimens represented 47.06% of all specimens collected in the ICU. This study demonstrated that most of these pathogens isolated from clinical samples were MDR. We conclude that the incidence of high rates of resistance is alarming high and is continuously increasing and spreading. Therefore surveillance of bacterial prevalence and susceptibility

patterns of the isolates from ICU is crucial in determining optimum empirical therapy of infections in critically ill patients.

Conflict of Interests

The authors have not declared any conflict of interests.

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Gut bacterial microbiota in psoriasis: A case control study

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Gut microbiota is mainly composed of four phyla; however, the human gut microbiota is dominated by only 2 of them and most of them are uncultivable. Psoriasis is an inflammatory skin disorder with associated inflammation of internal organs and musculoskeletal system. This study aimed to, identify numerically abundant bacteria phyla in fecal samples of patients with psoriasis, evaluate whether differences in fecal microbiota correlate with the occurrence of psoriasis and understand the possible pathogenesis behind psoriasis-related bacterial targets. From April, 2015 to 2016, 90 adults were selected prospectively to allocate 2 equal groups: Gr1 (45 cases) patients with psoriasis, and Gr2 (45 cases) healthy controls. Psoriasis Area and Severity Index (PASI) for each psoriasis patient was detected. All subjects were subjected to history taking, clinical examination, and fecal real time polymerase chain reaction (PCR) testing for the Firmicutes, Bacteroidetes, and Actinobacterial phyla. In both groups, Firmicutes were the most common detected phylum followed by Bacteroidetes and finally Actinobacterial phyla. High statistically significant difference was reported for the Firmicutes/ Bacteroidetes ratio between the psoriasis patients and the control group and showed statistically significant positive correlations with PASI. Actinobacterial count was significantly higher in the control group than in psoriasis patients and showed statistically significant negative correlations with PASI. It is believed that, there are fractions of the gut microbiota with the ability to counteract inflammation (Bacteroidetes and Actinobacterial), and others that are more prone to induce inflammation (Firmicutes) and the disturbed microbiome ratio may be the cause for inducing psoriasis.

Key words: Psoriasis, gut microbiota, real time polymerase chain reaction (PCR), firmicutes, bacteroidetes, actinobacteria.

INTRODUCTION

The collection of microorganisms that live in a peaceful coexistence with their hosts has been called the microbiota (Kunz et al., 2009), which colonizes every exposed body surface to the external environment such as skin, genitourinary, gastrointestinal, and respiratory tracts (Chiller et al., 2001; Verstraelen, 2008). The human

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Table 1. Method for calculating the Psoriasis Area and Severity Index (PASI).

Degree of Severity (per body region)	Value given
No symptoms	0
Slight	1
Moderate	2
Marked	3
Very marked	4

Surface involved (per body region)	Value given
<10%	1
10%-29%	2
30%-49%	3
50%-69%	4
70%-89%	5
90%-100%	6

gut has a major surface for microbial colonization and rich in molecules that can be used as nutrients by microbes, making it a preferred site for colonization, so the most heavily colonized organ is the gastrointestinal tract; and the colon alone is estimated to contain over 70% of all the microbes in the human body (Ley et al., 2006). Gut microbiota are mainly composed of four phyla, namely, Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria (Qin et al., 2010). The human gut microbiota is dominated by only 2 of them: the Bacteroidetes and the Firmicutes (>98%), whereas Actinobacteria, Proteobacteria, and others are present in minor proportions (Eckburg et al., 2005). Gut microbiota provides its host with a physical barrier to pathogens by competitive exclusion, such as occupation of attachment sites, consumption of nutrient, and production of antimicrobial substances. It also stimulates the host to produce various antimicrobial compounds. Healthy gut microbiota is essential to promote host health and disturbance of it results in a variety organ system diseases (Sekirov et al., 2010), as the intestinal microbiome is able to affect extra-intestinal distant sites (Eppinga et al., 2014).

Cultivation has great advantages in microbiology diagnosis; however, traditional bacteriological methods recover less than 40% of the total bacterial species of the GIT, and cultivable bacteria is not a representative of the total phylogenetic diversity (Eckburg et al., 2005). Rapid nucleic acid amplification and detection technologies quickly displace the traditional assays. Real-time PCR techniques with specific 16S ribosomal RNA (rRNA) gene-based oligonucleotide primers had been demonstrated to be powerful methods for detecting target bacteria in complex ecosystems (Layton et al., 2006). Several dermal diseases appear to have a gut-skin connection. Psoriasis is a systemic autoimmune inflammatory disease that shares some immunological aspects with other inflammatory based diseases, such as Crohn's disease (Moran and Shanahan, 2014). GIT disorders are present in 28% of patients with psoriasis (Krinitsina, 1998).

There is growing evidence for a gut-skin connection (Gueniche et al., 2010; Bowe and Logan, 2011). However, this is still an emerging field and there is much research that needs to be conducted, to have a better understanding of the relationship between the gut and the skin. Our hypothesis is that, the immune-mediated inflammatory pathway in psoriasis are induced or mediated by the disturbance in the gut microbiome.

MATERIALS AND METHODS

This study was conducted over the period from February 2015 to February 2016 on 90 adults attending University Hospital after IRB approval and informed consent. They were selected prospectively to allocate 2 equal groups: Gr1 (n = 45) patients with psoriasis (all of the patients had active psoriatic lesions), and Gr2 (n = 45) healthy controls, age, and sex matched individuals with no history of psoriasis. Exclusion criteria were as follows: oral antibiotic, systemic corticosteroids and immunosuppressive therapy within 3 month of sample collection, current extreme diet (e.g., parenteral nutrition or macrobiotic diet), history of IBD, current consumption of probiotics, gastrointestinal tract surgery leaving permanent residua (e.g., gastrectomy, bariatric surgery, or colectomy). Body Mass Index (BMI) was calculated using the following equation: weight (kg)/height (m^2). Subjects with BMI 19 to 25 are considered normal, 26 to 29 as overweight and >30 as obese (Daviglus et al., 2003).

All subjects were subjected to history taking and clinical examination Psoriasis patients were diagnosed in a dermatology clinic, Psoriasis Area and Severity Index (PASI) were recorded for each patient (Fredriksson and Pettersson, 1978), which involves the assessment over 4 body regions (head [h], trunk [t], upper [u] and lower [l] extremities) of erythema (E), infiltration (I), desquamation (D), and body surface area involvement (A), as in Table 1. Since the head, upper extremities, trunk, and lower extremities correspond to approximately 10, 20, 30, and 40% of body surface area respectively, the PASI score is calculated by the formula:

PASI = 0.1 (Eh + Ih + Dh) Ah + 0.2(Eu + Iu + Du)Au + 0.3(Et + It + Dt) At + 0.4(El + Il + Dl)Al

Samples

Fecal samples were collected from all participants in sterile collection containers. Undigested particles approximately 1 g stool were removed by washing, low-speed centrifugation of samples, followed by high centrifugation of the supernatant were used to form bacterial pellet which were stored frozen at –70°C until use.

Extraction of gut microbiota DNA

DNA was obtained from the samples bacterial pellet by QIAamp DNA Stool kit, according to the manufacturer's instructions (QIAGEN, Hilden, Germany).

Table 2. Specific primers pairs sequences used.

Target phylum	Target gene	Primers sequences	References
Total bacteria	16S rRNA	FP: ACTCCTACGGGAGGCAGCAG RP : ATTACCGCGG CTGCTGG	Fierer et al., 2005
Firmicutes	16S rRNA	FP: GGAGYATGTGGTTTAATTCGAAGCA RP:AGCTGACGACAACCATGCAC	Guo et al., 2008
Bacteroidetes	16S rRNA	FP: GGARCATGTGGTTTAATTCGATGAT RP: AGCTGACGACAACCATGCAG	Guo et al., 2008
Actinobacteria	16S rRNA	FP:TACGGCCGCAAGGCTA RP: TCRTCCCCACCTTCCTCCG	Bacchetti et al., 2011

Real time PCR

The real time PCR was carried out to detect the copy numbers of the 16S rRNA gene for all Bacteria, three dominant phyla were present in the gut by using specific primers which include: most Bacteroidetes, Firmicutes, and Actinobacterial division, as shown in Table 2. Amplification and detection of DNA by real-time PCR were performed with ABI-Prism 7500 Sequence Detection System (Applied Biosystems) using optical grade 96-well plates. Duplicate sample analysis was performed, in a total volume of 25 µl using SYBR Green PCR Master Mix (QIAGEN, Hilden, Germany). Each reaction contained 12.5 µl of SYBR green master mix, 0.3µl of each primer, and 2.5 µl of the DNA template. The PCR reaction conditions consist of an initial denaturation step of DNA at 95°C for 10 min followed by 40 cycles consisting of denaturation at 95°C for 15 s, and annealing-elongation step at 60°C for 1 min (Guo et al., 2008). A melting curve analysis was done, and the threshold cycle (CT) values and baseline settings were determined by automatic analysis settings. The data were analyzed using the Sequence Detection Software version 1.6.3 (Applied Biosystems).

Standard curves

Standard curves were generated by using 10-fold serial dilutions, (5X1015 to 5x106) of chimeric plasmid which was quantified using a spectrophotometry (NanoDrop ND-1000, USA) (Armougom et al., 2009). Each standard curve was generated by plotting Ct values versus the number of plasmid copies. When PCR was performed on tested fecal samples, we used this standard curve to quantify each bacterial phylum. The chimeric fragment sequence (362 bp) was constructed, and it contains parts of 16S rRNA genes of Bacteroidetes (sequence is in regular style), and Firmicutes (sequence is in bold style). The primers sequences were complementary to that in lowercase underlined: agcagccgcggtaatACGGAGGATCCGAGCGTTATCCGGATTTATT*GGGTTTAAGGG*AGCGTAGGTGGACTGGTAAGTCAGTTGTGAAA GTTTGCGGCTCAACCGTAAAATTGCAGTTGATACTGTCAGTCT TGAGTACAGTAGAGGTGGGAATTCGTGGTgtagcggtgaaatgcttagg tcagctcgtgtcgtgaGATGTTGGGTTAAGTCCGCAACGAGCGCAACC CTTATTGTTAGTTGCCATCATTTAGTTGGGCACTCTAGCGAGAC TGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC*GTCAAATC ATCATGCC*CCTTATGACCTGGGCTacacacgtgctacaatgg

Statistical analyses

The data were statistically analysed using the Statistical Package for Social Sciences version 20 (SPSS Inc., Chicago, IL, USA). Quantitative data were presented as, means and standard deviation and also described as numbers and percentages. Mann-Whitney test was used for comparing groups. All differences were considered statistically significant if $p<0.05$. Correlations were established by coefficient of correlation (r).

RESULTS

Patients with psoriasis and healthy control subjects, were matched in age (42.3 ± 10 years, vs 44.2 ± 7.1, respectively), and sex (27 females /45patient, vs 25females/45 subjects, respectively). No statistically significant difference was observed between the two groups regarding BMI (24.5 ±4.7, vs26.9 ±7.1, respectively). Firmicutes was the commonest detected phyla in psoriasis patients (83%), while Bacteroidetes and Actinobacterial phyla were accounted for 9.2 and 2.8%, respectively. In the control group the detected percentage were 70, 22, and 3.9% for Firmicutes, Bacteroidetes, and Actinobacterial phyla, respectively. There was non-significant difference between patients in GpI and GpII for total bacteria, Firmicutes and Bacteroidetes phyla counts. However, Actinobacterial phylum count was significantly ($p < 0.001$**) higher in the control group than in psoriasis patients, as in Table 3 and Figure 1.

For Firmicutes/Bacteroidetes ratio (F/B), we observed a high statistically significant difference ($p < 0.001$**) between the ratio in psoriasis patients (9.02) and in the control group (3.18), as showed in Figure 1. The mean value for PASI was 11± 9, and showed a statistically significant positive correlations with Firmicutes/ Bacteroidetes ratio (r=0.312, p=0.036*).

Statistically significant negative correlations were observed with Actinobacterial phylum (r=-0.298, p=0.047*). Non-significant correlations were detected with Firmicutes (r=0.264, p= 0.079), and Bacteroidetes (R=-0.292, P= 0.052). However, weak negative correlation was reported with Bacteroidetes.

Table 3. Comparison betweengut microbiota gene copies number/gm stool in GpIand GpII.

Phyla	GPI	GpII	P
All bacteria	1.1 × 1012±19.5	1.3 × 1012±16.88	0.96
Bacteroidetes	1.012 × 1011±8.2	2.86 × 1011±8.7	0.3
Firmicutes	9.13× 1011±7.62	9.1 × 1011±10.07	0.99
Actinobacterial	3.12 × 1010±1.46	5.06 × 1010±1.54	<0.001**

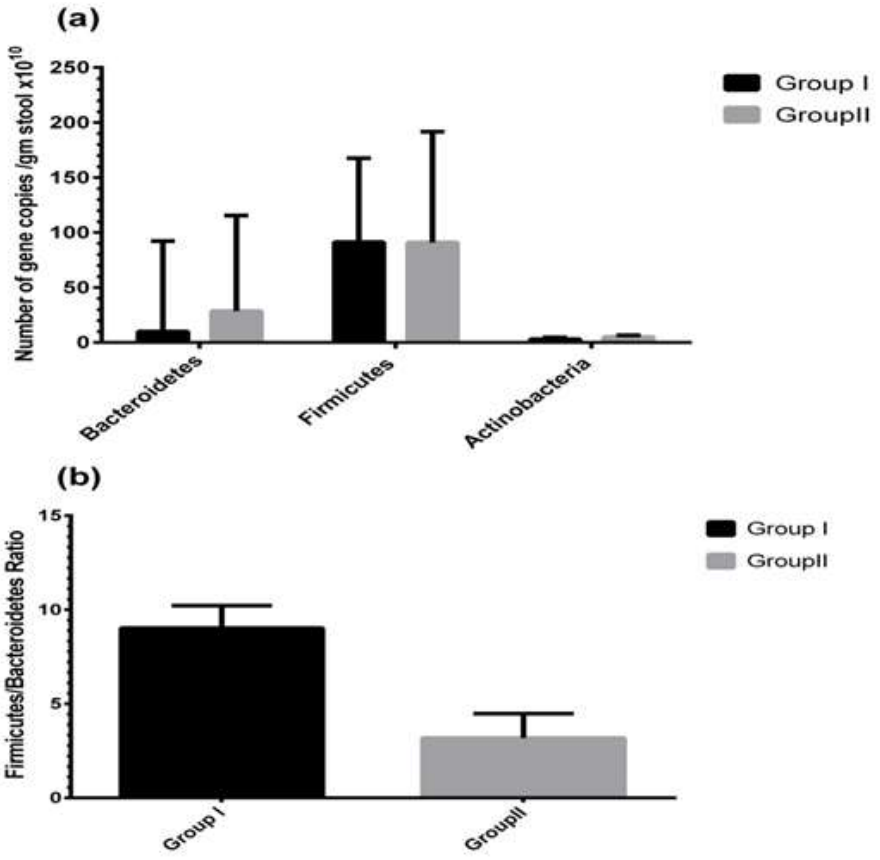

Figure 1. Box-and-Whisker plot of A) Gut microbiota gene copies number/gm stool in GpI and GpII b) The Firmicutes/Bacteroidetes ratio in GpI and GpII.

DISCUSSION

In the current study, in both GpI and GpII, Firmicutes was the commonest detected phyla (83 and 70%, respectively), followed by Bacteroidetes (9.2 and 22%, respectively). It was clear that both Firmicutes and Bacteroidetes were the dominant phyla in gut microbiome and accounted for more than 90% of the total detected bacteria (92.2% in GpI and 92% in GpII). These results were parallel to Rajilic-Stojanovic et al. (2007) who reported that Firmicutes and Bacteroidetes phyla were predominating and represent 90% of the total gut microbiota (that is, 65 and 25%, respectively). A lower percentage (80%) was reported for the three phyla by Lay et al. (2005). In contrary, Manson et al. (2008) reported a higher percentage (99%) for the two dominant intestinal microbiome phyla, also Marchesi (2010) mentioned that the large majority of microbes reside in our GIT belong either to the Firmicutes or Bacteroidetes phyla and that was similar to Koenig et al. (2011) results, who reported about 80 and 20% for Firmicutes and Bacteroidetes, respectively. This variation is accepted as

these proportions can vary greatly between individuals and even within single individual over time because peoples received antibiotic and reduced certain bacterial types according to the used antibiotic. Besides that, the dietary changes can influence the types of microbiota, as polysaccharide-rich diet significantly altered the microbiota composition, resulting in an increase in Firmicutes and decrease in Bacteroidetes, while high fiber diet has been associated with increases in Bacteroidetes and a lower abundance of Firmicutes in humans (Turnbaugh et al., 2009; Hakansson and Molin, 2011). In the present study, there was no significant difference between patients in GpI and GpII for total bacterial, Firmicutes and Bacteroidetes phyla counts. However, it was reported that, a highly significant difference was observed between the F/B ratios and was higher in psoriasis patients than in the control group. Also PASI showed statistically significant positive correlations with F/B ratio in spite of non-significant correlations that were observed with Bacteroidetes, and Firmicutes alone. It should be borne in mind that certain combination of the microbiota can enhance the pathogenic effects, and another combination can keep the person healthy, so it's all about the ratio of certain microbiota that have a beneficial effect when compared with the ratio of others that disturb the balance.

In some chronic diseases, as psoriasis, the pathologic agent might be the disturbed microbiota ratio, and this presumably means a decreased bacterial diversity and/or different degrees of overgrowth by bacteria inducing inflammatory responses by the immune system (Hakansson and Molin, 2011; Fry et al., 2013). The question is which bacteria are the most forceful in causing inflammation? Let's remember that CD4+ T helper cells can differentiate towards Th1, Th2, Th17 and Treg phenotypes (Gaboriau-Routhiau et al., 2009). Zambrano-Zaragoza et al. (2014) detected that the dys regulation of IL-17 (pro-inflammatory) has been implicated in psoriasis, while Tregs have an anti-inflammatory role, and its increase is an early predictive marker for clinical response in psoriasis as reported by Richetta et al. (2011). Some gut bacteria appear to drive Treg development preferentially, while others promote Th17 development (Ishikawa et al., 2008). Gut microbiota has a significant influence as a complex, on the development of inflammatory/autoimmune diseases but the specific mechanisms that lead to the induction of Tregs versus Th17 cells by various commensal bacteria are currently unknown. However, toll-like receptor 9 plays a major role (Dalpke et al., 2006). Mazmanian reported that B. fragilis (Bacteroidetes phylum) increases the suppressive capacity of Tregs and induces it to produce anti-inflammatory cytokine (Mazmanian et al., 2008).

Moreover, Oral administration of antibiotic which leads to a reduction in bacteria from the Firmicutes phylum, and a relative increase in bacteria from the Bacteroidetes phylum, suggested that antibiotic-mediated protection against inflammation mediate the increase in Bacteroidetes with enhancement of dendritic cells that converted the naïveTcells into IL-10-producing Tregs (Ochoa-Reparaz et al., 2010). Most of the Firmicutes are Gram positive bacteria, while most of the Bacteroidetes are Gram negative bacteria (Hakansson and Molin, 2011). Firmicutes was the most common phylum of the skin in psoriasis. Fry suggested that Crohn's disease (CD) occurs as a result of immune tolerance breakdown of the intestinal microbiota in genetically susceptible individuals and he also reported that CD patients are 5 times more likely to develop psoriasis, so psoriasis may occurs due to immune tolerance breakdown of microbiota (Fry et al., 2013). Many studies reported that the peptidoglycan (PG) is antigenic and triggers psoriasis, in which the T cell stimulation which occurs is proved by isolated PG-specific T cells from psoriatic skin lesions, furthermore there are four Peptidoglycan recognition proteins (PGRPs), which bind to the PG of Gram-positive bacteria. PGRP-3 and PGRP-4 are secreted in the gut, and skin. In psoriasis there are mutations in the genes for the PGRP-3 and PGRP-4 and this may lead to an abnormal response to bacterial PG in psoriasis, which may results in inflammation (Kainu et al., 2009; Dziarski and Gupta, 2010). In the current study, Actinobacterial phylum accounted for 2.8% in GpI, and 3.9% in GpII and showed a high statistically significant difference between the two groups. This was close to the results of Koenig et al. (2011), who reported that Actinobacterial phylum was 3% of the total microbiome in the gut. Also PASI showed statistically significant negative correlations with Actinobacterial phylum. This is in harmony with many studies who illustrated the complex relation between Actinobacterial phylum and inflammatory or immune related diseases as what we have here in psoriasis (Veiga et al., 2010; Kosiewicz et al., 2011). Studies reported that oral administration of probiotics, including the Bifidobacterium species (Actinobacterial phylum) reduced the intestinal inflammation in the colitis model and protect it against the development of various inflammatory and autoimmune diseases by, reducing the levels of other bacteria that cause inflammation. The authors also reported that the fecal levels of Bifidobacterium were inversely related to the inflammatory core (Calcinaro et al., 2005; Lavasani et al., 2010).

The inflammation-suppressing fractions of the microbiota may: (i) counteract some of the inflammation-aggravating bacteria, which will decrease the inflammatory tone of the system; (ii) improve the barrier effect of the GI mucosa, which allows less inflammation-inducing components in the lumen to translocate out into the body; (iii) directly interact with inflammation-driving components of the immune system. All three actions may be at work simultaneously (Fry et al., 2013). The limitations of this study are: relatively small sample size,

un-attainability of detecting the skin microbiome to explore the relation between the detected types of gut microbiome and skin microbiome and to clear the effect of gut microbiome in psoriasis skin plaques.

Conclusion

Firmicutes and Bacteroidetes were the dominant phyla in gut microbiome. F/B ratio was higher in psoriasis patients group than in the control group which showed positive correlation with PASI. It is believe that, there are fractions of gut microbiota which has the ability to counteract inflammation, and others which are more prone to induce inflammation (Firmicutes). The disturbance of microbiota ratio may be the cause for inducing inflammatory responses by the immune system in psoriasis. Actinobacterial group may be one of the bacteria that play an anti-inflammatory role as it was decreased significantly in psoriasis patients and negative correlations were reported with PASI. However, the relationship between psoriasis and gut microbiome should be dealt with caution for further investigations.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Removal of *Escherichia coli* in treated wastewater used for food production in Morogoro, Tanzania

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The aim of this study was to assess the removal efficiency of *Escherichia coli* at Mafisa and Mzumbe domestic wastewater treatment ponds in Morogoro, Tanzania. The study was done from October, 2013 to April, 2014. A total of 125 water samples from inlets and subsequent anaerobic, facultative and maturation ponds as well as treated wastewater were collected and analysed for *E. coli*. The estimated retention times of the wastewater treatment units were 19 and 22 days in Mafisa and Mzumbe ponds, respectively. The concentration of *E. coli* ranged from 4.70 to 5.60 log cfu/mL in untreated wastewater and was reduced to <1.00 to 2.00 log cfu/mL in the treated wastewater. During rainy and cold seasons, the effluent discharged out at Mafisa during August 2013; and March and April, 2014 was about 2 log cfu/mL while at Mzumbe *E. coli* concentration in effluent discharged out was up to 1.23 log cfu/mL. The concentration of *E. coli* in untreated and treated wastewater from the two wastewater treatment ponds study sites were comparable (P<0.05). Reduction of *E. coli* concentration in wastewater treatment ponds study sites was significant with less reduction seen at Mafisa, during rainy and cold seasons in March, April and August. To conclude, the simple wastewater treatment ponds in the study sites were effective and demonstrated potential for reduction of public health risks associated with use of treated wastewater in agricultural irrigation and aquaculture.

Key words: Agricultural irrigation, aquaculture, retention time, wastewater treatment.

INTRODUCTION

Water is an essential resource in supporting life of humans, animals, plants and other living organisms.

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In Sub-Saharan Africa many people struggle for access to a limited clean and safe drinking water. Currently, wastewater is widely used in food production systems such as in agricultural irrigation and aquaculture which has been compelled by the growing scarcity of clean water (WHO, 2006a; WHO, 2006b). Globally, households and commercial business points in urban and peri-urban areas increasingly produce wastewater from toilets, bathrooms, laundries and kitchens. Wastewater generated from these facilities may constitute major source of pollution of water bodies and environment (Senzia et al., 2009; Akpor and Muchie, 2011; Mkali et al., 2014). About 98% (Mateo-sagasta et al., 2015) to 99.9% (Pescod, 1992) of domestic wastewater constitutes of water and the remaining percent include organic matters and faecal pathogens of major public health concern such as *E. coli, Salmonella* and *Shigella*. For instances the *E. coli* pathotypes (Diarrheagenic *E. coli*) are among the leading bacteria causing infections in human and animals including sepsis/meningitis, urinary tract infection (UTI) and diarrhoea. These pathotypes *E. coli* include enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvassive (EIEC), enterohaemorhagic (EHEC), enteroaggregative (EAEC) and diffusely adherent *E. coli* (DAEC) (Hussain, 2015; Jafari et al., 2012; Kaper et al., 2004).

Wastewater and sludge in developing countries including Sub-Saharan Africa is mainly collected from pit latrines, septic tanks and a limited amount is collected through centralized piped networks. Pit latrines are common in rural and unplanned urban and peri-urban areas (Mara, 2013). Feacal sludge from poorly constructed pit latrines and wastewater disposal system in areas with high water table or lowland contaminates ground water and often predisposing to human, animals and environmental health risks (Jiménez et al. 2010; Mwang'onde et al., 2013). In urban areas, full pit latrines and septic tanks are emptied; the sludge is collected by sanitation trucks and delivered to the designated areas on land or in ponds (Tilley et al., 2014; Mateo-sagasta et al., 2015). However, wastewater can either be treated or not treated prior to discharge to receiving water bodies (Mateo-sagasta et al., 2015). Generally, in areas where wastewater treatment ponds are not in place, untreated wastewater or sludge is applied on bare or agricultural land (Jiménez et al., 2010).

Centralized wastewater treatment systems include natural biological treatment systems such as man-made wastewater treatment (stabilization) ponds which are common in tropical and subtropical countries where land is not a compromising factor (Mara, 2003; Naddafi et al., 2009). The stabilization ponds are characterized by a primary treatment including screening solid wastes, in anaerobic pond (s) for ≥1 day, in facultative pond (s) for ≥7 days and in maturation pond (s) for ≥12 days and sometimes supplemented with storage or treatment reservoirs (Mara, 2013). Total retention time in well designed treatment ponds are about 20 to 40 days (Mara, 2000). The anaerobic and facultative ponds are designed to ensure the removal of biological oxygen demand (BOD5) for five days, while maturation ponds are designed to remove the excretal pathogenic bacteria from 3 to 6 log units (Mara, 2013; Hwang, 2012). In addition wetlands systems are as well used to supplement for further reduction of pathogens (Mthembu et al., 2013; Kipasika et al., 2016) and or disinfection of treated wastewater (Silva et al. 2013).

The advantages of stabilization ponds include limited technological investment, low cost, cheap/unskilled labour, and minimal maintenance costs (Mara, 2003; Jiménez et al., 2010). However, the main disadvantage is the limitation of land availability in urban areas (Jiménez, 2006). In developing countries, Sub-Saharan Africa, data on generation, treatment, maintenance and wastewater use are limited (Jiménez, 2006; Sato et al., 2013). Sato et al. (2013) reported that only 3 countries (Senegal, Seychelles and South Africa) out of 48 countries had complete data on generation, treatment and use of wastewater; while 13 countries had incomplete data and the other 32 countries including Tanzania had no data. On the other hand, farmers from urban and peri-urban areas in developing countries depend on low quality irrigation water (LQIW) which is often in form of untreated wastewater, partially treated and haphazardly blended wastewater/ polluted surface water, ground water and well water (Mateo-Sagasta et al., 2013).

A driver for wastewater use is that; wastewater contains valuable resources such as nutrients (for example, nitrogen and phosphorus), organic matters and energy (Mateo-sagasta et al., 2015). It is available all year round and usually is free or available at very low cost (Jiménezet al., 2008). Furthermore, if treated wastewater or sludge is applied in agriculture, it provides nutrients required for growth of the plant (Kołodziej et al., 2016; Antonkiewicz, 2014).

The value of wastewater use has been recognized by farmers worldwide. For example irrigation agriculture plays a dominant role in increasing crop yields and sustainability of production throughout the year (Babayan et al., 2012). For instance, in Tanzania wastewater is used for horticulture production, though the data on the generation, treatment and use are not available (Sato et al., 2013). Wastewater has been used in many parts of Tanzania such as Kilimanjaro and Arusha (Senzia et al., 2009; Mkali et al., 2014) as well as in Morogoro urban and peri- urban areas. However, use of untreated wastewater, partially or even treated wastewater in food production may pose health risks to farmers, traders and consumers (Jiménez et al., 2010).

Although faecal coliforms (FC) count is used to monitor faecal pollution regardless of drawbacks like the growth of other thermotolerant non-faecal organisms at the same temperature of 44°C, but also the *E. coli* remains a good indicator bacterium for faecal pollution (Edberg et al.,

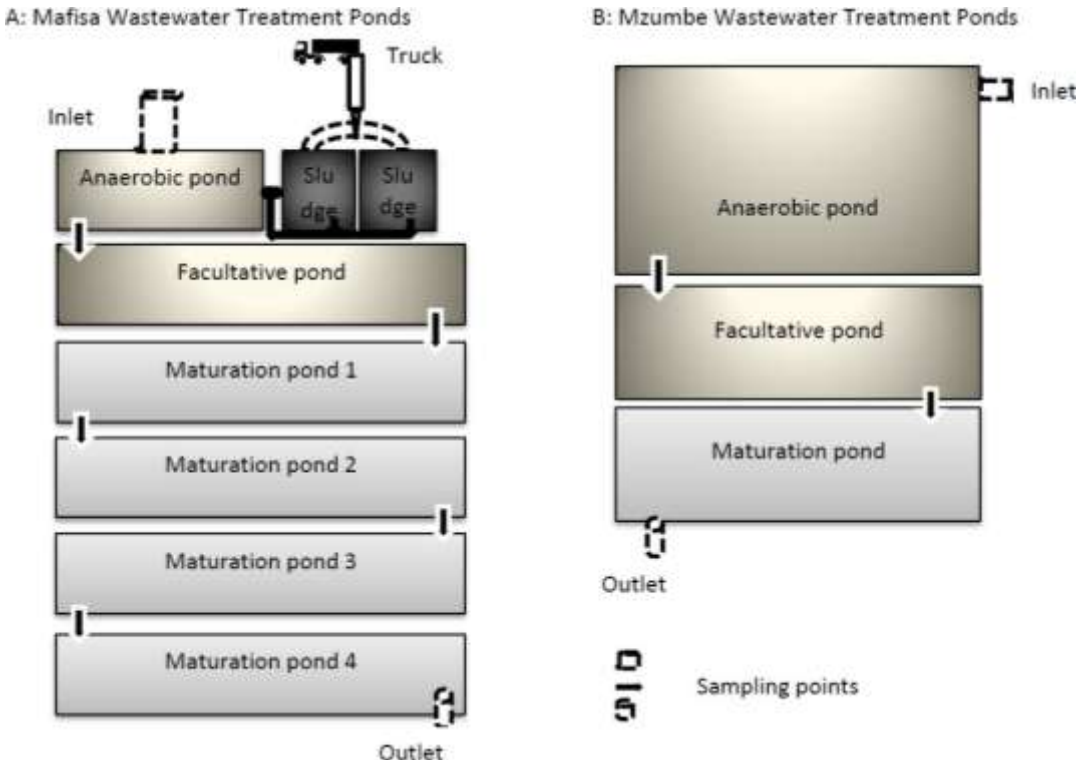

Figure 1. Schematic steps for Mafisa and Mzumbe wastewater treatment ponds.

2000; Okoh et al., 2007). Therefore, the present study was intended to assess the performance of wastewater treatment ponds in reducing the concentration of *E. coli* in treated wastewater to comply with the current WHO guidelines (WHO, 2006a; WHO, 2006b) and the Tanzania water quality standards (URT, 2007) for agricultural irrigation or aquaculture in urban and peri-urban areas of Morogoro.

MATERIALS AND METHODS

Study area

This study was conducted at Morogoro municipality, located at 06°49′20″S 037°39′55″E and Mzumbe is located at 06°53′29″S 37°33′37″E peri-urban of Mvomero district. According to the United Republic of Tanzania census of 2012 the Morogoro urban had about 320,000 inhabitants and Mvomero district had about 310,000 inhabitants. In Morogoro region, during March to August is usually the rainy and cold season with fewer daily hours of sunshine as compared to the dry season from September to February which is likely explaining the factors that may influence wastewater treatment ponds.

Study sites

Two wastewater treatment ponds units were selected; Morogoro urban water and sanitation authority (MORUWASA) - domestic wastewater treatment ponds unit (Mafisa) and Mzumbe wastewater treatment ponds (Figure1). Mafisa wastewater treatment unit consists of two ponds receiving faecal sludge delivered by sanitation trucks, a 9,000 m^3 anaerobic pond, a 12,000 m^3 facultative pond and four maturation ponds (10,000 m^3), all serial connected. Mafisa receives wastewater from residential areas, business areas, institutions and hospitals (Figure 1A). The sludge from sanitation trucks is pumped into the two small sludge ponds and then enters into an anaerobic pond which also receives wastewater from the municipality main sewer canal. The retention time in the Mafisa wastewater treatment unit is about 19 days. Treated wastewater is discharged through a concrete piped canal and passes through a wetland with about 20 acres of rice fields and is further used downstream for vegetable irrigation.

Mzumbe wastewater treatment system consists of an anaerobic pond (6,800 m^3), a facultative pond (2,400 m^3), and a maturation pond (1,600 m^3) serial connected with a retention time of about 22 days (Figure 1B). It serves about 10,000 people from university campus facilities, staff quarters and hospital. The quantity of received wastewater varies depending on the population of students and workers e.g. limited wastewater is generated during the periods of vacation and dry months (September to February) seasons. Treated wastewater is discharged through an earth canal and used downstream for vegetable irrigation. During dry season farmers compete on the limited available treated wastewater for irrigation and some farmers have to suspend growing vegetables.

Sample collection and handling

The purposive sampling technique was used. Samples were collected from the inlet and inlets-outlets of the anaerobic, facultative and maturation (final treated wastewater) ponds at the two study sites (Figure 1). In addition faecal sludge samples were collected from the trucks during emptying/delivering of the sludge from the trucks (Mafisa; Figure 1A). Sampling was done from

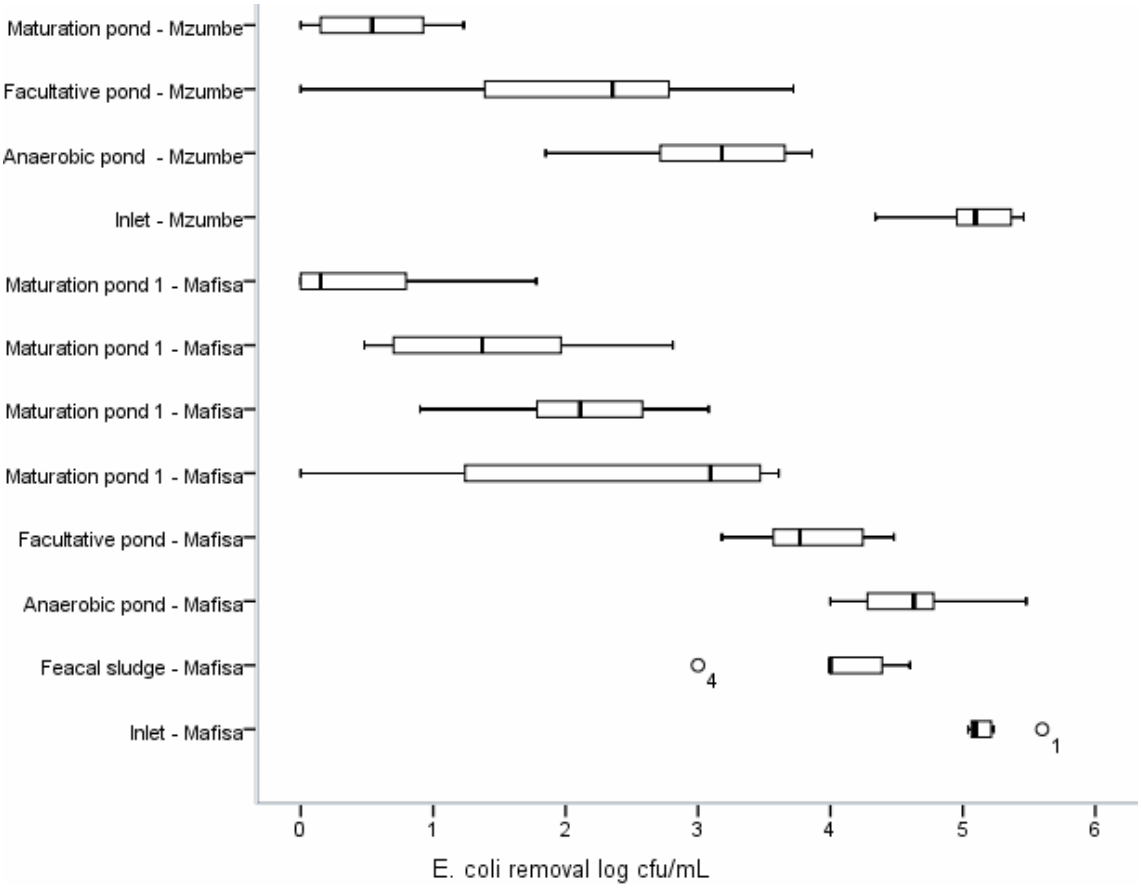

Figure 2. Reduction of concentration of *E. coli* at Mzumbe and Mafisa wastewater treatment ponds (O4 - and O1 - outliers).

August, 2013 to June, 2014 during dry (September to February) and rainy season (March to May). A total of 125 wastewater samples were collected during eleven sampling times. Eighty five wastewater samples were collected from Mafisa and 40 wastewater samples from Mzumbe wastewater treatment units. Wastewater samples were collected using sterile 250 mL glass bottles tied up with a rope. Sampling was done either in the morning or in the evening hours. Samples were immediately placed in an insulated box with cooling elements and transported to the Sokoine University of Agriculture in Morogoro for laboratory analysis on the same day, those were samples collected in the morning. While water samples collected during the evening were kept overnight in a refrigerator at 2 to 8°C prior the analysis on the following day.

Enumeration of *Escherichia coli*

Enumeration and isolation of *E. coli* was done using Petrifilm Select *E. coli* (SEC) plates (3M Microbiology Products, St. Paul, USA) as per manufacturer's instructions. In brief, one milliliter of sample from an appropriate serial 10 - fold dilutions in 0.1% buffered peptone water (BPW) (Oxoid Ltd, Hampshire, England) was inoculated onto SEC plates and incubated at 44°C for 24 h. All blue *E. coli* colonies on the SEC plates with entrapped gas, regardless of size or intensity of colour were counted and interpreted as *E. coli*. If there was no colony on the SEC plates, it was reported as less than 1 cfu/mL (detection limit) equivalent to 0 log cfu/mL.

Data analysis

The concentration of *E. coli* was calculated as colony forming units (cfu) per mL and transformed to $\log_{10}$ cfu/mL (log cfu/mL). Paired samples means of *E. coli* concentration in log cfu/mL between Mafisa and Mzumbe wastewater treatment units were analyzed by t-test using SPSS statistics 20.0 (IBM, California, USA) at $p<0.05$. Reduction of *E. coli* concentration in the different ponds was compared between the two study sites to ascertain if there were differences in removal efficiency of *E. coli*.

RESULTS AND DISCUSSION

Reduction of *E. coli*

The mean concentration of *E. coli* in untreated wastewater was 5.12 log cfu/mL and 5.08 log cfu/mL, which were reduced to 0.65 log cfu/mL and 0.55 log cfu/mL in treated wastewater at Mafisa and Mzumbe, respectively (Figure 2). The mean concentration of *E. coli* in faecal sludge from trucks at Mafisa was 4.05 log cfu/mL (Figure 2). The overall reduction of *E. coli* concentration in wastewater treatment ponds at the two

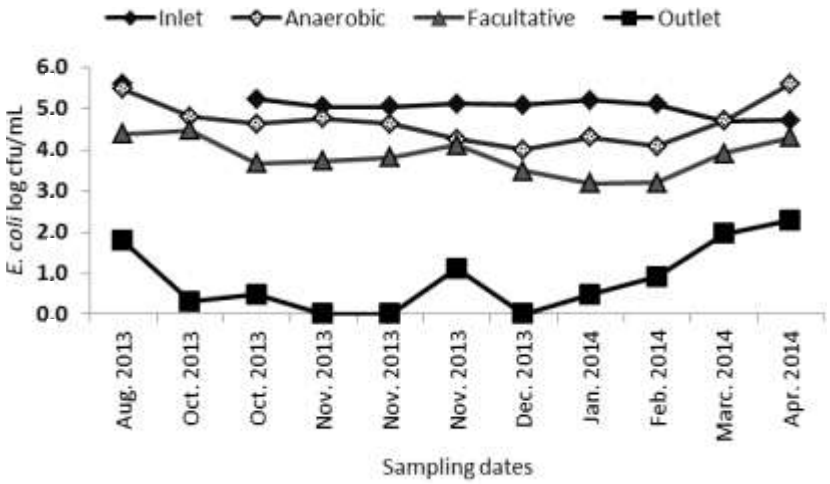

Figure 3. Effect of trend in concentration of *E. coli* at Mafisa wastewater treatment ponds.

study sites was approximately 4-log cfu/mL. The concentration of *E. coli* of about 5.0 log cfu/mL in untreated wastewater has been documented elsewhere (Hendricks and Pool, 2012; Farasat et al., 2012; George et al., 2007). Likewise a similar 4-log reduction of faecal indicators and pathogens was seen in a comparable wastewater treatment ponds systems treating municipal wastewater in Nigeria (Mohammed, 2006) and India (Tyagi et al., 2008). The removal of pathogenic bacteria in the biological wastewater treatment / wastewater stabilization- ponds has been reported as 3 to 6 log units (Jiménez et al., 2008).

Generally, wastewater stabilization ponds are low cost wastewater treatment systems and achieve high enteric pathogen removal in tropical and sub-tropical regions (Mara, 2003; Morris 2003). These systems are well-suited for developing tropical countries as they comprise of a simple technology which is easy to operate, maintain and often water is transported by gravity only. In contrast, modern or secondary and tertiary treatment technologies are unaffordable and complex to operate satisfactorily, thus requiring trained staff to operate, use chemicals, as well as maintenance and electricity is needed to pump and transport wastewater (Tilley et al., 2014). Consequently, such advanced treatment (Tertiary treatment) systems often break down and fail to remove faecal pathogens to acceptable levels allowing a safe use of wastewater in agriculture (Hwang, 2012; Jiménez et al., 2008).

The mean concentration of *E. coli* in untreated wastewater and treated wastewater at the two treatment systems was not different ($P>0.05$), while the E. coli reduction in the anaerobic and facultative ponds effluent was significantly higher at Mzumbe ($P<0.01$) (Figure 2). This was most likely due to longer retention times in the anaerobic pond (10 days), the facultative pond (6 days) and the maturation pond (6 days) at Mzumbe as compared to Mafisa anaerobic pond (2 days), facultative pond (4 days) and four maturation ponds (3, 3, 3 and 4 days). These findings are in agreement with the comparable studies elsewhere (Hwang, 2012; Mara, 2000). It is also well-established that retention time and pathogens (for example, *E. coli*) reductions in stabilization ponds are positively correlated (U. S. EPA, 2002). A similar reduction in *E. coli* concentration (<1 log cfu/mL) in effluent from the two study sites was due to an approximately 2-log reduction occurring in the four maturation ponds at Mafisa (Figure 2).

Maturation ponds generally show a high faecal indicator and pathogens reduction (Pescod, 1992; Hwang, 2012). A similar *E. coli* reduction (3.85±4.32 to 1.11 ±1.12- log *E. coli*) as seen in the present study was reported in a comparable wastewater treatment system in Thailand (Kantachote et al., 2009) consisting of an anaerobic pond (6 days), a facultative pond (9 days) and a maturation pond (4 days).

At Mafisa, the *E. coli* concentration in the wastewater treatment ponds was high in March, April and August (Figure 3). It should also be noted that rain events are so severe with extraordinary volumes of storm- and surface run-off water. However, no such seasonal variation was observed for *E. coli* concentration at Mzumbe which receives domestic wastewater and rain water only. There were no storm- and surface run-off water, contaminated with animal and human faeces entering to the wastewater treatment ponds (Figure 4). Generally, it is possible to increase environmental health risk if the receiving water bodies, flooding lowland crop/vegetables fields may be contaminated with faecal pathogens and may lead to the diarrheal diseases, such as, Cholerae outbreaks and typhoid fevers (Mwang'onde et al., 2013); and skin diseases (Trang et al., 2007), which is common when

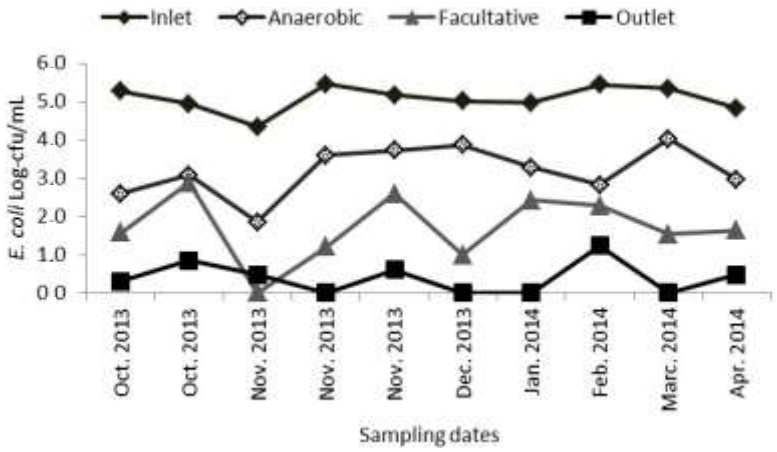

Figure 4. Effect of trend in concentration of *E. coli* at Mzumbe wastewater treatment ponds.

using untreated wastewater.

Use of treated wastewater for food production

The Mafisa and Mzumbe wastewater treatment units were able to reduce the *E. coli* concentration to less than 1 log cfu/mL a guideline value that is according to FAO (Pescod, 1992) and WHO guidelines (2006) allowing the treated wastewater to be used for unrestricted agricultural irrigation. Thus, the current agricultural use practices of the treated wastewater by the farmers at the two study sites would according to these guidelines not pose significant human health risks. Several cities and municipalities in Tanzania as well as other Sub-Saharan African countries have constructed wastewater treatment facilities similar to Mafisa and Mzumbe. Findings in this study thus demonstrate that, if the wastewater treatment ponds are well operated and maintained they have the capacity to effectively reduce faecal pathogens to a level where urban and peri-urban farmers can safely use treated wastewater to irrigate their crops that can feed the urban consumers (Senzia et al., 2009; Sato et al., 2013; Kulkarni, 2014). However, the *E. coli* concentrations seen at Mafisa in rainy and cold season during March, April and August where severe rain events occur with extraordinary volumes of storm - and surface runoff-water showed less reduction. This suggests that, even the treated wastewater may not be safe to use for unrestricted irrigation purposes, in particular if such events coincides with infectious disease outbreaks like dysentery or cholera. Specific health risk impact assessments are therefore, needed for such worst-case scenarios.

Conclusion

The findings from this study allow wastewater stakeholders to make informed decisions about the use of treated wastewater and the risk of contamination of downstream receiving water bodies and for use in food production. The performance of the two simple wastewater treatment units was satisfactory in reducing *E. coli* and potential risk from low quality irrigation water (e.g. treated wastewater) use during dry season. However, there were good estimated retention times of 19 and 22 days on both wastewater treatment ponds study sites. The concentration of *E. coli* was reduced to <1log cfu/mL, the level that is recommended by the current WHO guidelines (2006) for safe use in agricultural irrigation and aquaculture. Basing to the findings from the study sites on the quality of treated wastewater, it may be used for agricultural irrigation and aquaculture. The data generated from this study may contribute to the national and international policy and guidelines (e.g. FAO / WHO guidelines) with regard to the reduction of *E. coli* in wastewater and safe use of treated wastewater in agricultural irrigation and aquaculture.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Antimicrobial activities of a multi-species probiotic ingredient derived from opaque sorghum beer during its propagation in a starchy career model

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This study evaluates the antimicrobial activities of a multi-species probiotic ingredient derived from the African opaque sorghum beer during its propagation in a starchy career model. The aim was to establish the optimum growth conditions that warrant the optimum antimicrobial activities in the product. The antimicrobial activities were tested against Gram-positive bacteria (*Staphylococcus aureus* ATCC 27844, *S. aureus* MR 825), Gram-negative bacteria (*Escherichia coli* ATCC 25922, *E. coli* O157:H7 ATCC 700728, *Salmonella typhi* R 30951401, *Klebsiella pneumoniae* ATCC 35657), as well as against yeast (*Candida albicans* MHMR), using agar disc diffusion method. Also, the growth of viable cells and physicochemical parameters during the propagation were monitored. The results showed that the pH and dry matter content of the probiotic ingredient decrease significantly ($p<0.05$) during the propagation whereas the lactic acid, the titratable acidity, lactic acid bacteria (LAB), yeasts and moulds counts increase significantly ($p<0.05$). From 0 to 12 h, the product failed to inhibit the growth of all indicator strains. From 24 h and onward, the probiotic career inhibited all indicator strains except for *K. pneumoniae* (ATCC 35657) which could not be inhibited. Clearly, our study showed that 36 h of the propagation were sufficient to generate a probiotic ingredient with optimum antimicrobial activities.

Key words: Probiotic, antibiotic, opaque beer, antimicrobial activity.

INTRODUCTION

The use of antibiotics for diseases prevention or healing and as growth factors in animals feeding contributed to the development of breeding through health improvement and zootechnic performances of animals. The estimated

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antibiotic use in animal agriculture is 7.36 to 11.18 million kilogram per year (Khachatourians, 1998; Doyle, 2001). Globally, tonnes of antibiotics have been distributed in the biosphere during an antibiotic era of only about 60 years duration (Balcázar et al., 2006). However, these amounts of antibiotics have favored a strong selection among microorganisms, which developed resistance to these antibiotic chemicals, mainly by a horizontal and promiscuous flow of resistance genes (Aarestrup et al., 2000; Randall et al., 2003; SCAN, 2003 Currently, the potential for agricultural antibiotics to contribute to the development of antibiotic-resistant bacteria of human concern is the subject of intense debate and research (Wegener et al., 1999). Resistance mechanisms can arise by two ways: Chromosomal mutation or acquisition of plasmids. Chromosomal mutations cannot be transferred to other bacteria but plasmids can transfer resistance rapidly (Lewin, 1992). There is an increasing interest in finding alternatives to the use of antibiotics in animals feeding due to the ban of subtherapeutic antibiotic usage in Europe (Fajardo et al., 2012). Thus, several alternative methods are explored. Among them, probiotics are a subject of particular attention.

The probiotics with antimicrobial properties act by producing bacteriocins such as nicin (Yateem, et al., 2008) or by lowering the pH as a result of acidic compounds production such as lactic acid (Psomas et al., 2001). The progressive reduction of the use of antibiotics in animals feeding, as growth promoters, has raised renewed interest in the incorporation of microbial strains in animals feeding, in order to maintain the beneficial effect obtained with antibiotics (Guillot, 2001). Kpete-kpete is the starter culture used to ferment tchoukoutou, the most produced and consumed opaque sorghum beer in Benin.

Lactic acid bacteria and yeasts have been reported (Kayodé et al., 2007) to be the major microorganisms involved in the fermentation of tchoukoutou. These fermentation microorganisms have been reported to possess probiotic effects, to reduce the level of pathogenic bacteria occurring in beverages and to reduce the severity duration and morbidity of diarrhea (Mensah et al., 1991; Kimmons et al., 1999). Recent researches in Ivory Coast showed net body weight increase and increased feeding efficiency in broilers when settling of palm wine or yogurt probiotic were used as feeding supplementations (Bohoua, 2008). In Benin, an investigation by Houndonougbo et al. (2011) showed low mortality and increased body weight gain when chicken's feed was supplemented with starter culture harvested from opaque sorghum beer. In addition, a recent survey conducted by N'tcha et al. (2015) showed that Kpete-kpete is used to cure humans and animals diseases such diarrhoea, dysentery and wounds. The aim of the present study is to optimize the antimicrobial activity of Kpete-kpete during its propagation in a sorghum-based starch used as career.

MATERIALS AND METHODS

Starchy career model

Starch extracted from a red variety of sorghum (*Sorghum bicolor* (L) Moench) was used as starchy career. The starch was extracted following the process described by Kayodé et al. (2012). Ten kilogrammes (10 kg) of cleaned sorghum grains were dehulled using a mini-PRL dehuller (Thiès, Sénégal) and then ground into flour.

Propagation of the probiotic ingredient and sampling

The sorghum flour is mixed with distilled water to obtain a dough (45% w/w), which is inoculated with 10% (w/w) of Kpete-kpete, kneaded into dough and allowed to ferment in a plastic bucket with lid for 72 h. Samples were withdrawn at 0, 6, 12, 24, 36, 48, 60 and 72 h of propagation for analysis. At each time point, 12.5 g of sample were aseptically taken from the flask for microbiological analysis and antimicrobial activity (10 and 2.5 g, respectively). Another sample of 50 g was kept at -10°C for pH, titrable acidity and dry matter measurements within 4 h approximately after sampling. The remaining sample was frozen for further analysis. In other to check the effect of drying temperature on the functional properties of the ingredient, we dried (42°C for 24 h) the product obtained at 72 h of propagation and derived samples for analysis of antimicrobial activities.

Microorganism materials used for antimicrobial test

The assayed pathogens included Gram-positive (*Staphylococcus aureus* ATCC 27844*, S. aureus* MR 825), Gram-negative (*Escherichia coli* ATCC 25922*, E. coli* O157:H7 ATCC 700728*, Salmonella typhi* R 30951401, *Klebsiella pneumoniae* ATCC 35657) and one yeast (*Candida albicans* MHMR). *E. coli* O157:H7 ATCC 700728*, S. aureus* ATCC 27844, *S. typhi* R 30951401*, K. pneumoniae* ATCC 35657 were supplied by the Laboratory of Food Safety and Water Quality of Ministry of Health, whereas *E. coli* ATCC 25922*, S. aureus* MR 825 and *C. albicans* MHMR were obtained from the Laboratory of Biology and Molecular Typage in Microbiology. Each stock culture was maintained in the respective growth media, containing 30% of glycerol, and stored at -80°C. Before the use in experiments, the strains were transferred into fresh growth media and incubated at suitable temperature for 18 to 24 h. This was followed by two consecutive transfers in the medium and incubated under the conditions indicated. The antimicrobial activities of ingredients were evaluated by means of disc diffusion assays.

Physicochemical analysis

Water content was determined as described (AACC, 44-15 A, 1984). Titratable acidity and pH were determined as described by Nout et al. (1989). The pH was measured using a digital pH-meter (JENWAY, Model 3505, UK) calibrated with buffers at pH 4.0 and 7.0 (WTW, Weilheim, Germany).

High pressure liquid chromatography (HPLC) analysis of sugars and organic acids

Lactic acids and soluble sugars were determined following the method developed by Mestres and Rouau (1997). 50 mg of samples were extracted with 5 mM sulphuric acid in 1.5 mL centrifuge tubes under continues agitation for 30 min at room temperature. After extraction, samples were centrifuged at 3500 x g for 30 min and filtered through a 0.45 µm pore filter before quantifi-

cation by HPLC using an Aminex HPX-87H$^+$cation-exchange column (BioRad Hercules, USA) thermostated at 37°C. Detection was done at 210 nm with an IR-detector. Elution was with sulphuric acid 5 mM at a flow rate of 0.6 mL min^{-1}. The injection volume of the sample was 20 µL. Organic acids and sugars were expressed as g/kg dry matter. Analyses were performed in duplicate.

Counts of viable microorganisms

Total counts of lactic acid bacteria (LAB), yeasts and moulds were enumerated according to the method described by Nout et al. (1987). At each sampling time, duplicate samples (10 g) were diluted in 90 mL sterile peptone physiological saline solution (5 g peptone, 8.5 g NaCl, and 1000 mL distilled water, pH = 7.0) and homogenized with a Stomacher lab-blender (type 400, London, UK). Decimal dilutions were plated. Viable counts of LAB were determined on de Man, Rogosa and Sharpe Agar (MRSA, CM 361, Oxoid, Hampshire, England) containing 0.1% (w/v) natamycin (Delvocid, DSM, The Netherlands) with incubation in anaerobic jar (Anaerocult A, Merck KGaA, Germany) at 30°C for 72 h. Yeasts and moulds were enumerated using Malt Extract Agar (MEA, CM 59 Oxoid, Basingstoke, Hampshire, England). MEA plates were incubated at 25°C for 72 to 120 h. The colonies were then counted and expressed as logarithmic colony forming units per gram (log_{10} CFU/g) of the sample.

Agar disc-diffusion assay

The capacity of the ingredients to inhibit a representative group of pathogens and other was determined by modifying the disc diffusion method of NCCLS (2003). Twenty milliliters (20 mL) of molten Mueller-Hinton Agar (MHA, CM 337, Oxoid, Basingstoke, Hampshire, England) were poured into sterile Petri dishes and allow to solidify. 100 µL of the overnight Mueller-Hinton broth (MHB, CM 405, Oxoid, Basingstoke, Hampshire, England) culture of each pathogen strain, which have been adjusted to 0.5 McFarland-turbidity, was spread on the plates. Once the plates were dried aseptically, five blank discs papers (6 mm in diameter) were placed onto the surface of the agar. The moist or dried sample of probiotic ingredient was reconstituted with sterile distilled water to obtain a solution of 500 mg mL^{-1}. This solution was stirred vigorously using a magnetic stirrer for 30 min and then centrifuged at 3 500 x g for 30 min. Forty microliters (40 µL) of each supernatant were placed into the discs. The plates were left at room temperature for 1 h so that the absorbed supernatant become diffused into the agar, and then incubated at 37°C for 24 h. The tests were carried out in duplicate.

Statistical analysis

The propagation trials were carried out in triplicate. Mean values and standard deviations were calculated from the experimental data. Statistical Package for Social Science (SPSS), version 16.0 (Chicago, IL, USA) was used. Data analyses involved one-way analysis of variance (ANOVA). Significant difference was established at 5%.

RESULTS AND DISCUSSION

Changes in pH, titratable acidity, lactic acid, maltose and glucose contents of the ingredient during the propagation

The pH value decreased significantly ($p<0.05$) from 5.63 to 4.03 within 24 h of propagation (Table 1). After 24 h, the pH remains relatively stable around a value of pH = 3.84. The titratable acidity increased significantly ($p<0.05$) from 8.95 g/kg at 0 h (calculated as lactic acid) to 40.21 g/kg at 72 h of propagation. The progressive fall in pH and increase in titratable acidity during the fermentation process is typically characteristic of lactic acid fermentation of cereal grains (Singh et al., 2003; Vieira-Dalodé et al., 2007).

According to Tharmaraj and Shah (2009), the production of organic acids such as acetic, citric and lactic acids are responsible for the decrease of pH in such product. Interestingly, Nout (1991) and Steinkraus (1996) reported that a pH of 3.5 to 4.0 is sufficient to inhibit Enterobacteriaceae and other Gram-negative bacteria. Concomitantly to these modifications, we observed a decrease in the dry matter content of the ingredient which shifted from 51.98% at 0 h to 48.89% at 72 h of propagation.

The changes in lactic acid, maltose and glucose concentrations of the ingredient during propagation are also reported in Table 1. The amount of lactic acid in the dough increased rapidly to reach a maximum at 36 h of propagation. Thereafter, a significant decrease was observed until the end of propagation. The maltose content increased from 2.13 g/kg at 0 h to 3.84 g/kg at 24 h of fermentation and then decrease to 1.85 at 36 h of propagation and no significant decrease was observed afterward. Similar trend was observed for the glucose content with the difference that the most significant change occurred at 36 h of propagation. Water and volatile compounds production during aerobic and anaerobic catabolism by yeasts and LAB might be responsible for these changes (Hounhouigan et al., 1993). Similar modifications were reported in other African fermented cereal products (Muyanja et al. 2002; Sefa-Dedeh et al., 2003; Vieira-Dalodé et al., 2007).

Changes in lactic acid bacteria, yeasts and moulds counts during propagation

The changes in microbial count of the ingredient during the propagation are shown in Figure 1. The LAB counts increased from 6.47 to 10.44 log cfu/g. The most significant ($p< 0.05$) increase in the numbers of LAB was noted during the first 36 h of propagation. No significant growth was noted between 36 and 48 h. Further incubation led to a significant decrease of LAB counts. Similar trend was observed for yeast and mould counts. The symbiotic relationship between LAB and yeasts during the fermentation process of starch-based product is well established (Nout, 1991). Indeed, the development of yeasts is favored by the acidic environment created by LAB, while the growth of bacteria is stimulated by the presence of growth factors such as vitamins and soluble nitrogen compounds provided by yeasts.

Table 1. Trends of pH, titratable acidity, lactic acid, maltose, glucose and dry matter during propagation.

Time (h)	pH	Titratable acidity	Lactic acid	Maltose	Glucose	Dry matter
0	5.63±0.12*[a]	8.95±1.06[a]	0.69±0.22[a]	2.13±0.44[a]	3.73±1.02[a]	51.98±0.62[a]
12	4.55±0.32[b]	22.76±5.58[b]	7.43±0.90[b]	2.48±1.15[ab]	3.56±0.49[a]	51.17±1.46[abc]
24	4.03±0.07[c]	28.56±2.82[bc]	16.33±1.94[c]	3.84±0.89[b]	3.51±0.20[a]	50.45±0.95 [abc]
36	3.89±0.02 [c]	35.26±3.04[c]	24.88±2.20[d]	1.85±0.21[a]	1.34±0.69[bc]	50.25±0.97 [abc]
48	3.87±0.10 [c]	37.36±4.91[d]	20.21±0.20[e]	1.63±0.57[a]	1.68±0.84[b]	49.62±1.61[bc]
60	3.80±0.06 [c]	40.22±5.95[d]	20.61±0.88[e]	1.21±0.32[a]	0.26±0.05[c]	49.10±1.59[c]
72	3.84±0.13 [c]	40.21±6.05[d]	18.10±1.47[ce]	1.16±0.16[a]	0.25±0.11[c]	48.89±1.71[c]

*Values are means ± standard deviations. Values bearing different letters in a column are significantly different (p <0.05).

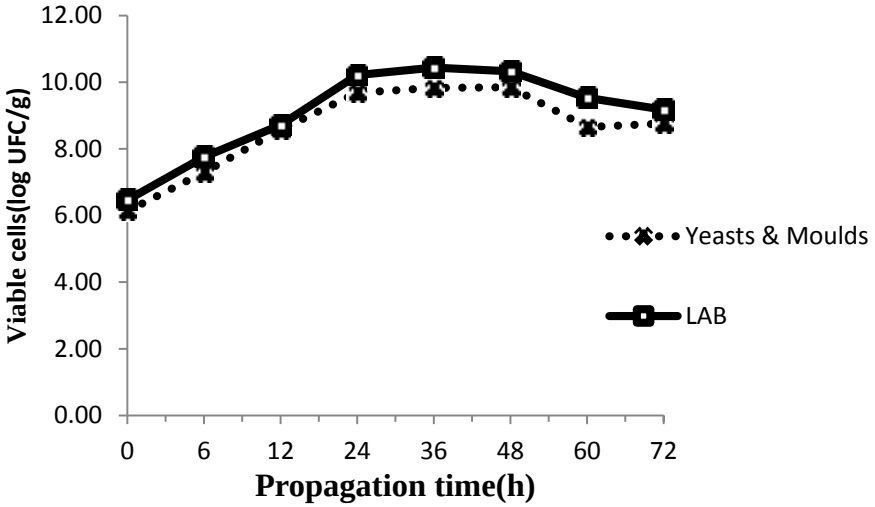

Figure 1. Changes in viable cells counts during probiotic ingredient production.

Antimicrobial activity

The antimicrobial activity was assessed during the probiotic ingredient propagation against indicator strains by Agar disc diffusion method. Figure 2 showed the inhibition zone diameter resulting from the antimicrobial activity of the probiotic ingredient against Gram positive, Gram negative bacteria and one yeast. A superior diameter to 1 mm around the disc was considered as a positive result. Thus from 0 to 12 h, the probiotic ingredient failed to show any inhibitory activity against all indicator strains. It is most likely that enough antimicrobial compounds were not produced by the functional microorganisms during this propagation time. Ahmad et al. (2014) reported that the antimicrobial effect of *Lysinibacillus* jx416856 started after 15 h of incubation in MRS broth and growth dependent bacteriocin activity was observed at early log phase (18 h). However, at 24 h of propagation, our probiotic ingredient inhibited indicator strains broadly except *K. pneumoniae (*ATCC 35657). After 24 h of propagation, all indicator strains were inhibited by the probiotic ingredient. The inhibition zone diameters observed at 36 h of propagation were significantly higher for all indicator strains. The antimicrobial effect increased until a stationary phase which occurs at 36 h and remained constant till 72 h. Ahmad et al. (2014) reported similar trend for *Lysinibacillus* jx416856 and even though indicated that its antimicrobial activity decreased eventually with a constant level. In another study, Djadouni and Kihal (2012) recorded optimal bacteriocin production in MRS after 24 h of incubation. These results supported our findings since the growth of all indicator strains tested were inhibited after 24 h of the propagation time. Interestingly, the lactic acid production, and probably bacteriocin, significantly increased after 24 h of the propagation. Between 24 and 36 h the inhibition zone diameters increase steadily and remains constant (P> 0.05) till 72 h of propagation. This suggested that 36 h of propagation is enough to inhibit all pathogen tested. Among the indicator strains, *C. albicans* MRMH (16±1.41 at 72 h), *S. aureus* RM 825 (15±1.41 at 48 h), *E. coli* O157: H 7 ATCC 700728 (14.75±0.35 at 36 h) were extensively inhibited by the probiotic ingredient while *S. typhi* R30951401 (12.5±2.12) and *K. pneumoniae* ATCC 35657 (11.5±2.12) were weakly inhibited. Clearly, the

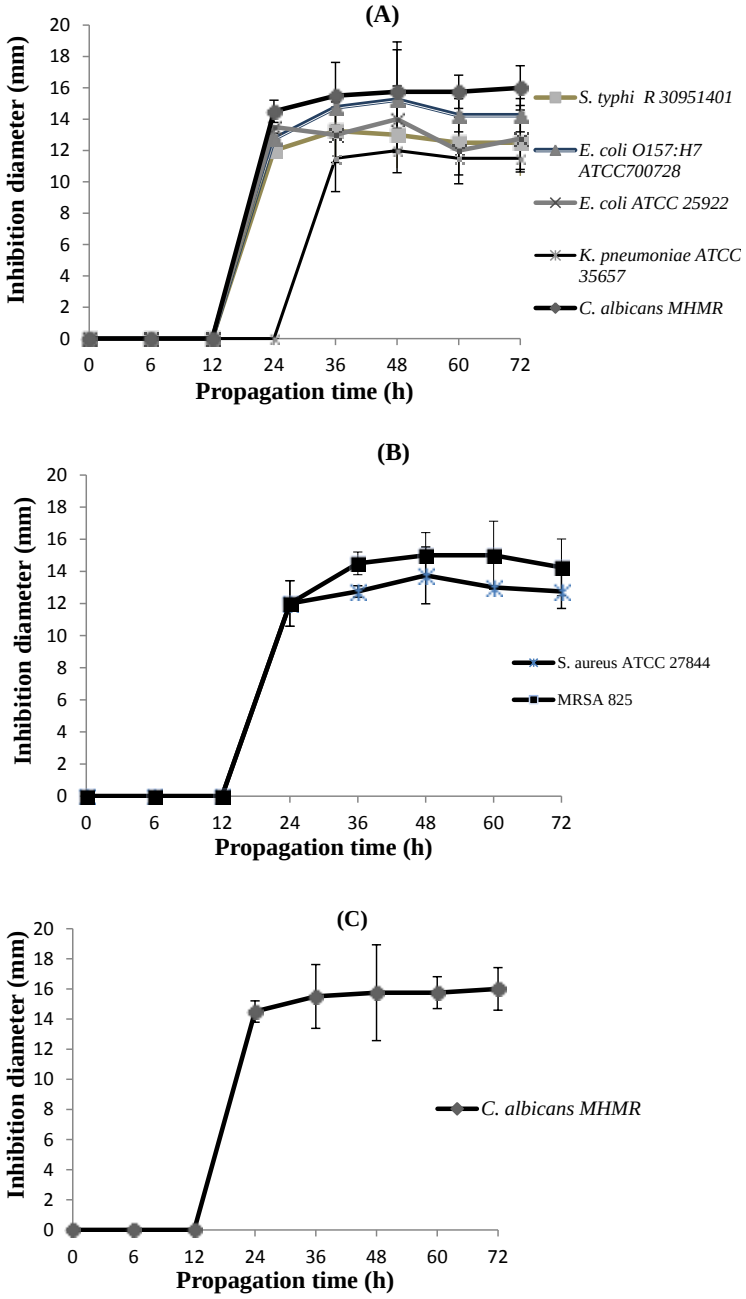

Figure 2. Changes in antimicrobial activity of probiotic ingredient against Gram negative (A) and Gram positive (B) bacteria and yeast (C) during propagation time. The inhibition diameter was measured after 24 h of incubated at 37°C by disc diffusion method.

inhibitory activity depends on the propagation time and on the type of microorganism considered. In other to check the effect of drying temperature on the functional properties of the ingredient, we dried the product at 42°C for 24 h. Thus, after drying, the probiotic ingredient at 72 h also exhibited antimicrobial activities against all pathogen strains (Figure 3). There was no significant difference between inhibition zone diameters recorded for the dried and undried probiotic ingredient.

We studied the relationship between some measured parameters (Table 2). High negative correlation exists between the lower pH and the antimicrobial activity (r = -0.932). In a similar study, Tejero-Sariñena et al. (2012) reported that the pH was inversely correlated with the diameter of inhibition against the pathogenic indicator strains. Level of lactic acid is in high positive correlation with the antimicrobial activity. The pH-lowering effect resulting from acid production is most likely responsible

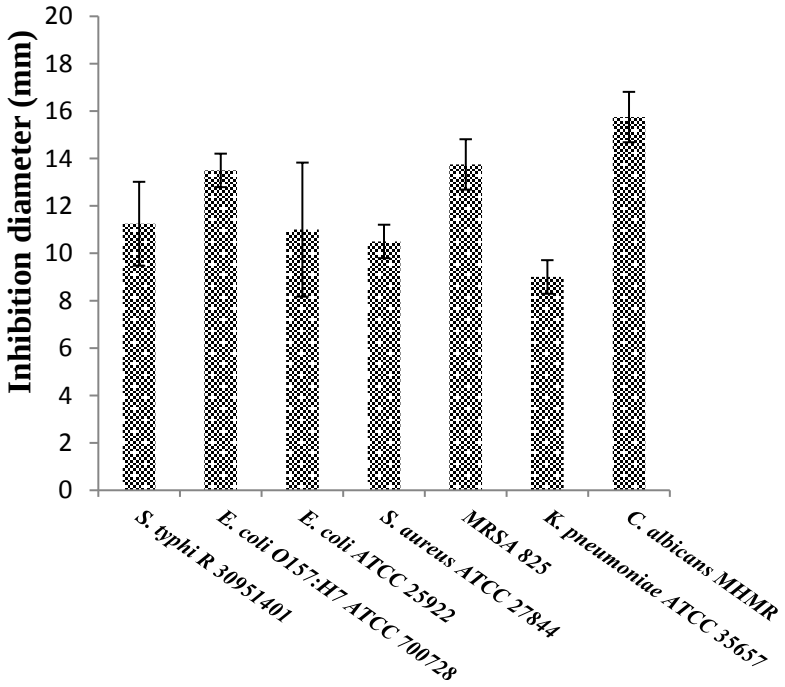

Figure 3. Antimicrobial activity of dried probiotic ingredients against indicator strains by agar disc diffusion method.

Table 2. Pearson correlation matrix between physicochemical, microbiological and antimicrobial activity.

Activity	pH	Dry matter	Lactic acid	Maltose	Glucose	Yeasts and moulds	LAB
Dry matter	0.617*						
Lactic acid	-0.944**	-0.439					
Maltose	0.228	0.222	-0.220				
Glucose	0.705**	0.522	-0.719**	0.602*			
Yeasts and moulds	-0.772**	-0.280	0.758**	0.064	-0.191		
LAB	-0.861**	-0.193	0.856**	0.008	-0.288	0.942**	
Antimicrobial activity	-0.932**	-0.639*	0.931**	-0.345	-0.770**	0.644**	0.779**

**Correlation is significant at the 0.01 level; *Correlation is significant at the 0.05 level; antimicrobial activity (expressed in mm of inhibition diameter).

for the inhibitory mechanism. Indeed, the inhibitory effects of lactic acid bacteria might be due to either individual or joint production of organic acids, hydrogen peroxide, or bacteriocins (Ennahar et al., 2000; Villamil et al., 2003; Vázquez et al., 2005). Moreover, it has been reported that LAB produce a large number of antimicrobial compounds such as organic acids, H_2O_2, diacetyl, enzymes, bacteriocin, and biosurfactants which are effective against food spoilage and pathogenic bacteria (Sharma and Saharan, 2014).

Conclusion

The probiotic ingredient derived from the African opaque sorghum beer exhibited antimicrobial activity with a large spectrum being effective against Gram negative, Gram positive and the yeast microorganisms. This antibacterial activity is preserved during the propagation process of the functional microorganisms in a cereal-based starchy career. 36 h of propagation is enough to inhibit all pathogens tested. The microorganisms involved in the starter of African sorghum beers could an alternative to antibiotic chemicals. More in-deep researches are ongoing on the molecular and functional characterization of these microorganisms.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Drug-resistant bacteria in frozen and fresh marine shrimp

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This study aimed to evaluate the bacteriological quality of frozen and fresh shrimp samples by *Salmonella* detection and the quantification of staphylococci and coliforms. Antimicrobial susceptibility profile of staphylococci isolates was also determined. A total of 30 shrimp samples commercialized in Sobral-CE - 15 fresh and 15 frozen, each one weighing 500 g - was analyzed. There was no contamination by *Salmonella* and/or coliform, but the *Staphylococcus* quantification showed that 12 samples (80.0%) of the frozen and 10 (66.7%) of the fresh shrimp presented a bacterial load above 3.0 log_{10} CFU g^{-1} - a limit recommended by the current legislation in Brazil. 17 drug-resistant staphylococci strains were isolated, and the following antimicrobial resistance profiles were detected: monoresistance (n=4), cross-resistance to beta-lactam (n=4), and multidrug resistance to: Oxacillin+ampicillin+tetracycline (n=1), oxacillin+tetracycline+ penicillin+chloramphenicol+vancomycin (n=1). The findings indicate that frozen and fresh shrimps may act as vehicles for the spread of staphylococci resistant to drugs of clinical interest.

Key words: Enterobacteria, *Staphylococcus* drug-resistant, shrimp.

INTRODUCTION

Seafood contaminated with foodborne bacterial pathogens is a worldwide problem. In this context, food outbreak cases involving the consumption of shrimp have been reported (Jiménez et al., 2011), serving as an alert to the need to ensure a more thorough quality of the product.

In shrimps, the most commonly used indicators for bacterial quality are the enteric bacteria (fecal coliforms and *Salmonella*) (Koonse et al., 2005) and *Staphylococcus* (Noor et al., 2014). Fecal coliforms and bacteria from the genus *Salmonella* are indicators of fecal contamination, and its occurrence in seafood is associated with food poisoning by infectious agents. On the other hand, the presence of staphylococci in food is associated with inadequate handling practices, causing food poisoning by intoxication.

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Other problem that has been associated with shrimp consumption is the emergence of drug-resistant microorganisms in these invertebrates. The data obtained from Nawaz et al. (2015) indicate that the use of antibiotics in shrimp aquaculture ponds may select bacteria resistant to these drugs. Furthermore, these authors alert to the fact that imported shrimp can be a reservoir of multiple antibiotic-resistant bacteria.

Food contamination with multidrug-resistant bacteria is considered a potential source for the wide dissemination of resistant-bacteria in communities (Le et al., 2015). Resistance to 16 drugs (ampicillin, ciprofloxacin, polymixin B, cefixime, amoxicillin, ceftriazone, penicillin, chloramphenicol, trimethoprime-sulfomethoxazole, gentamycin, nalidixic acid, kanamycin, vancomycine, erythromycin, tetracycline, streptomycin) in pathogenic bacteria isolated from shrimps was reported by Noor et al. (2014), indicating that these invertebrates can be vehicles in the transmission of drug resistant strains.

Thus, this study aimed to evaluate the bacteriological quality of frozen and fresh shrimp samples by (1) *Salmonella* detection, (2) quantification of staphylococci and coliforms, and (3) assessment of susceptibility profiles of staphylococci strains.

MATERIALS AND METHODS

Samples of *Litopenaeus vannamei* shrimp (15 fresh and 15 frozen, each sample consisting of a shrimp pool weighing 500 g) were purchased at local retail markets in Sobral, Ceará, Brazil. All samples were stored in isothermal boxes and taken to the Laboratory for Bioprospecting and Applied Molecular Experimentation (NUBEM) at INTA College. The time gap between sampling and analysis took no longer than one hour.

Preparation

For *Staphylococcus* analysis and coliform quantification, 25 g of each sample were aseptically weighed and mixed in 225 mL of saline solution at 0.85%. This homogenate corresponded to a 10^{-1} dilution, and it was the standard set for subsequent series of decimal dilutions up until 10^{-3} in a 0.85% saline solution, at a 1:9 ratio.

Quantification and isolation of staphylococci

Staphylococcus Standard Plate Count (SPC) was performed in Agar Baird-Paker (BP - Difco) enriched with an egg yolk solution at 50 and 1% potassium tellurite, as described by Bennett and Lancette (2001). Colonies in the BP medium grown with typical *Staphylococcus* features (black, and with halo) were isolated in Brain Heart Infusion broth (BHI- Difco). All strains were subjected to biochemical screening and were identified as *Staphylococcus aureus* when presented the following profile; (1) Gram-positive cocci; (2) ability to coagulate rabbit plasma; (3) mannitol (+) in mannitol salt agar (10% NaCl); (4) oxidase (-) in oxidase strips (Laborclin); (5) acetoin production (+) in Voges-Proskauer. SPC calculation was performed by multiplying the colony count of *Staphylococcus* by its correspondent dilution, expressed as $\log_{10}$ colony forming units (CFU) per gram.

Coliform quantification

Quantification of coliforms was performed by the multiple-tube fermentation method according to recommendations of Feng et al. (2002), using presumptive test and confirmed test for fecal coliform. For presumptive test, 1 mL aliquots from each dilution (10^{-1} to 10^{-3}) were inoculated into 3 lauryl tryptose broth tubes incubated at 35 ± 0.5°C. Confirmed test for fecal coliform was performed from inoculation of positive (fermentation of lactose) tubes to EC broth and incubated at 44.5 ± 0.2°C for 24 ± 2 h.

Salmonella detection

25 g of each sample were aseptically weighed and inoculated in lactose broth, with incubation for 24 h at 35°C. After incubation, selective enrichment was performed in Tetrathionate Broth (Difco) for 24 h at 35°C, followed by plating on MacConkey agar (Difco) and Brilliant Green Bile Agar. After this procedure, colonies with *Salmonella* characteristics were plated on tryptone soy agar medium (TSA) for biochemical screening in lysine iron agar, sulfide indole motility agar and triple sugar iron agar (Andrews et al., 2014).

Antibiogram

Staphylococcus colonies isolated in TSA were selected and put to antimicrobial susceptibility testing using disk-diffusion technique in Mueller-Hinton agar. All colonies were suspended in a 0.85% saline solution until a 0.5 turbidity in the McFarland scale. This suspension was then homogenized, and colonies were plated with a sterile swab on Mueller-Hinton agar. After this procedure, the following antimicrobial discs were applied: Imipenem 10 µg (IMP); cefepime 30 µg (CPM); chloramphenicol 30 µg (CLO); streptomycin 10 µg (EST); ceftriaxone 30 µg (CTX); oxacillin 1 µg (OXA); tetracycline 30 µg (TET); gentamicin 10 µg (GEN); penicillin 10 µg (PEN); ciprofloxacin 30 µg (CRO); vancomycin 30 µg (VAN); ampicillin 10 µg (AMP). All plates were incubated at 35°C for 24 h. Diameter of the inhibition zone was then measured, and the strains were classified according to the standard established by CLSI (2012).

Statistical analysis

Data were analysed by software GraphPad Prism 5.0. ANOVA followed by Student-Newman Keuls as *post hoc* test. The values of $p<0.05$ were considered statistically significant.

RESULTS

Salmonella was not detected in any of the 30 samples analyzed: 15 samples of frozen shrimp and 15 of fresh shrimp. Index <3.0 MPN mL^{-1} of fecal coliform was detected for all samples. The results for the quantification of coagulase-positive *Staphylococcus* (CPS) are shown in Figure 1. For frozen shrimp samples, an oscillation of 2.43 to 5.28 $\log_{10}$ CFU g^{-1} was observed. In fresh shrimp, staphylococci population ranged from 2.19 to 5.39 $\log_{10}$ CFU g^{-1}. There was no significant statistical difference ($p>0.05$) between frozen shrimp versus fresh shrimp. Table 1 shows the results for antimicrobial resistance profiles of 76 staphylococci strains: 10 profiles were

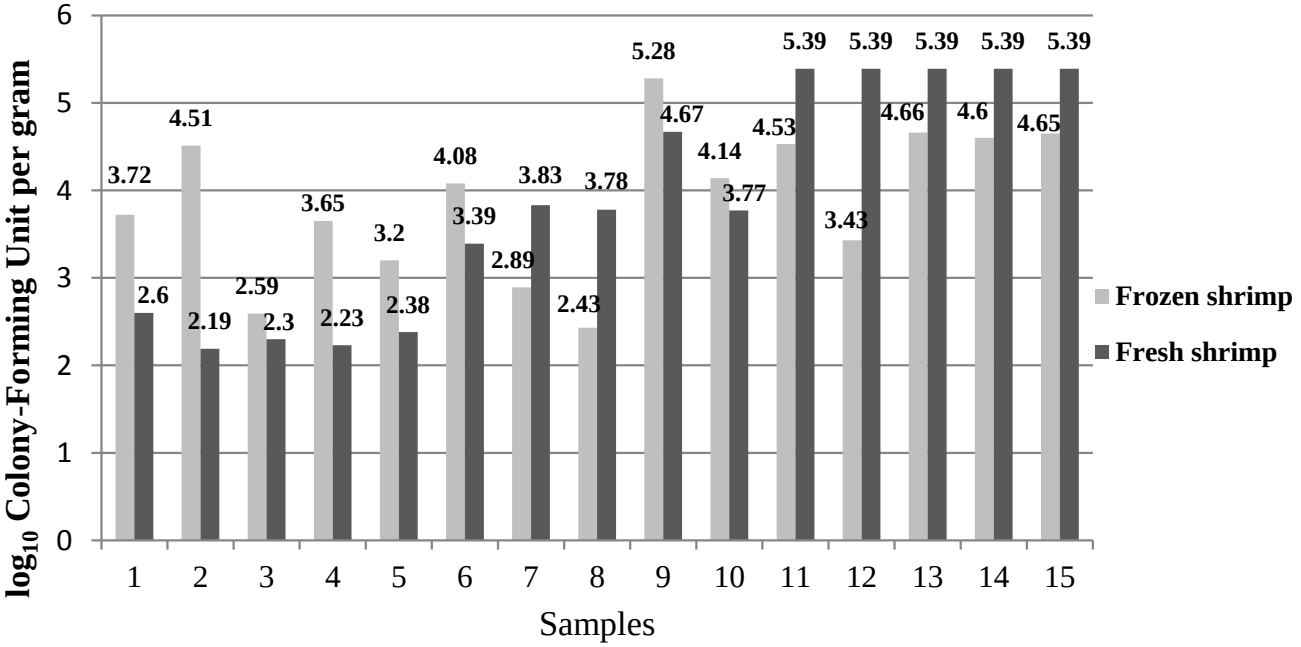

Figure 1. Quantification of *Staphylococcus* (log_{10} Colony Forming Unit per gram) in frozen and fresh shrimp samples marketed in Sobral-CE.

detected, and we highlight the number of strains with monoresistance to oxacillin (n=5), cross-resistance to Oxa+Cpm (n=3), and multidrug resistance to Oxa+Amp+Tcy (n=1) and Oxa+Tcy+Pen+Clo+Van (n=1).

DISCUSSION

The *Salmonella* data are in agreement with the Brazilian current legislation - Resolution RDC n° 12/2001 from the National Health Surveillance Agency (Brasil, 2001), which determines the absence of *Salmonella* in 25 g of frozen and fresh shrimps as a quality criteria. Absence of *Salmonella* and fecal coliforms indicates that samples of frozen and fresh shrimps (n=30) were not contaminated in the aquatic environment and/or during the handling process by feces of warm-blooded animals.

In the present study, the fact that no fecal indicator was not detected is in accordance with the findings of Koonse et al. (2005), who examined the prevalence of *Salmonella* and coliform bacteria in shrimp aquaculture farms, and found a significant relationship (p = 0.0342) between the log number of fecal bacteria and the probability of detecting *Salmonella* in any given sample.

On the other hand, *Salmonella* contamination in shrimp has been previously reported (Akiyama et al., 2011; Banerjee et al., 2012). In this context, Zhang et al. (2015) investigated the incidence of *Salmonella* in 730 samples aquaculture products from China, and found discordant results with this present study, since 217 (29.7%) of samples were positive for this bacteria. For these authors, aquaculture products, including shrimps, can become sources of *Salmonella* by exposure to contaminated water or through processing practices. Absence of fecal coliform (FC) in both types of samples (fresh and frozen) also contrasts with recent studies. Parente et al. (2011) evaluated the bacteriological quality of 28 shrimp samples cultivated in Brazil, and in 22 samples a quantification of CF was detected, with oscillation of 3.6 to 2.1 × 10^4; only 6 samples had <3.0 MPN/CF. For the authors, the presence of FC in the shrimps is related to the quality of the water from the pond.

The results of *Staphylococcus* quantification (Figure 1) showed that 12 samples (80.0%) of frozen shrimp and ten (66.7%) sample units of fresh shrimp presented a bacterial load above 3.0 log_{10} CFU g^{-1}; the recommended limit by Brazil (2001). These results serve as a warning about the quality of seafood, since the occurrence of coagulase-positive staphylococci in foods is associated with improper handling practices (Kadariya et al., 2014), indicating inadequate hygiene in any of the stages from capture to distribution of the end product to the consumer.

Comparing the values of the bacterial count of sample units, it is clear that the detection of staphylococcal rates above those permitted by the current legislation in Brazil was more frequent in frozen shrimp samples (80%). This may be related to the quality of raw material, since it is expected that the freezing decrease the microbial load on foods.

The detection of bacteria in frozen seafood is not an

Table 1. Antimicrobial resistance profile of staphylococci isolated from frozen and fresh shrimp.

Resistance profile	Frozen shrimp (n=39)	Fresh shrimp (n=37)
Monoresistance		
Oxa	4	1
Tcy	1	1
Cro	-	1
Pen	-	1
Cross-resistanceto beta-lactam		
Oxa+Com	2	1
Oxa+Amp	-	1
Oxa+Amp+Pen	-	1
Oxa+Amp+Cpm+Ctx	-	1
Multidrugresistance		
Oxa+Amp+Tet	-	1
Oxa+Tcy+Pen+Clo+Van	1	-
Total (%)	8 (20.5)	9 (24.3)

n, Isolates number. Oxa, oxacillin1 µg; Tcy, tetracycline 30 µg; Cro, ciprofloxacin 30 µg; Pen, penicillin 10 UI; Cpm, cefepime 30 µg; Amp, ampicillin 10 µg; Ctx, ceftriaxone 30 µg; Clo, chloramphenicol30 µg; Van, Vancomycin 30 µg.

unusual fact. Noor et al. (2014) evaluated frozen shrimps in Bangladesh, and found samples contaminated with a huge bacterial load (10^6-10^8 CFU/g). Among the specific pathogens, staphylococci were detected. Noor Uddin et al. (2013) researched the bacterial flora in raw frozen cultured seafood (raw frozen cultured and wild-caught shrimp and fish) imported to Denmark and identified 6% of bacterial isolates as *Staphylococcus*.

In the present study, resistant staphylococci strains (n=17; 22.3%) were isolated from both types of shrimp (Table 1). This fact may be related to the indiscriminate use of antibiotics in shrimp farming. Intensified aquaculture includes the use of antimicrobials for disease control (Noor Uddin et al., 2013). For Duran and Marshall (2005), the occurrence of antibiotic-resistant bacteria in food products of animal origin is a potential health threat because resistance might be transferred among bacteria, and antibiotic-resistant pathogens may not respond to antibiotic treatments.

Detection of staphylococcal strains resistant to beta-lactams can be related to: β-lactamase acquisition, modification of penicillin-binding proteins, or acquisition of low-drug-affinity penicillin-binding proteins (Krupa et al., 2014).

Staphylococci resistant strains to penicillinase-stable penicillins- oxacillin (n=13) (Table 1) - may constitute a potential health risk for consumers, considering that oxacillin and methicillin resistant strains are resistant to all β-lactam agents (Matouskova and Janout, 2008). In this study, 5 strains resistant only to oxacillin were detected (Table 1). This may be explained by the fact that methicillin/oxacillin-resistant staphylococci are heterogeneous in their expression of resistance to β-lactam agents, and test conditions have a major effect on the expression (Brown, 2001).

Besides beta-lactams, tetracycline resistance was detected (Table 1). Tetracyclines (TCAs) are a broad spectrum of drugs that have been successfully used worldwide in both veterinary medicine and in aquaculture (Andersen et al., 2005). The data of this research related to tetracycline resistance are not an unusual fact. Tuševljak et al. (2013) researched the antimicrobial use and resistance in aquaculture through the opinion of aquaculture-allied professionals around the world. The questionnaire was administered to 604 professionals in 25 countries, and pointed out that the use of tetracycline was reported by 9% of respondents working with shrimp. Resistance to tetracycline in one or more species of bacteria was reported as 'frequent-to-almost always' for shrimp species by 36% of respondents.

Two strains presented as multi-drug resistant to Oxa+Amp+Tet (n=1) and Oxa+Tet+Pen+Clo+Van (n=1) (Table 1). Isolation of multidrug-resistant bacteria from aquaculture products has been reported (Zhang et al., 2012; Nawaz et al., 2015), emphasizing the importance of controlling the use of these drugs in the farming of aquatic organisms.

The findings of this study serve as a warning to the need of good handling practices implementation in all shrimp production stages, from capture to marketing, in order to ensure food safety for its consumers. In addition, the study of phenotypic profile of strains indicates that frozen and fresh shrimps may act as vehicles for the spread of staphylococci resistant to drugs of clinical significance. However, the determination of genetic origin (chromosomal or mobile genetic elements) by the new molecular methods is a vital aspect for research of bacterial drug resistance.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Resistance genes to sulphonamide in commensal *Escherichia coli* isolated from stool of patients in Mansoura University Children Hospital

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Commensal bacteria have a great impact on the emergence and spread of antibiotic resistance. This emphasizes a great need to underscore the magnitude of this problem in our locality, and children are taken as a sector in this research because they are usually subjected to heavy load of antibiotic usage. This study aimed at determining sulphonamide resistance genes presence among fecal isolates of commensal *Escherichia coli* detected in patients attending Mansoura University Children Hospital (MUCH) and to check the value of these commensals in the appearance and transmission of antimicrobial resistance. Forty five (45) co-trimoxazole resistant *E. coli* were haphazardly chosen for detection of resistant determinant to sulphonamide. The methods used were antibiotic sensitivity tests by disc diffusion, detection of *sul* and *int1* genes by PCR and conjugation assay. Co-trimoxazole resistance was found in 80.3% of the examined fecal commensal *E. coli*. *sul2* gene recorded the highest prevalence in the examined co-trimoxazole resistant *E. coli* strains (73%). *int1* gene was found in 62% of those isolates. 35.5% of the studied isolates had the ability to transmit genes of resistance to the recipient susceptible isolates by conjugation experiment. The recorded great prevalence of resistance genes to sulphonamide in commensal isolates of *E. coli* among children seems to be alarming which may indicate the future increase in the prevalence of those resistant genes in our community. This problem underlines the necessity of limitation of antibiotic usage, particularly among children.

Key words: Sulphonamide resistance, *Escherichia coli*, *sul* genes, integrons.

INTRODUCTION

The dissemination of antimicrobial resistance was found to be an important problem that worsens the outcome of antibiotic therapy and leads to more duration of the diseases periods, high mortality rates in addition to increased hospital related payment (WHO, 2015). In developing localities, a great effect of this problem was

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found. Actually, a great prevalence of decreased response to antibiotics is usually recorded in screening assays of different bacterial strains (Shears, 2001), and in researches that examined normal bacterial flora as an important index for distribution of genes that are responsible for decreased response to antibiotics (Alves et al., 2014). In addition, developing localities usually suffer from the bad impact of antibiotic resistance on disease outcome and rates of mortality which is due to recurrent infections by bacteria and the great value of antibiotics in fighting them (Adefisoye and Okoh, 2016). Treatment of many human diseases was greatly dependent on sulphonamid drugs, but, sulphonamides were usually added to trimethoprim in order to decrease the appearance of resistance, this usually limits the prescription of these drugs that have the advantage of being low cost. Sulfamethoxazole plus trimethoprim (co-trimoxazole) was still found to be one of the major antibiotics used in dealing with many diseases caused by bacterial infection and WHO reports this antimicrobial as the only one that should be used in management of certain serious diseases (Perreten and Boerlin, 2003).

The decreased response to sulphonamides specially by *Escherichia coli*, is usually due to genetic alteration of dihydropteroate synthase gene (*folP*) in the chromosome, that limit the binding ability of this enzyme with the inhibitory agents, also it may be due to gaining of *sul* type determinants encoding enzymes with lower sulphonamides binding ability (Sk¨old, 2000).

Three genetic determinants have been described, *sul1*, *sul2* and *sul3*. *sul1* is commonly found in association with integrons of class 1 (Deng et al., 2015). *sul2* gene is usually found to be controlled by various plasmids, on the other hand, *sul3* is a new sulphonamide genetic determinant, that carry several enzyme variants (Singha et al., 2015). All of these genetic determinants have been found in the isolates of *E. coli* recovered from human sources (Perreten and Boerlin, 2003).

The important value of normal bacterial flora in the appearance and dissemination of antibiotic unresponsiveness is globally observed (APUA, 2008). Certain strains of the normal bacterial flora, like *E. coli* of stool were studied as an important determinant in the assays of antibiotic limited response (Osterblad et al., 2000). So, this research aimed at checking the presence of *sul*-type genes in the commensal *E. coli* recovered from stool samples of patients attending MUCH, and also determined the value of these commensal pathogens in emergence and transmission of antimicrobial unresponsiveness.

MATERIALS AND METHODS

Design of the study

Cross sectional descriptive study was conducted on 173 patients between 6 month and 6 years of age attending the outpatient clinics of MUCH. The duration of the study was six months, starting from first of May to the end of October, 2015. The protocol of this study was approved by the ethical committee in the Faculty of Medicine, Mansoura University.

Clinical samples

Stool samples were collected from all the children under complete aseptic condition.

Microbiologic studies

Stool samples were processed in Microbiology Diagnostic and Infection Control Unit in the Department of Medical Microbiology and Immunology, Faculty of Medicine, Mansoura University. The collected specimens were cultivated on MacConkey's agar and Eosin Methylene Blue (EMB) agar media.

Strains identification

E. coli bacterial isolates were identified by Gram stained films, appearing as Gram-Negative rods. They produced deep red colonies on MacConkey's agar and gave characteristic greenish metallic sheen on EMB agar. Further identification was done by conventional biochemical IMViC (Indole, Methyl red, Voges Proskauer and Citrate) tests. As they were Indole and Methyl red positive, Voges Proskauer and Citrate negative (Cheesbrough, 2002) identification was confirmed by (API) 20 E analytical profile index (Bio-merieux SA, Montalieu Vercica and France). *E. coli* ATCC 25922 was used as an organism for quality control. The isolated strains were stored on fresh Nutrient agar slopes for antimicrobial sensitivity testing.

Testing for antimicrobial sensitivity

Antimicrobial susceptibility tests were carried out on the identified *E. coli* as recorded by the recommendations of CLSI. Disc diffusion on the agar of Mueller-Hinton (MHA; Bio -Rad, Marnes –La -Coquette, France) was done to determine the sensitivity to co-trimoxazole (SXT) (25 µg) [sulfamethoxazole in combination with trimethoprim], ampicilline (AMP) (10 µg), amoxicilline/clavulinic acid (AMC) (30 µg), azteronam (ATM) (30 µg), cefotaxime (CTX) (30 µg), imipenem (IPM) (10 µg), netilmicin (NET) (30 µg), chloramphenicol (C) (30 µg), kanamycin (K) (30 µg), amikacin (AK) (30 µg), gentamicin (CN) (30 µg), (Oxoid AB) (Koneman et al., 1997). The inhibitory zone limits of the tested antimicrobials were referred to CLSI (2014).

Co-trimoxazole resistant strains were determined in 139 specimens among the studied 173 subjects (80.3%). Forty five isolates were selected randomly for detection of *sul* and *int1* genes and conduction of conjugation assay.

Detection of *sul* and *int1* genes by polymerase chain reaction

PCR was used to detect *sul1, sul2, sul3* and *int1* genes. Freshly isolated colony of each bacterial isolate was added to distilled sterile water (100 mL) and boiled for 10 min at a temperature of 100°C. Centrifugation was done, PCR assays were performed with the supernatant using primers shown in Table 1. A total volume of 50 µL reaction mixture had these reagents: Primers (1 µM), DNA

Table 1. The primers used for polymerase chain reaction detection of *sul1, sul2, sul3* and *int1* genes.

Gene	Primer	Size (bp)	Annealing temperature (°C)	Reference
sul1	F: 5'-CGGCGTGGGCTACCTGAACG-3' R: 5'-GCCGATCGCGTGAAGTTCCG-3'	432	55	Arabi et al., 2015
sul2	F: 5'-GCGCTCAAGGCAGATGGCATT-3' R: 5'-GCGTTTGATACCGGCACCCGT-3'	293	53	Arabi et al., 2015
sul3	F: 5'-CAGATAAGGCAATTGAGCATGCTCTGC-3' R: 5'–GATTTCCGTGACACTGCAATCATT-3'	569	55	Arabi et al., 2015
int1	F: 5'-GCCTGTTCGGTTCGTAAGCT-3' R: 5'-CGGATGTTGCGATTACTTCG-3'	585	56	Ma et al., 2009

(100 ng), Tris-HCl (10 mM; pH 8.3), KCl (50 mM), dNTP (200 µM), 1 U of Taq DNA polymerase and MgCl2 (1.5 mM) (Frank et al., 2007). DNA amplification was done in DNA Thermal cycler (peltier-Effect cycling- MJ Researches, INC.). PCR temperature conditions and genes band size are shown in Table 1. Agarose gel (1.5%) was used to electrophorese the PCR products. Bands were detected in comparison with DNA standard marker: #SMO323 marker (Fermentas) and visualized under UV light (Van Tongeren et al., 2011).

Conjugation experiment (Sunde and Sørum, 2001)

For detection of transferability of *sul* genes, conjugation experiment was done as follows: co-trimoxazole resistant *E. coli* isolates were used as donor for mating experiment, and *E. coli* BM21 (resistant to nalidixic acid, positive for lactose fermentation, and free of plasmide) was used as the recipient for conjugation experiment with co-trimoxazole resistant *E. coli* isolates (Vacsera, Cairo, Egypt). The recipient and donor isolates were cultured in broth of brain heart infusion (BHI) for 5 h at 37°C, then the recipient (50 µL) and donor (25 µL) were mixed in fresh BHI broth (3 mL), after that it was kept in the incubator overnight at 37°C. The transconjugants were detected on agar plates of Mueller-Hinton that contained 40 mg/L nalidixic acid and 256 mg/L sulfamethoxazole.

Confirmation of resistance features in the conjugated isolates

PCR assays with the same previous techniques were carried out to confirm the existence of resistant genetic determinant in the conjugated isolates, and this was done using DNA of those isolates as a template.

Also, confirmatory antibiotic sensitivity testing was done to the transconjugants to phenotypically check the transmission of the antibiotic resistance pattern in those isolates.

Analysis of data

The data were entered and analyzed statistically with Statistical Package of Social Science (SPSS) using software version 17. Qualitative data was described as numbers and percentages. Inter-group comparison of categorical data was done using Chi-square test (X^2-value). *P*-value <0.05 was considered to be statistically significant.

RESULTS

Forty five (45) isolates, haphazardly chosen from 139 co-trimoxazole resistant *E. coli* strains isolated from the studied stool samples of children attending the outpatient clinics of MUCH, were examined as regarding antibiotic sensitivity, PCR for detection of *sul1, sul2, sul3* and *int1* genes and conjugation assay.

Antibiotic sensitivity tests revealed that all the examined strains except three were nonresponsive to two distinct family of the tested antibiotics. Fourteen (14) isolates exhibited identical pattern of resistance, as they showed antibiotic non responsiveness to ampicilline (AMP), amoxicilline/clavulinic (AMC) and chloramphenicol (C) in addition to co-trimoxazole. The examined isolates showed a great resistance to augmentin (32 isolates, 71%), ampicillin (26 isolates, 58%), and chloramphenicol (21 isolates, 47%). The pattern of antimicrobial resistance exhibited by the examined 45 strains is shown in Table 2.

PCR checking for *sul1, sul2* and *sul3* genes showed the existence of one form of *sul*-genes at least in 41 of the studied 45 isolates (91%). Only one form of *sul*-gene was detected in 33 strains (*sul2* in 25 strains and *sul1* in 8 strains), on the other hand, 8 strains were found to harbor 2 distinct forms of *sul* genes (*sul2* with *sul1* in 6 strains, and *sul3* with *sul2* in 2 strains). Totally, the percentages of different *sul* genes types in the examined strains were 73% for *sul2*, 31% for *sul1* and 4% for *sul3*. Regarding PCR results for *int1* gene, it was revealed that 28 isolates (62%) were positive for this gene by PCR (Figures 1 and 2).

Regarding the distribution of *sul* genes in relation to *int1* gene (Table 3), it was found that *sul1* gene was more frequently associated with the presence of *int1* gene than *sul2* and *sul3*, as 79% of strains that harbor *sul1* gene were found to be positive for *int1*, whereas 61 and 50% of strains that harbor *sul2* and *sul3* genes respectively, were positive for that gene.

The conjugation experiments showed that 16 (35.5%)

Table 2. Resistance phenotypes of the examined 45 Co-trimoxazole resistant *E. coli* strains isolated from stool samples.

Isolate	Other resistance	Isolate	Other resistance	Isolate	Other resistance
1	AMP, AMC,C	16	AMC, C	31	AMC, NET
2	AMC, NET	17	AMP, AMC	32	AMP, AMC, C
3	AMP, C	18	AMP, AMC, C	33	AMC, C
4	AMP,AMC,C	19	AMP, K	34	_
5	AMC, CN	20	AMP, AMC, C	35	AMC
6	_	21	AMC, C, CN	36	AMP, AMC, C
7	AMC	22	AMP	37	AMP, AMC, C
8	AMP	23	AMC	38	AMC, CTX
9	AMC, C, CN	24	AMC, C	39	AMP, AMC, C
10	AMP, AMC, C	25	AMP, AMC, C	40	AMP, AMC, C
11	AMP, AMC, C	26	AMP, K	41	AMP, K
12	AMC	27	AMP	42	AMP
13	AMP, AMC, C	28	AMC, CN	43	AMP, AMC, C
14	AMC, NET	29	AMP, AMC	44	_
15	AMP	30	AMP	45	AMC,C, CN

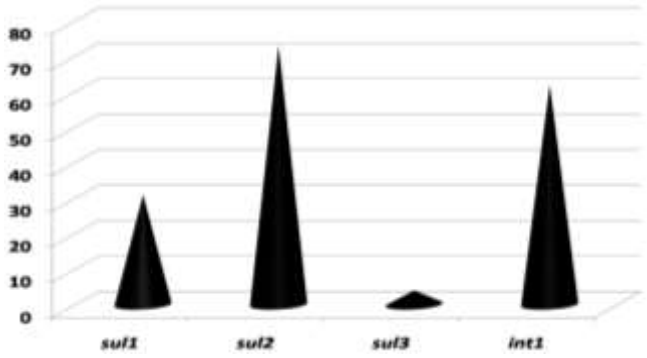

Figure 1. PCR results revealing the percentage of *sul1*, *sul2, sul3* and *int1* genes in the examined isolates.

of the studied isolates had the ability to transmit the detected resistant genes to the receiver isolates as revealed by the confirmatory PCR. Nearly all the strains had the ability to transmit their pattern of antibiotic resistance to the receiver isolates, except 2 strains that could not transmit all their antibiotic resistance profile as revealed by the confirmatory antibiotic sensitivity testing (one isolate not to ampicillin and the other not to gentamicin).

Transfer of resistance features was found to be significantly associated with the existence of *int1* gene in the examined strains ($P < 0.05$), as 15 (94%) of the strains that were positive by conjugation experiment were found to harbor class 1 integrase gene.

DISCUSSION

The persistent increased resistance to sulphonamide compounds among different infectious agents is aggravating. Novel resistant genetic determinant that are responsible for non-responsiveness to 'outdated' antimicrobials, like those compounds, are continuously being detected (Arabi et al., 2015).

Previous studies reported a great incidence of *E. coli* in stool samples with gained resistant determinant to different antibiotics, particularly the outdated anti-microbials (for example, penicillin and co-trimoxazole) especially in young age (Niaz et al., 2016).

Commensal bacteria are similar to pathogenic ones in being subject to heavy load of antibiotics. Normal bacterial flora like *E. coli* is usually used as an index of transmission and spread of the gained resistant determinant (Adefisoye and Okoh, 2016).

To the authors' knowledge, this study is the first study in Egypt that assessed the presence of sulphonamide resistant determinant among commensal *E. coli* in children.

This study revealed that the randomly selected 45 co-trimoxazole resistant faecal *E. coli* strains showed great resistance to augmentin, ampicillin and chloramphenicol (71, 58 and 47%, respectively). This antibiotic resistance profile was previously detected in healthy individuals by Bartoloni et al. (1998) in Bolivia, van de Mortel et al. (1998) in Venezuela and Okeke et al. (2000) in Ile-ife, Nigeria. These data are also consistent with the finding of further studies that showed a great antibiotic resistance pattern recorded by the commensal *E. coli* from low-resource settings (Bailey et al., 2010).

Ampicillin and chloramphenicol are among the older generations of antibiotics which are used in children and high resistance observed in them may be due to selective pressure from their inappropriate and excessive uses in our locality which by cross-resistance affected

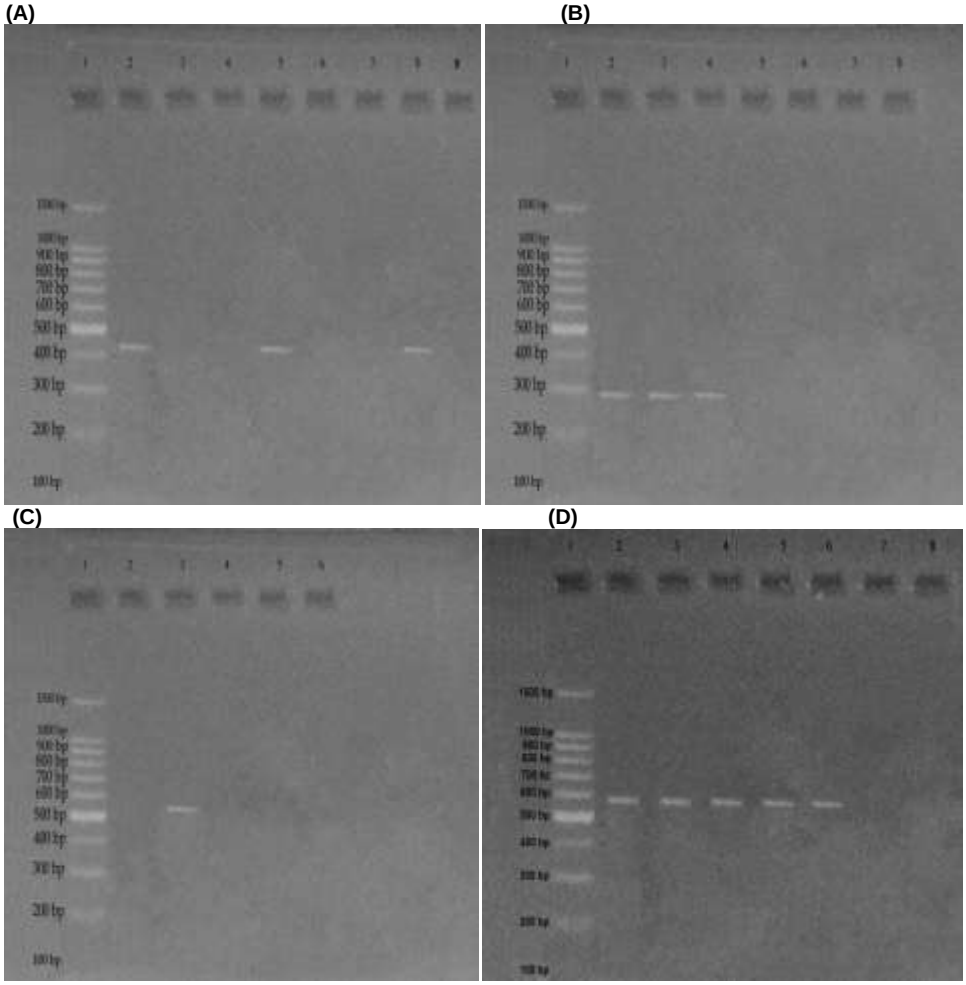

Figure 2. PCR results of *sul1*, *sul2, sul3* and *int1* genes of the studied isolates. Lane 1 shows molecular size marker #SMO323. (A): lanes 2, 5 and 8 show bands of 432 bp from positive strains that carry the *sul1* genes; (B): lanes 2, 3 and 4 show bands of 293 bp from positive strains that carry the *sul2* genes; (C): lane 3 shows band of 569 bp from positive strain that carry the *sul3* gene; (D): lane 2,3,4,5 and 6 show bands of 585 bp from positive strains that carry the *int1* genes.

Augmentin. Based on the PCR results, the *sul2* gene has the highest prevalence in the examined co-trimoxazole resistant *E. coli* strains. Frequency of *sul2* (73%) was higher than that of *sul1* (31%) and *sul3* (4%), which is in accordance with other studies conducted by Grape et al. (2003), Infante et al. (2005) and Wu et al. (2010).

This study is in parallel with earlier researches that determine the prevalence of sulphonamide resistance genes *sul2* and *sul1* among the commensal isolates of *E. coli* in different localities and recorded a great incidence of *sul* genes resistant determinant in the majority of the studied isolates mainly *sul2* or *sul1* alone or the two genes together (Rådström et al., 1991). Although, Frank et al. (2007) demonstrated an elevated prevalence of *sul1* than *sul2* gene among their studied isolates, the study is still in agreement with recent studies that recorded *sul2* gene with a higher existence rate than *sul1* and *sul3* in commensal isolates of *E. coli* from studied individuals in Denmark and other localities (Trobos et al., 2008); also in the UK, non-responsiveness to sulfonamide in *E. coli* of human source remains high and *sul2* is still the most prevalent one although the prescription of sulphonamide drugs has been ended many years ago (Bean et al., 2009).

sul3 is a new genetic resistance determinant to sulphonamides. It has genetic relatedness to *sul2* and *sul1*, it was firstly detected in pigs in 2003 (Perreten and Boerlin, 2003). It has been commonly observed in *E. coli* isolated from pigs in Switzerland. In the same year, Grape et al. (2003) detected this gene in *E. coli* recovered

Table 3. Distribution of *sul1*, *sul2* and *sul3* in the examined isolates and its relation to *int1* gene presence.

Isolate	Type of gene			
	sul1	***sul2***	***sul3***	***int1***
1	+	+		+
2		+		+
3		+		
4	+			+
5		+		+
6				
7	+			+
8		+		+
9	+	+		+
10		+		
11		+		+
12	+			+
13		+		+
14		+	+	+
15		+		
16	+			+
17		+		+
18	+	+		+
19		+		
20		+		+
21	+	+		
22				+
23		+		+
24		+		+
25		+		
26		+		+
27	+			
28		+		
29		+		+
30	+			+
31		+		
32	+	+		
33		+		
34				
35		+		+
36		+		
37	+			+
38		+		
39	+	+		+
40		+		+
41		+		+
42				
43		+	+	
44	+			+
45		+		+

from human samples in Sweden. *sul3* has an amino acid identity of about 40% relatedness to the already present genes (*sul2* and *sul1*). It was first detected in conjugative plasmid of 54 kb weight, also it could be carried by another huge plasmid in addition to the first one (Perreten and Boerlin, 2003). According to the studies of Wu et al. (2010) and Ziembińska-Buczyńska et al. (2015), *sul3* gene is the least prevalent one in *E. coli* strains recovered from human and animal resources, this data is in parallel with the present results. Although, *sul3* gene was rarely detected in the studied isolates (4% only), this small percentage should be considered as it may be a warning sign revealing that its existence can be widespread in the locality.

Resistance to various antibiotic is usually caused by integrons which are harbored by bacterial chromosome or carried by plasmids (Tajbakhsh et al., 2015). These antibiotic resistance determinants are able to hold antibiotic resistant genes by site-specific recombination system. Also, they gain novel genes in various types of bacteria (El-Sokkary and Abdelmegeed, 2015). Integrons of class 1 are movable elements which were found to be effective in transmission of antimicrobial resistant genes due to presence of mobile gene cassettes (Ammar et al., 2016). As approved, transmission of *sul* genetic determinant among different bacterial strains is usually accompanied by integration of genetic cassettes into the integrons (Sobia et al., 2016). This study assessed the presence of *int1* gene among the studied isolates, and detected the gene in 28 (62%) of them. These results approximates the finding of Infante et al. (2005), who found *int1* gene in 9 (45%) of their studied 20 isolates and Lavakhamseh et al. (2016) who recorded the presence of the same gene in 47% of their studied isolates. On the other hand, higher percentage of *int1* gene (95%) was recorded by Frank et al. (2007).

The discrepancy among different studies could be attributed to different localities where each one has its own pattern of pathogens resistance genes. Also, the studied isolates were different, as Frank et al. (2007) conducted study on different strains of *Enterobacteriaceae* not *E. coli* alone. The remaining isolates in this research that were found to be negative for *int1* gene may carry other mobile genetic elements, that could act as sources of *sul* genes.

In this study, it was reported that 79, 61 and 50% of the isolates which harbor *sul1, sul2* and *sul3* genes, respectively, were found to be positive for *int1* gene, with *sul1* being the most frequent one found in association with that gene. These findings are in harmony with that of Antunes et al. (2005), who observed great association between *sul1* and *int1* gene. Similar to this study results, Shehabi et al. (2006), recorded that, *sul1* was more frequently associated with *int1* gene than *sul2*, also Khamesipour and Tajbakhsh (2016) recorded the association of *sul1* genes with class 1 integrons in 66.66%

of their examined strains which was more than *sul2* and *sul3.* However, Infante et al. (2005) and Wu et al. (2010) observed *sul3* gene, as the most frequent one that was found in association with *int1* gene. These different studies observations suggest the necessity of spending more efforts to do future researches on the new *sul3* gene in relation to *int1*.

The conjugation testing used in this research demonstrated that the resistant genes were mostly present in conjugative plasmids, as it was successfully transferred in 35.5% of the studied isolates approximating the results of Antunes et al. (2005), who stated that, sulfonamide resistance was transferred in 43% of their studied isolates. The significant association that was found between *int1* gene occurrence and transfer of resistant features, as 94% of strains that were positive by conjugation experiment were found to harbor *int1* gene indicates the high prevalence of conjugative plasmids carrying *int1* gene, this significant association was also confirmed by Sunde and Norstro¨m (2006) and Ravi et al. (2015). In those studied isolates, this may be due to presence of certain powerful plasmid harboring *int1* gene and it has a high transmission ability or it may be that *int1* gene represents a portion of an 'antimicrobial resistance island' which has the ability of incorporation in different types of conjugative plasmids. Briefly, resistance determinant can be transmitted by conjugal transfer, indicating the association of the responsible resistant genes with mobile elements like plasmids.

Furthermore studies seem to be necessary to describe the plasmids and the genetic characters of *int1* gene which they harbor.

Conclusion

Commensal isolates of *E. coli* that shows resistance to co-trimoxazole were proved to be prevalent among children in this study locality. The three *sul*-genetic variants (*sul1, sul2* and *sul3*) were detected in those isolates, indicating the high prevalence of such resistant elements. *sul2* gene was higher in prevalence than *sul1 and sul3*. The heavy presence of sulphonamide resistance genes in the enteric *E. coli* highlights the role of normal bacterial flora as a significant source of genetic determinants that encodes resistance to various antimicrobials. The existence of different types of *sul* genes seems to be due to the heavy load of sulfonamides and other antibiotics which are usually prescribed.

High prevalence of *int1* gene was found in resistant strains indicating widespread distribution of resistant determinants in the community. Restrictive utilization of all antimicrobials is recommended in order to minimize the expansion of antibiotic resistance problem among different bacterial strains, particularly in children.

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