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ARTICLES

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Full Length Research Paper

Evaluation of microbiological quality of raw milk, sour milk and artisanal yoghurt from Ouagadougou, Burkina Faso

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The present study was undertaken to investigate the microbiological quality of milk and artisanal dairy products sold in Ouagadougou markets. Forty-five (45) samples of milk and dairy products including raw milk, sour milk and yoghurt were collected and analyzed for aerobic mesophilic bacteria (AMB), total coliforms (TC), thermotolerant coliforms (TTC), *Staphylococcus aureus (S. aureus)*, lactic acid bacteria (LAB) and yeasts and moulds (YM). The coliforms bacteria were then purified and identified by using API 20E system. The mean values of TC, TTC, *S. aureus* LAB and YM in the raw milk were 8.95, 6.43, 6.21, 4.83, 8.01 and 4.78 log cfu mL^{-1} respectively, In sour milk they were 10.44, 5.62, 4.12, 6.98, 7.38 and 3.75 log cfu mL^{-1} respectively, while in yoghurt samples the mean values of TC, TTC, *S. aureus* LAB and YM were 9.98, 5.77, 5.50, 6.12, 9.78 and 4.86 log cfu mL^{-1} respectively. The dominant coliforms isolated from raw milk, sour milk and yoghurt samples were found to be *Escherichia coli*, *Klebsiella pneumonia* and *Enterobacter cloacae*. *Citrobacter* spp was not found in any of the analyzed samples. The wide range of the samples analyzed did not comply with the standards quality. Thus it is necessary to set up an awareness program including capacity building of all the actors working in the sector of milk and dairy products.

Key words: Raw milk, sour milk, yoghurt, microbiological quality, Ouagadougou.

INTRODUCTION

Milk and dairy products are important components of a healthy diet. However, they can present a health hazard due to the possible contamination with pathogenic bacteria when there are consumed unpasteurized or expose to environment, (Angulo et al., 2009). More than 200 known diseases are transmitted through food contaminated by pathogenic bacteria, fungi, viruses, and parasites (Oliver et al., 2005). The prevalence of food

borne pathogens in milk is influenced by numerous factors such as farm size, number of animals on the farm, hygiene, farm management practices, variation in sampling and types of samples evaluated, differences in detection methodologies used, geographical location, and season (Oliver et al., 2005). In Burkina Faso, livestock production increase results in the diversification of supply especially in the dairy sector (Hamadou et al., 2004). There is an important demand of milk and dairy products although the dairy potential is particularly important with 250 million liters of milk per year of bovine origin mainly (Hamadou and Sanon, 2005; Corniaux, 2013). A significant part of production sold to people is found in the informal sector without any control. Milk and its derivatives are sold in dubious circumstances in the local markets. The actors of this form of marketing of the products are often both producers and sellers and the sale of milk is a cultural activity. It is practiced generally by the Fulani ethnic group whose main activity is farming. Raw milk is processed at home, and thereafter left for spontaneous fermentation or transported to the market in calabashes. Other dairy products such as dégué, yoghurt, gappal, tchobal are also sold (Hamadou and Sanon, 2005; Hama et al., 2009). Sour milk is mostly packaged in recycled bottles and sold by street food vendors resulting from the fermentation of unpasteurized raw milk (Bagré et al., 2014). Several studies reported the presence of enterobacteria, *Salmonella,* yeast and *Staphylococcus aureus* in the raw milks and sour milk sold in Burkina Faso markets and their consumption can seriously affect the health of consumers (Barro et al., 2002; Savadogo et al., 2004; Bagré et al., 2014; Sissao et al., 2015). These studies showed that *Escherichia coli* and *Salmonella* strains isolated from raw milk and sour milk consumed in Ouagadougou and Ziniaré were resistant to antibiotics. All of the isolates were resistant to amoxicillin-clavulanic acid, 90% of the isolates were resistant to erythromycin and 75 to 78.26% to amoxicillin (Bagré et al., 2014).

The objective of this study is to assess the hygienic quality of commercially available dairy products in Ouagadougou markets and to identify the eventual coliforms and *Staphylococcus* found.

MATERIALS AND METHODS

Sampling

The collection of the samples took place from October 2013 to May 2014 at five markets in Ouagadougou, Burkina Faso. Raw milk, sour milk and yoghourt sold in five markets of Ouagadougou

(codified market A, market B, market C, market D and market E) were concerned by the sampling. Three (03) samples of each product were collected by market. A total of forty five samples were collected in sterile bottles and transported to the laboratory in cold chain under temperature 4°C and analyzed within 24 h of sampling.

Physico-chemical analysis

The physico-chemical analysis was measured according to standard methods. pH values of the samples were measured with an electronic pH meter by homogenizing 10 mL of product and 10 mL of distilled water, (CONSORT P901, Belgium). Total titratable acidity (TA) was measured by titrating 10 mL of sample against N/9 sodium hydroxide (NaOH) solution using phenolphthalein as indicator and dry matter (DM) content was measured using standard procedure (AOAC, 2005).

Microbiological analysis

For preparation of stock solutions, there were tenfold dilutions and inoculation on agar plates. For all the determinations, 10 g of the samples were homogenized in a stomacher with 90 mL of sterile peptoned buffered water. Tenfold serial dilution was prepared and spread-plated for microorganisms count. 1 mL of suitable diluted was used for spreading except *S. aureus* enumeration. Aerobic mesophilic bacteria (AMB) were enumerated on pour plates of Plate Count Agar (Liofilchem, Italy) incubated at 30°C for 72 h (ISO 4833, 2003).

Yeasts and moulds were counted by cultivation on Sabouraud-Chloramphenicol Agar (Liofilchem, Italy) after incubation at 25°C for 4 to 5 days according to ISO 7954 (1988) standard. Lactic acid bacteria (LAB) were counted by cultivation on De Man, Rogosa and Sharpe Agar (Merck, Germany) incubated anaerobically in an anaerobic jar at 37°C, for 3 days according to ISO 15214 (1998) standard.

S. aureus counts were determined by spreading 0.1 mL of a suitable diluted sample onto the surface of Baird-Parker agar (Liofilchem, Italy) containing Egg Yolk Tellurite Emulsion. The inoculated plates were incubated at 37°C for 48 h. For confirmation, one vial of coagulase test (Liofilchem, Italy) was aseptically reconstituted with 4 mL of physiological solution. Presumed colonies were purified and transferred in tubes containing 5 mL of brain-heart infusion and incubated for 6 h at 37°C. 0.5 mL of culture broth were then mixed with 0.5 mL of coagulase test and incubated at 37°C for 6 h in order to observe coagulation (positive result). For negative result after 6 h, the tube was incubated overnight for a new observation (French Standard EN ISO 6888-2, 2003). Coliforms were enumerated on Violet Red Bile Agar (VRBA) (Liofichem, Italy), incubated at 37°C (TC) or 44°C (TTC) for 24 h according to International standard ISO 4832 (2006)..

Identification of coliforms bacteria and determination of pathogenic germs prevalence

For identification of coliforms, the colonies on VRBA were picked were obtained. A total of 181 coliforms bacteria were isolated. The isolates were first characterized based on colony and cell

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Table 1. Physico-chemical and microbiological (log cfu.mL^{-1} ± SE) characteristics of raw milk.

Characteristics	Standard values*	Markets					Mean
		A	B	C	D	E	
pH	6.6-6.8	6.34±0.09[a]	6.14±0.13[a]	6.31±0.04[a]	6.27±0.00[a]	6.39±0.27[a]	6.29±0.05
TA (°D)	15-17	26.25±5.75[b]	69.50±13.00[a]	23.50±7.50[b]	30.25±0.00[b]	21.50±5.00[b]	33.84±5.86
DM (%)	12.8	8.89±2.07[a]	8.16±4.01[a]	7.34±3.62[a]	5.54±0.00[a]	7.58±4.59[a]	7.32±1.07
AMB	<5.48	8.20±0.92[b]	8.17±0.55[b]	9.57±0.01[a]	7.64±0.00[b]	5.83±0.69[b]	8.95±0.43
TC	<3	6.08±1.57[b]	7.00±0.26[a]	5.57±0.12[b]	0.00±0.00[b]	4.46±0.58[b]	6.43±0.84
TTC	<2	5.93±1.62[ab]	6.80±0.41[a]	5.20±0.76[ab]	0.00±0.00[b]	4.41±1.57[ab]	6.20±0.82
YM	<3.3	5.41±0.34[a]	4.48±0.81[ab]	2.74±0.03[b]	4.23±0.00[b]	3.87±2.09[b]	4.83±0.46
LAB	nd	7.45±0.00[a]	7.64±0.66[a]	8.58±2.00[a]	7.43±0.00[a]	5.15±1.89[a]	8.00±0.60
S. aureus	<2	4.17±0.70[bc]	3.28±0.61[c]	5.26±0.27[a]	5.15±0.00[ab]	3.43±1.04[c]	4.78±0.39

AMB: aerobic mesophilic bacteria, TC:total coliforms, TTC : thermotolerant coliforms *S. aureus: Staphylococcus aureus* LAB : lactic acid bacteria YM: yeasts and moulds. The same letter (a, b, c) in the same column indicated no statistical difference (p≥0.05). Results are expressed as the mean of three independent determinations with the standard error (SE). cfu refers to Colony Forming Unit. *FAO (1978).

morphology (Parkouda et al., 2010), Gram staining, catalase test, 3-hydroxy-butanone production (VP test), acids mixt fermentation (RM test), oxidase test. Gram reaction was carried out by the KOH (3%) method (Gregersen, 1978), catalase was determined by adding to a colony on a glass slide a drop of H_2O_2 solution (30%). Oxidase reaction was carried out by using oxidase disc. VP reaction and RM reaction was carried out on the Clark and Lubs broth (Clark and Lubs, 1915; Sevastianos, 1977). The analytical profile index (API) tests kits were used for identification of presume coliforms (*E. coli; Enterobacter* spp*, Citobacter* spp and *Klebsiella* spp). The API test kits used were API 20 E Kit, which were prepared and performed according to the manufacture manual bioMerieux (system for the identification of *Enterobacteriaceae*). Apiweb STAND ALONEV.1.1.0 software (Biomérieux) was used for isolates identification.

Statistical analysis

Each sample was analyzed in triplicate. Microbial counts were converted to log cfu mL^{-1}. All the results were subjected to Analysis of variance (ANOVA) using SPSS (version 20). Means, standard error of means and the least significant difference between the means were determined (p < 0.05).

RESULTS AND DISCUSSION

The present study investigated the microbiological and physico-chemical quality of artisanal milk products (raw milks, sour milks and yoghurts) sold in Ouagadougou. The results from the raw milks are presented in Table 1. The pH values of raw milk from the 5 markets varied from 6.14 to 6.39. The mean value (6.29) is lower than FAO standard and the pH value (6.60) reported by Akabanda et al. (2010). This low pH prevents the growth of most spoilage and pathogenic organisms (Varga, 2007). The titratable acidity (TA) values ranged from 21.50°D to 69.50°D corresponding to 33.84±5.86°D as mean value. This value is higher than FAO standard and those reported by Varga (2007) where the milk titratable acidity

varied from 18.1°D to 21.0°D.

The dry matter contents in raw milk samples varied from 5.54 to 8.89% with a mean value of 7.32±1.07%. The mean value was lower than those recommended by FAO (1978) which is 128 g L^{-1} (12.8%).

Concerning the aerobic mesophilic bacteria of raw milk from the 5 markets, the values varied from 5.83 log cfu mL^{-1} (market E) to 9.57 log cfu mL^{-1} (market C) corresponding to 8.95 log cfu mL^{-1} as mean value. This value is higher than FAO standard (5.48 log cfu mL^{-1}). Torkar et al. (2008) and Aaku et al. (2004) reported 4.51 log cfu mL^{-1} and 6.74 log cfu mL^{-1} as total number of micro-organisms in raw milk, respectively, which is lower than in our experiment (8.95 log cfu mL^{-1} as mean value). According to the Regulatives EU (Regulation 853, 2004) the rolling geometric average of total number of micro-organisms should not exceed 5 log cfu mL^{-1} of raw cow's milk from primary production.

Total coliforms counts ranged from 0.00 (market D) to 7 log cfu mL^{-1} (market B) corresponding to 6.43 log cfu mL^{-1} mean value. The number of thermotolerant coliforms varied from 0.00 (D) to 6.80 log cfu mL^{-1} (B) corresponding to 6.20 log cfu mL^{-1} as mean value. Kas et al. (2013) reported 2.7 log cfu mL^{-1} as thermotolerant coliforms concentration in milk, which is lower than our mean value. Moreover it is important to note that no coliform (both total and thermotolerant) was detected in D market samples. In this market standards are respected (<3 log cfu mL^{-1}).

The incidence of coliforms in raw milk has received considerable attention, partly due to their association with contamination of fecal origin and the consequent risk of more pathogenic fecal organisms being present, partly because of the spoilage their growth in milk at ambient temperatures can produce. Coliform counts regularly in excess of 2 log are considered as evidence of

Table 2. Physico-chemical and microbiological quality (log cfu mL^{-1} ± SE) of sour milk by market.

Characteristics	Standard values	Market					Mean
		A	B	C	D	E	
pH	≤ 4.5[+]	5.47±0.60[a]	4.37±0.43[a]	4.36±0.59[a]	4.50±0.55[a]	4.17±0.58[a]	4.57±0.24
TA (°D)	≤ 30*	82.69±9.00[a]	115.88±29.70a	29.56±5.83[a]	84.13±32.17[a]	92.69±40.62[a]	80.99±12.59 [b]
DM (%)	nd	4.98±0.79[a]	6.55±2.27[a]	6.22±0.65[a]	6.71±0.39[a]	7.84±1.93[a]	6.46±0.60
AMB	≥7*	11.00±1.21[a]	8.23±0.08[a]	6.91±0.67[a]	7.57±0.12[a]	10.55±0.76[a]	10.44±0.36
TC	<2[ʌ]	0.00±0.00[b]	5.11±1.20[a]	6.07±0.31[a]	5.90±5.90[a]	1.68±0.54[a]	5.63±0.59
TTC	<1	0.00±0.00[a]	4.44±1.09[a]	3.75±0.89[a]	1.28±1.28[a]	0.40±0.25[a]	4.11±0.40
YM	nd	7.23±1.08[a]	6.56±0.74[a]	6.08±0.85[a]	7.23±0.56[a]	6.69±0.38[a]	6.98±0.32
LAB	≥7*	7.08±1.43[b]	7.84±0.32[a]	6.57±0.48[b]	7.14±0.09[b]	7.36±0.30[ab]	7.38±0.35
S. aureus	<2[ʌ]	1.15±0.32[a]	1.40±0.39[a]	3.43±0.46[a]	4.39±0.47[a]	2.04±0.16[a]	3.75±0.29

AMB: aerobic mesophilic bacteria, TC: total coliforms, TTC : thermotolerant coliforms *S. aureus: Staphylococcus aureus* LAB : lactic acid bacteria YM: yeasts and moulds. The same letter (a, b, c) in the same column indicated no statistical difference (p≥0.05). Results are expressed as the mean of three independent determinations with the standard error (SE). cfu refers to Colony Forming Unit. +FAO (1978), *Codex Standard for Fermented Milk (243-2003), ʌ ANZFA (2000).

unsatisfactory production hygiene (Directive 92/46/EEC). Sporadic high coliform counts may also be a consequence of unrecognised coliform mastitis, mostly caused by *E. coli* (Torkar et al., 2008).

The concentration of yeasts and moulds in milk samples ranged from 2.74 to 5.41 log cfu mL^{-1}. The mean number of yeasts and moulds found in raw milk samples of this study (4.83 log cfu mL^{-1}) is higher than the mean yeast count (2.64 log cfu mL^{-1}) in raw milk from farms located in different areas of Sardinia (Fadda et al., 2004). It is also worth noting that two out of five market (C and E) samples examined had viable yeast counts lower than 4 log cfu mL^{-1}, a level required by Codex Alimentarius (Codex, 2004). In that case, it is documented that yeasts occur in raw milk at insignificant numbers probably due to competitive utilization of the growth substrates by psychrotrophic bacteria of milk or owing to inhibition by metabolites excreted by bacteria (Viljoen, 2001).

LAB counts ranged between 5.15 log cfu mL^{-1} (market E) and 8.58 log cfu mL^{-1} (market C). The mean value (8.00 log cfu mL^{-1}) is lower than those obtained by Akabanda et al. (2010) in Fulani raw milk from northern Ghana (4.69 log cfu mL^{-1}).

The concentration of *S. aureus* in raw milk varied from 3.28 log cfu mL^{-1} (market B) to 5.26 log cfu mL^{-1} (market C) and the mean value was 4.78 log cfu mL^{-1}. No sample contained a concentration of *S. aureus* lower than 2 log cfu mL^{-1}, a limit required by FAO (1978). The presence of those bacteria in milk indicated the contamination from various sources, such as animal, human, environment, utensils and others (Mubarack et al., 2010; Tankoano, 2014). The high numbers of the microorganisms indicated that milk is contaminated by external microorganisms as well as internal microorganisms. This might be due to the fact that milk is a good nutritive medium for the growth of microorganisms, especially with poor sanitary procedures and lack of the cooling facilities (Mubarack et al., 2010). *S. aureus* has been linked to gastroenteritis by producing enterotoxins, boils, skin infections, pneumonia, deep abscesses and meningitis in debilitated persons (Okpalugo et al., 2008).

The physico-chemical and microbiological quality of sour milk and yoghurt collected from Ouagadougou markets are shown in Tables 2 and 3, respectively. The results showed that the physic-chemical and microbiological quality of sour milk and yoghurt varied according the markets.

The pH of sour milk ranged between 4.17 (market E) and 5.57 (market A) corresponding to 4.57 as mean value; the pH for yoghurt samples varied from 3.98 (C) to 4.80 (B) corresponding to 4.33 as mean value. Similar values (3.84 to 4.48) were reported by Omafuvbe and Enyioha (2011) for yoghurt. According to Codex Alimentarius (2004), the pH of fermented milks and yoghourt should not exceed 4.5.

The titratable acidity of sour milk varied from 29.56°D to 115.88°D leading to 80.99°D as mean value while the titratable acidity for yoghurt ranged from 96.83 to 191.50 corresponding to 93.08 as mean value. These mean values were above minimum admitted limit 30°D and 60°D by Codex alimentarius (2004) respectively for fermented milks and yoghourt. Our results were similar to those reported by Kantinan et al. (2012) and Tidjani (2013).

The total bacterial count in sour milk varied from 6.91 to 11.00 log cfu mL^{-1}; the mean concentration (10.44±0.36 log cfu mL^{-1}) was higher than those obtained by Kantinan et al. (2012). In yoghurt samples, the total bacterial ranged between 7.64 and 10.69 log cfu mL^{-1} with a mean value of 9.98±0.58 log cfu mL^{-1}. According to Samet-Bali

Table 3. Physico-chemical and microbiological (log cfu mL^{-1} ±SE) quality of yoghurt by market.

Characteristics	Standard values	Market					Mean
		A	B	C	D	E	
pH	≤ 4.5+	4.67±0.70[a]	4.80±0.18[a]	3.98±0.03[a]	4.22±0.25[a]	4.04±0.14[a]	4.33±0.15
TA (°D)	≥60*	96.83±8.35[c]	158.83±19.22[ab]	191.50±11.25[a]	172.10±9.65[a]	117.67±26.24[bc]	148.93±10.72
DM (%)	nd	19.54±7.11[a]	26.40±1.98[a]	24.09±5.24[a]	31.87±5.23[a]	27.43±6.94[a]	26.24±2.41
AMB	≥7*	8.36±0.45[a]	8.30±0.19[a]	7.64±0.94[a]	8.08±1.60[a]	10.69±0.95[a]	9.98±0.58
TC	<2^	4.25±0.22[b]	2.11±0.76[b]	6.47±0.53[a]	4.92±1.34[b]	0.00±0.00[b]	5.777±0.6
TTC	<1	4.00±0.16[b]	0.00±0.00[b]	6.20±0.66[a]	4.83±1.30[b]	0.00±0.00[b]	5.50±0.6
YM	nd	5.81±0.55[a]	5.62±0.58[a]	4.11±0.30[a]	5.36±0.29[a]	6.76±1.35[a]	6.12±0.29
LAB	≥7*	8.17±0.53[a]	7.07±0.61[a]	7.44±0.91[a]	8.25±1.02[a]	10.50±0.98[a]	9.78±0.42
S. aureus	<2^	3.63±0.21[a]	2.36±0.56[a]	1.72±0.73[a]	5.45±0.79[a]	1.38±0.20[a]	4.86±0.42

AMB: aerobic mesophilic bacteria, TC: total coliforms, TTC : thermotolerant coliforms *S. aureus: Staphylococcus aureus* LAB : lactic acid bacteria YM: yeasts and moulds. The same letter (a, b, c) in the same column indicated no statistical difference (p≥0.05). Results are expressed as the mean of three independent determinations with the standard error (SE). cfu refers to Colony Forming Unit. +FAO (1978), *Codex Standard for Fermented Milk (243-2003), ^ ANZFA (2000).

et al. (2012), the dominance of mesophilic bacteria may be explained by the fact that the ambient temperature at which the natural fermentation of the samples took place is favorable for the proliferation of mesophilic bacteria.

LAB counts ranged from 6.57 to 7.84 log cfu mL^{-1} in the sour milk samples with a mean value of 7.38 log cfu.mL^{-1} and from 7.07 log cfu.mL^{-1} to 10.50 log cfu mL^{-1} in yoghurt samples with a mean value: 9.78 log cfu mL^{-1}. These data showed that LAB are the dominating microorganisms in sour milk and in yoghurt. Our results are similar to those reported by Akabanda et al. (2010) in Ghana which was 4.00 to 9.00 log cfu mL^{-1} and Nyambane et al. (2014) in Kenya which was 7.26 to 8.08 log cfu mL^{-1} in traditionally fermented milk products.

Yeasts and moulds counts in sour milk samples varied from 6.08 log cfu mL^{-1} with a mean value of 6.98 log cfu mL^{-1}. Abdalla and Ahmed (2010) reported on 4.17 to 5.70 log cfu mL^{-1} of the concentration of yeast and mold in Sudanese fermented dairy product, which is lower than in our results.

The concentration of yeasts and moulds in yoghurt samples ranged from 4.11 log cfu mL^{-1} to 6.76 log cfu mL^{-1} corresponding to 5.10±0.24 log cfu mL^{-1} as mean concentration. These values were higher than 2 log cfu mL^{-1} the limit recommended by Egyptian standards (2005) for yoghurt. Contamination due to yeast is still one of the major limiting factors for shelf life and commercial value of yoghurt. Moulds and yeasts growing in yoghurt and sour milk utilize some of the acid and produce a corresponding decrease of the acidity, which may favorable for the growth of putrefactive bacteria (Oyeleke, 2009).

S. aureus counts in sour milk varied from 1.15 to 4.39 log cfu mL^{-1} corresponding to a mean concentration of 3.75 log cfu mL^{-1}, which is higher than those reported by Abdalla and Ahmed (2010). Staphylococcal count in yoghurt ranged between 1.38 and 5.45 log cfu mL^{-1} with a mean concentration of 4.86 log cfu mL^{-1}. This value is higher than standard (< 2 log cfu mL^{-1}) and those obtained by Bonfoh et al. (2002) result which was 4.5 log cfu mL^{-1}.

A total of 182 coliforms isolates were regrouped according to morphological, physiological and biochemical characteristics and 41 isolates were identified.

The study indicates (Table 4) that the dominant coliforms associated with the dairy products were *Klebsiella* spp, *Enterobacter* spp, and *E. coli*. Here, no *Citrobacter* was identified in isolates. Other bacteria such as *Brucella, Serratia* spp*, Pantoea* spp*, Pasteurella* spp were also observed among the isolated stocks. The presence of those bacteria in dairy products indicated the contamination from human or by animals. The presence of coliforms in dairy products is not acceptable by safe food consumption standards. These organisms are highly pathogenic and may cause serious diseases for human. The frequency of *E. coli* was similar to those obtained by Bagré et al. (2014) (38% for raw milk and 44% for sour milk). The presence of *Serratia* and *Brucella* indicated the presence of mastitis in the cow milk (Bonfoh et al., 2002). The identification of these germs thus justifies the need for a follow-up of the animals, the screening of the pathogenic bacteria in the dairy products as well as the evaluation of their resistance to antibiotics usually used in Burkina Faso.

Conclusion

The results of the present work provide an overview on

Table 4. Frequency percentages of some coliforms isolated from milk, sour milk and yoghurt collected from local markets at Ouagadougou (Burkina Faso).

Isolates	Raw milk	Sour milk	Yoghurt	Total
E. coli	(5/15) 33.33	(2/15) 13.33	(1/15) 6.67	(8/45) 17.78
Klebsiella spp	(15/15) 100	(5/15) 33.33	(4/15) 26.66	(24/45) 53.33
Klebsiella pneumoniae	(10/15) 66.67	(3/15) 20.00	(4/15) 26.68	(17/45) 33.78
Enterobacter spp	(13/15) 86.66	(3/15) 20.00	(2/15) 13.33	(18/45) 40.00
Enterobacter cloacae	(10/15) 66.67	(1/15) 6.67	(2/15) 13.33	(13/45) 28.89

the microbiological quality of raw milk, sour milk and artisanal yoghurt sold in Ouagadougou markets. Raw milk, sour milk and yoghurt samples collected from the five markets were found to contain total coliforms, thermotolerant coliforms, *S. aureus*, lactic acid bacteria and yeasts and moulds at concentrations varying according to the markets as well as the microorganisms. Most of the samples examined were contaminated with microbes at the levels exceeding regulatory limits thus making these products inappropriate for human consumption. This study revealed that artisanal milks and dairy products from Ouagadougou markets including the raw milk, sour milk and yoghourt represents a risk for consumers and also for public health. The hygienic quality of commercial milk and dairy products must be improved considerably. Further studies should be undertaken to determine the source of the spoilage organisms. Therefore, it is necessary to set up an awareness program including capacity building of all the actors working in the sector of milk and dairy products.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Antimicrobial resistance of potential pathogenic strains isolated from eggs produced by informal farms and sold in Abidjan, Ivory Coast

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The aim of this study was to investigate the microbial quality of eggs produced by informal farms and sold in most Abidjan's markets. Thus, a preliminary investigation was conducted to evaluate the frequency of eggs consumption by the population of the study area (Abidjan). After this investigation, an analysis of the eggs microbiological quality was performed both on the shell and the eatable (egg yolk) part. The eggs were categorized into two groups: i- eggs with shell covered with droppings and ii- eggs with shell not covered with droppings. Four samples of eggs were randomly taken in the same batch collected from 10 districts of Abidjan. Therefore, a total of 90 samples of each category were taken for microbial analyses. The classical method of enumeration in food bacteriology was used for the research of total aerobic mesophilic flora, *Enterococcus*, total and fecal coliforms, *Enterobacteriaceae*, the golden *Staphylococcus* and sulphite-reducing anaerobic bacteria. The present study data show that the consummation of eggs varies according to the area. None of the eatable part contained the investigated bacteria. In general, bacterial counts were higher on shells covered with droppings. Total aerobic mesophilic counts of ~5x10^7 CFU/g *vs.* 10^5 CFU/g was observed on not covered samples. Similarly, *Enterococci*, *Enterobacteriaceae* and *Staphylococcus* reached ~10^4 CFU/g on shells covered with dropping, about 1 log higher than the values observed for non-covered shells. This study suggests that the contamination of the eggs by the investigated microbial parameter is mainly observed on the shell and not on the eatable part. Also, the highest contamination levels were observed in eggs with dropping on the shells.

Key words: Bacterial load, eggs, shell, market, dropping, Abidjan.

INTRODUCTION

Eggs constitute a rich food with high quality biological protein. These egg proteins are used by Food and Agricultural Organization (FAO) of the United Nation to estimate the quality of the other protein sources

(Faverger, 2005). Besides high content in proteins, eggs also play an important part in high digestibility of denatured lipids, many vitamins and mineral salts. They also present technological, emulsifying, foaming, gelling and coagulating properties (AFSSA, 2007).

In Ivory Coast, it was reported that about 60% of consumed eggs are supplied by informal chain (FAO, 2008). In such chains, eggs are provided by traditional or family farms in which they are laid directly on the ground and most time in contact with emitted droppings. So, after their collection, these eggs are delivered on the markets for consumption without any form of hygiene such as preliminary elimination of droppings on the shells. In this case, eggs are delivered to hawkers, semi-fixed and fixed dealers in markets or roads sides. As the droppings are reported to be a source of microbial contamination (Guinebretière et al., 2009), their presence can be harmful not only to the consumer but also the eggs collector.

Thus, the presence of droppings on the egg-shell would be a vector of potentially pathogenic microorganisms to man and can represent a source of food poisoning in a population. Indeed, Since the eighties, there has been a recrudescence of food collective toxi-infections associated with egg in shell and egg product consumption (Lahellec and Salvat, 2004). The bacterium involved in those infections are *Salmonella*, *Escherichia coli* and *Staphylococcus* (De Buyser et al., 2001; Chahed et al., 2007; Kouamé-Sina et al., 2010). However, thought eggs are largely consumed in the megalopolis of Abidjan, there is a lack of data concerning the microbial risk to the populations. So, this study was conducted with the aim of evaluating the bacterial loads of eggs provided by traditional farms and sold in the various markets of Abidjan, Ivory Coast.

MATERIALS AND METHODS

Study area

All the samples were collected in ten different markets (Abobo, Adjamé, Anyama, Attécoubé, Cocody, Koumassi, Marcory, Port-Bouët, Treichville and Yopougon) of Abidjan located at the southeast of Ivory Coast (Figure 1).

Sample collection

The egg samples were collected among the dealers in various markets ("Cocovico market" of Cocody Angré, "big market" of Treichville, "big market" of Abobo, "small market" of Marcory, "Gouro market" of Adjamé, "big market" of Port-Bouët, "Banco market" of Yopougon II, "big market" of Koumassi, "local markets" of Anyama and of Attécoubé) in Abidjan. In this market, eggs supplied

by the informal farmers to the dealers were randomly collected. Two categories of eggs were used as shown in Figure 2: eggs with shell covered with droppings (Figure 2a) and eggs with shell not covered with droppings (Figure 2b).

Each sample was composed of four eggs randomly taken from the same batch. So for the two kinds of collected eggs, a total of 180 (90 for each) samples were collected for microbial analyses. In terms of egg shell, 720 (180x4) were thus collected for the study. The egg collection was conducted from March 2014 to 2014 (3 months). This period represents the new-laid phase in such traditional farms. All the eggs were collected aseptically and taken to the laboratory in a clean refrigerator for proximate analysis.

Investigation

The investigation was conducted using the active participatory research methods among a specific group (young, old, men, women, etc). Semi-structured talks were used and the talks were inspired by the "snowball" method previously used by Subedi et al. (2003) and Delaunay et al. (2008). It indicates other surrounding farms apart from the first one fall back on the farm already visited (Thierry, 2009).

Microbiological analysis

The plate count agar was used to count total aerobic mesophilic flora according to French standard: V08-051: 2009. Enumeration of *Enterobacteriaceae* was performed on VRBG agar media according to ISO 21528-2: 2004. For enumeration of *Salmonella*, the SS agar media was used according to the standard described in the ISO 6579: 2002 Amd 1: 2007, whereas the Baird-Parker medial was used for the identification of *Staphylococcus* (37°C for 48 h) according to the French standard V08-057-1: 2004. The TSN agar media was used for the enumeration of Clostridium and sulphite-reducing anaerobic microorganisms (ASR) following the prescriptions of the ISO 7937: 2004 standard at 45°C for 24 to 48 h. Total and fecal coliforms was enumerated (37 and 44°C for 24-48 h) with rapid *E. coli* 2 as suggested in the 2011 version of AFNOR BIO-12 / 5-01 / 99 (2) and the BEA agar was used to investigate the presence of *Enterococcus* (37°C for 24-48 h) according to the ISO 7899/1: 1998 recommendations.

For all the above cited tests, a preparation of the initial eggshells suspension was made by introducing the sample (4 eggs) in 225 ml of sterilized buffered peptone water (BPW, Merk). The four egg shells were dabbled in the diluents for about 2 min (Protais et al., 2003) to obtain the stock solution (dilution 10^{-1}). This stock solution was used to prepare work solutions (10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) in aseptic conditions after decimal dilutions.

Analysis of the egg eatable part was performed after immersing the whole eggs in alcohol (70%) for 10 min to destroy the bacterial flora present all over the surface of the shells. After this operation, the content of each egg was put in a sterile glass jar and mixed with a sterile spatula. This egg solution (25 ml) was mixed with sterile distilled water (225 ml) in aseptical conditions to get a stock solution (dilution 10^{-1}).This stock solution was used to prepare work solutions (10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) in aseptic conditions after decimal dilutions. All the different solutions were inoculated in the appropriate media for a particular test as mentioned above. This stock solution was used to prepare work solutions(10^{-2}, 10^{-3}, 10^{-4}

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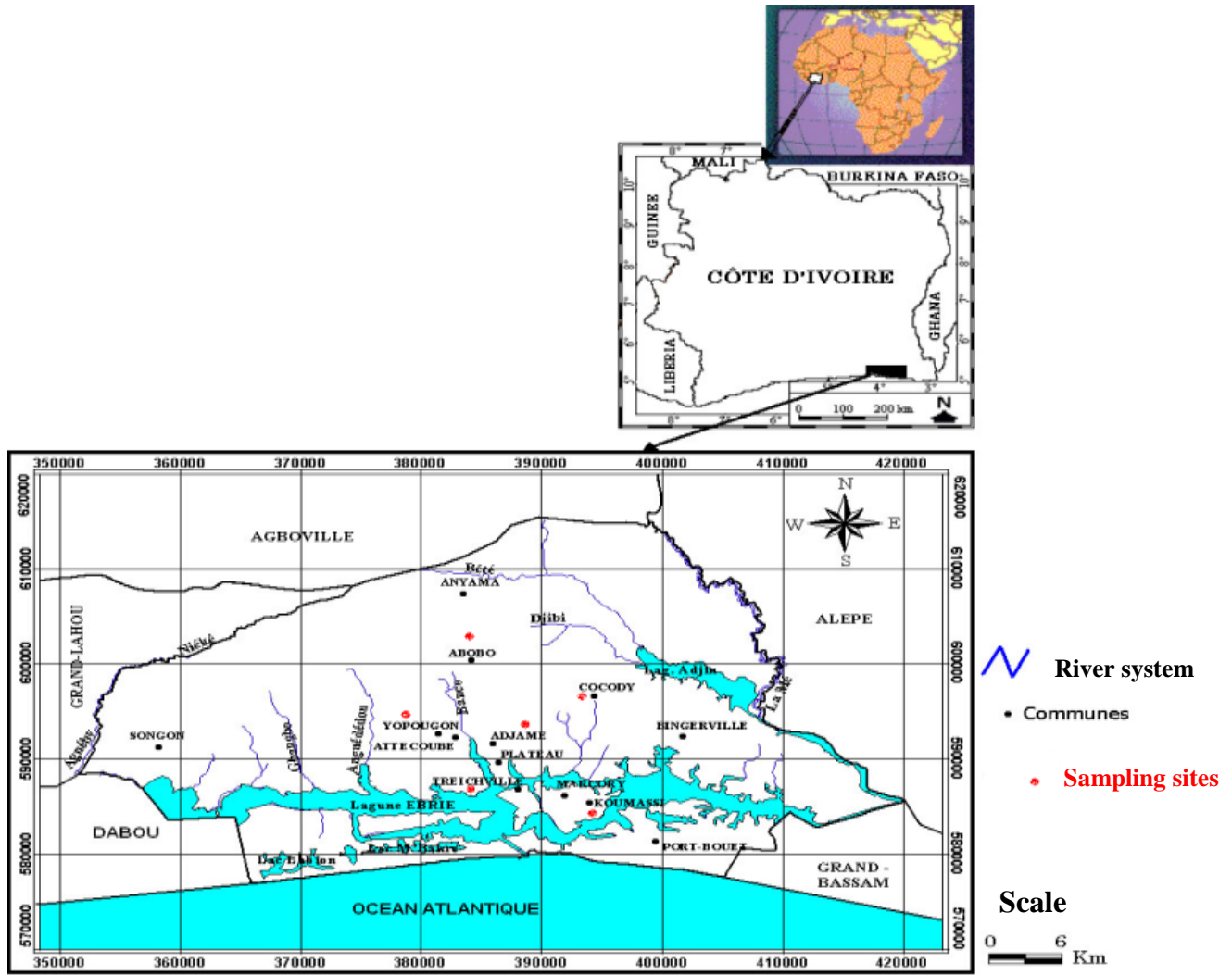
Figure 1. Study area showing the sample collection sites in the map of Abidjan.

Dropping on the eggs
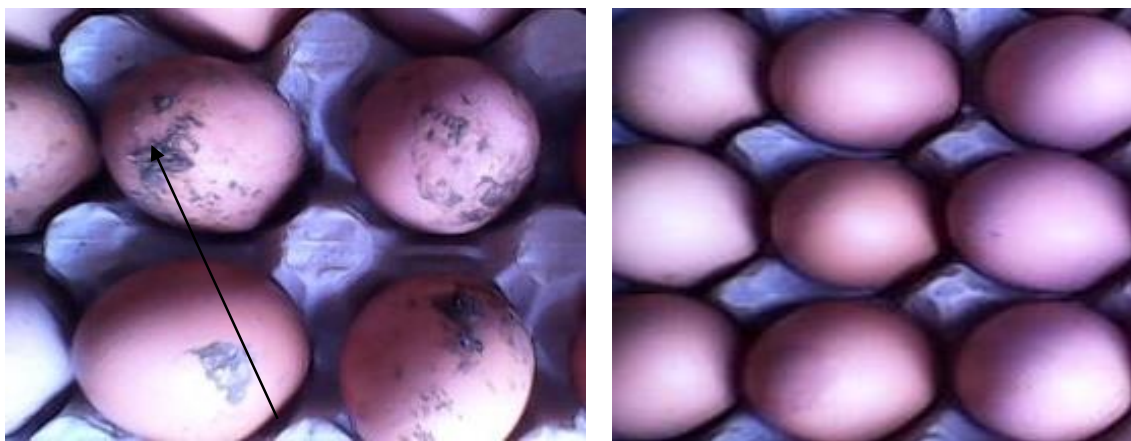
a.   Eggs covered with droppings (Ya)   **b.**   Eggs not covered with droppings (Yb)

Figure 2. The two categories of eggs collected for this study.

Table 1. Percentage of eggs consumption in the municipalities of Abidjan.

Municipalities	Quantity regularly consumed per day					Trend of consumption these the last three months		
	1	2	3	4	≥5	Constant	Evolutionary	Regressive
Abobo	50.7[b]	28[c]	20[b]	1.3[d]	0.0	76.4[d]	2.8[c]	20.8[b]
Adjamé	52.4[b]	33.3[a]	14.3[d]	0.0[e]	0.0	80.9[c]	4.8[b]	14.3[c]
Yopougon	57.4[b]	25.5[c]	10.6[d]	6.4[c]	0.0	85.1[bc]	4.2[b]	10.6[d]
Attécoubé	37.5[c]	37.5[a]	12.5[d]	12.5[a]	0.0	100.0[a]	0.0[d]	0.0[e]
Koumassi	41.2[c]	35.3[a]	17.6[c]	5.9[c]	0.0	94.1[b]	0.0[d]	5.9[de]
Marcory	81.8[a]	27.3[c]	0.0[e]	0.0[e]	0.0	100.0[a]	0.0[d]	0.0[e]
Cocody	50.0[b]	33.3[b]	8.3[de]	8.3[b]	0.0	66.7[e]	8.3[a]	25.0[a]
Anyama	40.9[c]	31.8[b]	13.6[d]	13.6[a]	0.0	77.3[d]	4.5[b]	18.2[bc]
Treichville	50.0[b]	33.3[b]	16.7[c]	0.0[e]	0.0	100.0[a]	0.0[d]	0.0[e]
Port-Bouët	42.9[c]	28.6[c]	28.6[a]	0.0[e]	0.0	100.0[a]	0.0[d]	0.0[e]
Average	51.3[b]	30.4[b]	14.7[d]	4.4[c]	0.0	89.1[bc]	2.2[c]	8.6[dc]

Numbers in the table are in %; in a column, the values with the same letters are not statistically different ($p > 0.05$).

and 10^{-5}) in aseptic conditions after decimal dilutions. All the different solutions were inoculated in the appropriate media for a particular test as mentioned above.

Antibiotic susceptibility

Antibiotic susceptibility pattern of the *E. coli* and *Staphylococcus* isolates was determined using the disk diffusion method on Mueller-Hinton agar. Inhibition zone diameter values were interpreted as recommended by the Committee of Antibiogramme of the French Society of Microbiology (CASFM, 2015). The tested antibiotics are Amoxicillin (AMX), Amoxicillin + clavulanic Acid (AMC), Imipeneme (IPM), Cefuroxime (CXM), Cefepime (FEP), Cefalotine (CTN), Aztreonam (ATM), Nalidixic acid (NA) and Ciprofloxacin (CIP).

Statistical analysis

The data analysis was done according to the objectives of the study. So, for the quantitative information, the descriptive statistical analysis (average, percentage, etc)

were done using the Graph pad 6.0 (Prism) software. With regards to the qualitative data, the method of analysis of contents was used. This method allows a systematic and rigorous analysis empirical results resulting from the semi-structured talks. For comparison, p < 0.05 was considered statistically significant.

RESULTS

The quantity of eggs regularly consumed per day and per capita was one (51.3%) followed by two (30.4%), three (14.7%) and four (4.4%). Among the interviewed people, none declared consuming more than 4 eggs daily. The trend of consumption during the last three months is dominated by a constant consumption (89.1%) (Table 1).

This study did not detect any microorganism from the eatable part of sampled eggs, suggesting bacteria were either absent or their level was below the detection limit. Consequently, further microbiological data were gotten from the

shells. Total aerobic mesophilic flora of egg shells was higher among shells with droppings with an average of 4.9×10^7 CFU/g and lower in the samples of shells without dropping (1.1×10^5 CFU/g). Considering the Ya category, the highest load in total aerobic mesophilic flora was obtained in the municipality of Port-Bouët (4.3×10^8 CFU/g) and the lowest was recorded in Cocody (2.6×10^5 CFU/g). In eggs shells without dropping (Yb), the highest load was obtained in the smallest municipality of Marcory (2.8×10^5 CFU/g) and in Port-Bouët (2×10^4 CFU/g). The lowest load in total aerobic mesophilic flora among the Ya categories (10^5 CFU/g) is equivalent to the highest load of the Yb category (Table 2). The research of *Enterococcus* displays an average charge of 5.5×10^4 and 2.3×10^4 CFU/g, respectively for Ya and Yb eggs categories. However, in the Ya category, the highest load was obtained in the smallest Marcory municipality (1.3×10^5 CFU/g)

Table 2. Load of total aerobic mesophilic flora and *Enterococcus* on the egg shells collected in the markets of Abidjan.

Municipalities	Bacterial loads (CFU/g)			
	Total aerobic mesophilic flora		*Enterococcus*	
	Ya	Yb	Ya	Yb
Abobo	$4.1*10^{5} \pm 2.8.10^{4}$[d]	$2.4.10^{4} \pm 2.6.10^{3}$[b]	$1.5.10^{4} \pm 9.8.10^{2}$[b]	$5.6.10^{3} \pm 8.1.10^{1}$[cd]
Adjamé	$3.6*10^{6} \pm 5.2.10^{5}$[c]	$2.6.10^{4} \pm 7.2.10^{3}$[b]	$1.1.10^{5} \pm 3.2.10^{3}$[a]	$7.7.10^{3} \pm 2.1.10^{2}$[c]
Yopougon	$2.4*10^{6} \pm 2.1.10^{5}$[c]	$2.2.10^{4} \pm 9.8.10^{2}$[c]	$1.8.10^{4} \pm 5.2.10^{2}$[b]	$4.5.10^{3} \pm 7.2.10^{1}$[cd]
Anyama	$1.8*10^{6} \pm 7.8.10^{5}$[c]	$3.5.10^{4} \pm 1.3.10^{3}$[b]	$1.2.10^{5} \pm 3.7.10^{3}$[a]	$5.7.10^{3} \pm 7.4.10^{1}$[cd]
Cocody	$2.6*10^{5} \pm 1.5.10^{4}$[d]	$2.5.10^{4} \pm 6.4.10^{2}$[b]	$5.6.10^{4} \pm 1.1.10^{3}$[b]	$1.5.10^{3} \pm 3.8.10^{1}$[d]
Attécoubé	$2.7*10^{6} \pm 1,1.10^{5}$[c]	$3.6.10^{5} \pm 5.10^{3}$[a]	$7.4.10^{4} \pm 8.10^{2}$[b]	$1.7.10^{5} \pm 2.4.10^{4}$[a]
Treichville	$2.6*10^{7} \pm 5.10^{5}$[b]	$3.5.10^{5} \pm 1.5.10^{4}$[a]	$1.3.10^{4} \pm 8.8.10^{1}$[b]	$1.3.10^{4} \pm 2.5.10^{2}$[b]
Marcory	$2.6*10^{7} \pm 2.5.10^{5}$[b]	$2.8.10^{5} \pm 8.6.10^{3}$[a]	$1.3.10^{5} \pm 1.2.10^{3}$[a]	$1.4.10^{4} \pm 5.3.10^{2}$[b]
Koumassi	$2*10^{6} \pm 2.7.10^{4}$[c]	$2.3.10^{4} \pm 1.8.10^{3}$[c]	$1.4.10^{4} \pm 2.9.10^{2}$[b]	$1.4.10^{3} \pm 4.9.10^{1}$[c]
Port-Bouët	$4.3*10^{8} \pm 6.10^{6}$[a]	$2.10^{4} \pm 3.4.10^{2}$[c]	$3.7.10^{3} \pm 3.8.10^{1}$[c]	$1.6.10^{3} \pm 4.5.10^{1}$[c]
Average	$4.9*10^{7} \pm 4.2.10^{7}$[b]	$1.1.10^{5} \pm 4.8.10^{4}$	$5.5.10^{4} \pm 1.6.10^{4}$[b]	$2.3.10^{4} \pm 1.7.10^{4}$[b]

CFU: Colony forming unit. Ya: eggs with droppings on the shell; Yb: eggs without droppings on the shell. In a column, the values with the same letters are not statistically different ($p>0.05$).

Table 3. Total coliforms load and *E. coli* on the egg shells collected in the markets of Abidjan.

Municipalities	Bacterial loads (CFU/g)			
	Total coliforms		*Escherichia coli*	
	Ya	Yb	Ya	Yb
Abobo	$2.6.10^{3} \pm 3.9.10^{1}$[c]	$1.5.10^{3} \pm 1.2.10^{1}$[b]	0[d]	0
Adjamé	$1.6.10^{4} \pm 7.4.10^{2}$[b]	$2.7.10^{3} \pm 1.3.10^{2}$[a]	0[d]	0
Yopougon	$1.5.10^{3} \pm 1.1.10^{2}$[d]	$1.6.10^{3} \pm 3.7.10^{1}$[b]	0[d]	0
Anyama	$1.5.10^{3} \pm 5.9.10^{1}$[d]	$1.9.10^{3} \pm 1.10^{2}$[b]	0[d]	0
Cocody	$2.4.10^{4} \pm 5.8.10^{1}$[a]	$2.2.10^{2} \pm 6.9$[c]	0[d]	0
Attécoubé	$2.2.10^{4} \pm 6.2.10^{2}$[a]	$2.2.10^{2} \pm 1.9.10^{1}$[c]	0[d]	0
Treichville	$1.9.10^{3} \pm 3.5.10^{1}$[c]	$1.8.10^{3} \pm 2.7.10^{1}$[b]	$3.3.10^{3} \pm 3.3.10^{3}$[a]	0
Marcory	$2.4.10^{4} \pm 3.8.10^{2}$[a]	$2.1.10^{3} \pm 3.5.10^{1}$[a]	$1.7.10^{1} \pm 1.7.10^{1}$[c]	0
Koumassi	$1.6.10^{3} \pm 3.8.10^{1}$[d]	$2.5.10^{2} \pm 2.1$[c]	$1.6.10^{2} \pm 1.6.10^{2}$[b]	0
Port-Bouët	$1.7.10^{4} \pm 3.2.10^{2}$[b]	$2.5.10^{2} \pm 6.5$[c]	0[d]	0
Average	$1.1.10^{4} \pm 3.2.10^{3}$[b]	$1.2.10^{3} \pm 3.10^{2}$[b]	$1.8.10^{1} \pm 1.6.10^{1}$[c]	0

CFU: colony forming unit. Ya: eggs with droppings on the shell; Yb: eggs without droppings on the shell. In column, the values carrying the same letters are not statistically different ($p>0.05$).

and the lowest at Port-Bouët (3.7×10^{3} CFU/g). In the second category, Attécoubé displays the greatest average charge (1.7×10^{5} CFU/g), whereas the lowest load (1.5×10^{3} CFU/g) was observed at Cocody. The differences of *Enterococcus* counts observed between samples were not statistically significant ($p >0.05$).

The recorded load in total coliforms on the egg shells was higher in Ya samples with an average of 1.1×10^{4} CFU/g when compared with the Yb ones (average 1.2×10^{3} CFU/g). Considering the Ya category, the highest value was observed in in the municipality of Marcory (2.4×10^{4} CFU/g) and the lowest at Koumassi (1.6×10^{3} CFU/g). In the Yb category, the highest load was

obtained in the municipality of Adjamé (2.7×10^{3} CFU/g). *E. coli* was detected in 3 out of the 10 collected samples and these samples were all issued from Ya category with an average value of 1.8×10^{1} CFU/g. The positive samples were collected at Treichville (3.3×10^{3} CFU/g), Koumassi (1.6×10^{2} CFU/g) and Marcory (1.7×10^{1} CFU/g) (Table 3).

Data obtained for *Enterobacteriaceae* suggest that the shells Ya categories were more contaminated (1.7×10^{4} CFU/g) than the Yb category's (1.5×10^{3} CFU/g). These germs were more detected at Marcory ($6.7x10^{4}$ CFU/g) and less in the municipality of Anyama ($1.6x10^{3}$ CFU/g). In addition, no significant difference was observed

Table 4. Load in *Enterobacteriaceae* and gilded *Staphylococcus* on the egg shells collected in the markets of Abidjan.

Municipalities	Bacterial loads (CFU/g)			
	Enterobacteriaceae		Gilded *Staphylococcus*	
	Ya	Yb	Ya	Yb
Abobo	$2.7.10^3 \pm 9.10^{1c}$	$2.1.10^3 \pm 2.10^{1cd}$	$1.8.10^4 \pm 5.6.10^{2bc}$	$2.10^4 \pm 9.10^{2a}$
Adjamé	$1.8.10^4 \pm 4.10^{2b}$	$2.7.10^3 \pm 4.7.10^{1b}$	$2.4.10^4 \pm 1.5.10^{3b}$	$1.4.10^3 \pm 1.2.10^{2d}$
Yopougon	$5.4.10^3 \pm 1.6.10^{2bc}$	$1.5.10^3 \pm 1.10^{2d}$	$2.3.10^4 \pm 1.1.10^{3b}$	$1.9.10^3 \pm 2.6.10^{1dc}$
Anyama	$1.6.10^3 \pm 4.7.10^{1c}$	$2.5.10^3 \pm 45.1^b$	$1.4.10^5 \pm 5.2.10^{3a}$	$1.3.10^3 \pm 6.7.10^{1d}$
Cocody	$2.5.10^4 \pm 9.8.10^{2b}$	$2.7.10^2 \pm 1.2^c$	$2.2.10^4 \pm 5.6.10^{2b}$	$2.2.10^3 \pm 3.4.10^{1d}$
Attécoubé	$2.4.10^4 \pm 5.2.10^{2b}$	$2,5.10^3 \pm 6.4.10^{1b}$	$2.5.10^4 \pm 1.7.10^{3b}$	$4.7.10^3 \pm 9.4.10^{1c}$
Treichville	$2.10^3 \pm 3.2.10^{1c}$	$2.1.10^3 \pm 3.3.10^{1c}$	$1.3.10^5 \pm 2.2.10^{3a}$	$1.3.10^3 \pm 4.10^{1d}$
Marcory	$6.7.10^4 \pm 6.9.10^{2a}$	$3.5.10^3 \pm 2.3.10^{1a}$	$2.2.10^5 \pm 4.7.10^{3a}$	$2.3.10^3 \pm 3.7.10^{1dc}$
Koumassi	$1.7.10^3 \pm 1.7.10^{3c}$	$2.7.10^2 \pm 1.5.10^{1c}$	$1.8.10^3 \pm 2.7.10^{1c}$	$1.3.10^3 \pm 5.5.10^{1d}$
Port-Bouët	$2.5.10^4 \pm 6.10^{2b}$	$2.6.10^2 \pm 2.3^c$	$2.6.10^4 \pm 7.6.10^{2b}$	$7.3.10^3 \pm 3.3.10^{2b}$
Average	$1.7.10^4 \pm 6.4.10^{3b}$	$1.5.10^3 \pm 4.10^{2d}$	$6.4.10^4 \pm 2.4.10^{4b}$	$4.3.10^3 \pm 1.8.10^{3b}$

CFU: Colony forming unit. Ya: eggs with droppings on the shell; Yb: eggs without droppings on the shell. In a column, the values with the same letters are not statistically different (p>0.05).

between the loads of Koumassi (1.7×10^3 CFU/g), Treichville (2×10^3 CFU/g), Abobo (2.7×10^3 CFU/g) and Anyama (1.6×10^3 CFU/g) (p>0.05). Considering the Yb category, the maximal and minimal loadings of the eggs shell were obtained at Marcory (3.5×10^3 CFU/g) and Yopougon (1.4×10^3 CFU/g), respectively (Table 4). The gilded *Staphylococcus*, were detected with average charges of 6.4×10^4 (for Ya) and of 4.3×10^3 CFU/g (Yb). Among the samples of Ya category, the municipality of Marcory recorded the highest load (2.2×10^5 CFU/g). A non-significant difference was observed between the load of Marcory and the one of Treichville (1.3×10^5 CFU/g) (p<0.05). With the Yb category, the maximum loadings and minimal were obtained respectively at Abobo (2×10^4 CFU/g) and Koumassi (1.3×10^3 CFU/g). Also, it should be noted that the average charge in gilded *Staphylococcus* is higher on the eggs shells of Ya category than on the Yb one (Table 4).

The average load of the tested shells in sulphite-reducing anaerobic microorganisms, was 10 CFU/g for Ya samples and 20 CFU/g for Yb ones. The largest load (20 CFU/g) among the Ya samples was obtained at Yopougon, Koumassi, Anyama and Adjamé, whereas the largest values (50 CFU/g) with Yb series was recorded at Anyama. The Yb egg samples collected at Port-Bouët did not reveal any presence of ASR strains. The comparison between the two categories of egg show that the Yb category has a more important (20 CFU/g) average charge in germs (Table 5). A compilation of all our results on sampled egg shells shows that various kinds of microorganisms (including aerobic mesophile bacteria, *Enterococcus* and *Staphylococcus*) were present at different proportions (Figure 3). The frequency of isolation of sulphite-reducing anaerobic microorganisms was lower by 48.4% followed by *E. coli* contamination at a frequency of 5.2%. None of the *E. coli* strains isolated from chicken egg shells presented resistance to carbapenem and cephalosporins. These strains were also sensitive to other families of antibiotics tested including monobactam and quinolones. However, the recorded data shows that all the *E. coli* strains isolated from the Ya eggs shells were resistant to amoxicillin, whereas 20% were resistant to the association amoxicillin/clavulamique acid (Table 6).

All *Staphylococcus* strains isolated from chicken eggs shells were resistant to cephalosporins. These bacteria are resistant at various proportions to the other tested antibiotics such as carbapenems and quinolones.

DISCUSSION

The analysis of the egg shells shows the presence of several germs such as total aerobic mesophilic flora, total coliform and *E. coli*, *Staphylococcus*, *Enterobacteriaceae* and the anaerobic sulphite-reducing bacteria. A previous work performed on egg shell reveals that about 40 different groups of bacteria can be isolated (Saver, 1991). But, fortunately, most of those bacteria are not pathogenic. Similarly, Protais et al. (2006) showed that the most frequently microorganisms found on the egg shells are total aerobic mesophilic flora and *Enterobacteriacea*. However, among these germs, the major agents of collective food poisoning such as *Salmonella*, etc can be noted (Messens et al., 2005). It should be noted that the presence of pathogenic materials on egg shell may be dangerous to the consumers because the egg contamination is reported to be either horizontal or vertical (Fuller, 1984; Bouedo et al., 1993; Gabriel et al., 2005). The different types of

Table 5. Sulphite-reducing anaerobic microorganisms' load on the shells of egg collected in the markets of Abidjan.

Municipalities	ASR Bacteria loads (CFU)	
	Ya	Yb
Abobo	4 ± 4^d	7 ± 7^c
Adjamé	$2.10^1 \pm 8^a$	$2.10^1 \pm 2.10^{1b}$
Yopougon	$2.10^1 \pm 2.10^{1a}$	$2.10^1 \pm 9^b$
Anyama	$2.10^1 \pm 8^a$	$5.10^1 \pm 2.10^{1a}$
Cocody	4 ± 4^d	6 ± 4^c
Attécoubé	10 ± 6^b	$2.10^1 \pm 2^b$
Treichville	4 ± 4^d	$2.10.^1 \pm 2.10^{1b}$
Marcory	8 ± 7^c	5 ± 3^c
Koumassi	$2.10^1 \pm 10^a$	4 ± 4^c
Port-Bouët	4 ± 4^d	0 ± 0^d
Average values	10 ± 2	$2.10^1 \pm 4$

ASR: Sulphite-reducing anaerobic microorganisms; CFU: colony forming unit. Ya: eggs with droppings on the shell; Yb: eggs without droppings on the shell. In column, the values with the same letters are not statistically different ($p>0.05$).
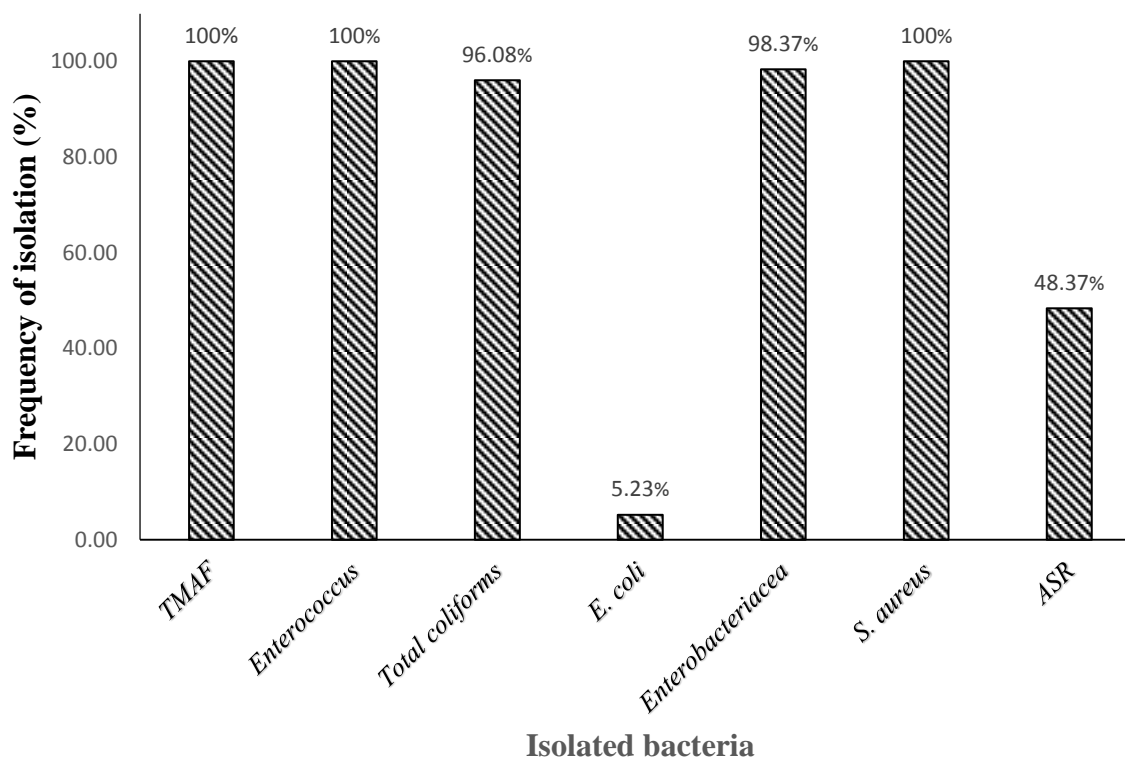
Figure 3. Frequency of some bacteria isolates on eggs shell collected in ten market of Abidjan. TMAF: total aerobic mesophilic flora, ASR: sulphite-reducing anaerobic microorganisms.

contamination are determined by the egg production environment (hen house, eggs storage room and others) and various handling (Florian and Trussel, 1958; Mayes and Takeballi, 1983; Stadelman and Cotterill, 1986; De Reu et al., 2005). Indeed, the presence of bacterial on the egg shells may be due to the intestinal flora of the good layers (Gabriel et al., 2005) because their digestive flora are a veritable nest of bacteria and fungi (Gabriel et

Table 6. Antibiotic resistance profile of *E. coli* and *Staphylococcus* isolates from egg shells collected in some markets of Abidjan.

Antibiotic resistance profile		*Escherichia coli*(n = 5)		*Staphylococcus*(n = 180)	
		Resistance	Sensibility	Resistance	Sensibility
β-lactams	**Penicillin**				
	AMX	100	0	65	35
	AMC	20	80	55	45
	Carbapenem				
	IMP	0	100	0	100
	Cephalosporin				
	CXM	0	100	45	55
	FEP	0	100	60	40
	CTN	0	100	35	65
	Monobactam				
	ATM	0	100	30	70
Quinolone	NA	0	100	15	85
	CIP	0	100	0	100

AMX: Amoxicillin; AMC: Amoxicillin + clavulanic acid; IPM: Impénem; CXM: Cefuroxime; FEP: Cefepime; CTN: Cefalotine; ATM: Aztreonam; NA: Nalidixic acid; CIP: Ciprofloxacin.

al., 2003). So, there is an established link between the layers breeding system and the produced eggs microbial load (Mallet et al., 2005). For example, the free-range farming are favorable to a high contamination of egg shell and its eatable part (Wall et al., 2008).

The present study results show that various kinds of bacteria are found on the egg shell. The diversity of bacterial loads on the sampled eggs could also be related to the use of old cells because the conditioned eggs bacterial loads increase when the cells are used too many times (Huneau-Salaün et al., 2009). In addition, the level of eggs-laying surfaces cleanliness could affect the bacterial load of these eggs (Mallet et al., 2005). Attention should be paid during the manipulations of eggs as it was established that the bacterial load of egg shell influences the penetration of potentially harmful pathogens in egg through the shell (Schoeni et al., 1995; Braun et al., 1999; Raghiante et al., 2010).

However, in spite of the remarkable presence of various bacteria on the analyzed shells, their eatable parts were free from microorganism. This result is contradictory to that of other authors who observed an *E. coli* prevalence between 26.29 and 90% in the eatable part of eggs (Lakehal, 2006; Protais et al., 2006). Such differences between the present study result and those reported by other authors may be due to the age of the analyzed eggs. Indeed, the inside content of old eggs is more likely to be contaminated by contaminants from the shell. One of the reasons that can explain the absence of

microbial contaminants in the present study samples may be the bactericidal effect of the white eatable part of egg (Vidal et al., 2003). So, microorganisms may be destroyed by this barrier with aim to protect the content of the eggs. Considering the shells, total coliform and of *E. coli* contaminants were present on the analyzed samples. The coliforms are naturally present in the intestinal flora of chicken and thus can easily contaminate egg shell via droppings (Gabriel et al., 2005). This result corroborates that of Bouedo et al. (1993).

The presence of *Staphylococcus* and anaerobic sulfite-reducing germs in this investigation is similar to the result reported by Elasri and Afilal (2014). These authors also suggest that the droppings may be the source of egg shells contamination with such microorganisms. These results show that the presence of dropping on egg shells is correlated to a higher contamination level. So with this high contamination level among samples with dropping on shells, their use without any sanitary precautions can be dissuaded. These high loads show the extent of the fecal contamination in the cases of free-range farming (Wall et al., 2008; Bouedo et al., 1993) because droppings are very charged in microorganisms in such kind of farming (Dougnon et al., 2014). The *Staphylococcus* load is higher in this study among egg shells with droppings.

The incidence observed is lower than that reported by several authors with 45% of *Staphylococcus* reported on samples from poultry farms or chicken meat (Hassen et

al., 2003; Ifesan et al., 2009).

Antimicrobial susceptibility analysis showed *Staphylococcus* strains resistant to several antibiotics. These strains of *Staphylococcus*, regardless of their origin, are mostly resistant to methicillin. Indeed, *Staphylococcus* strains isolated during this study showed a general resistance to β-lactam family and high sensitivity to carbapenems and monobactams. Thus, 65% of the *Staphylococcus* strains are resistant to oxacillin, 55% to oxacillin + clavilamique acid.

Egg shell isolates displayed a methicillin-resistant suggesting a human (clinical or environmental) origin. Indeed, the presence of methicillin-resistant strains in foods was reported in Germany by van den Broek (2003). The detection of resistant *Staphylococcus* in this study is of concern for food safety as this genus encompasses pathogenic species and in particular *Staphylococcus aureus* which can be resistant to antibiotics such as oxacillin and can produce toxin (Baba-Moussa et al., 2011, Attien et al., 2014). In 2010, an EFSA study at Abidjan reported similar results by showing that the main reservoir of methicillin-resistant *Staphylococcus* are pigs, calves and broilers (EFSA, 2010). This presence of methicillin-resistant *Staphylococcus* in food could be of human origin and linked to contamination of meat products during processing, transportation or sale.

Conclusion

The microbiological analyses of the egg shells, independently of the collection site showed the presence of many bacteria such as total aerobic mesophilic flora, *Enterococci*, total coliforms, *E. coli*, *Enterobacteriaceae*, gilded *Staphylococcus* and anaerobe sulfite-reducer (ASR) germs. Although, the eggs shells without droppings are less contaminated than those with dropping, the two groups of egg remain "not conforms" because of the presence of ASR germs that represent food poisoning risk. So, these results suggest a systematic cleaning (with adequate tools) of eggs in the traditional (informal) farms before markets supply.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Diversity of lactic acid bacteria isolated from "*kpètè-kpètè*" a ferment of traditional beer "*tchoukoutou*" produced in Benin

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The counts and identification of lactic acid bacteria isolated from the ferment "*kpètè-kpètè*" of the traditional beer (*tchoukoutou*) collected from nine large producing cities of Benin were carried out. Out of 209 strains isolated, 135 strains were purified, identified and were member of four different types based on their morphological traits, biochemical tests, API 50CH gallery and genotypic characterization (PCR technique). They are of the genus *Lactobacillus* (72), *Enterococcus* (38), *Leuconostoc* (11), *Streptococcus* (7) and *Pediococcus* (7). But the study of the biochemical and physiological characteristics identified 128 strains of the following species: *Lactobacillus fermentum* (47), *Lactobacillus divergens* (17), *Lactobacillus bifermentum* (2), *Lactobacillus fructuvoans* (2), *Lactobacillus casei* (2), *Lactobacillus acidophilus* (2), *Enterococcus faecium* (33), *Enterococcus faecalis* (5), *Streptococcus thermophilus* (7) and *Leuconostoc mesenteroides* (11). The genus *Lactobacillus* (56.25%) was dominant followed by the genus *Enterococcus* (25.78%).

Key words: Tchoukoutou ferment, lactic acid bacteria, *Lactobacillus* characterization, Benin.

INTRODUCTION

The lactic acid fermentation is a biological process in which a group of gram positive bacteria, growing under anaerobic conditions and using the carbon sources to produce lactic acid as a major organic acid is involved. In West Africa, the lactic acid fermentation has traditionally been developed for a wide range of raw materials consisting essentially of starch (≥80% of dry matter) (Ketiku and Oyenuga, 1970; Mugula et al., 2003).

Therefore, cassava and cereals such as maize, sorghum and millet are crushed and fermented to obtain

non-alcoholicproducts (paste and porridge) and alcoholic beverages that are differently named according to the countries (Odunfa, 1985). For example, the sorghum beer is named "*pito*" or "*burukutu*" in Ghana and Nigeria, "*otika*" in Nigeria, "*dolo*" in 'Burkina Faso, "*tchapalo*" in Ivory Coast and "*tchoukoutou*" in Benin (Ekundayo, 1969; Chavan and Kadam, 1989; Demuyakor and Ohta, 1991; Iwuoha and Eke, 1996; Sawadogo-Lingani et al., 2007; Amane et al., 2005; Kayodé et al., 2007; Dje et al., 2008).

Generally, fermented foods are prepared exclusively using bacteria or bacteria-yeasts mixed culture. Yeasts and lactic acid bacteria plays an important role in the production of many traditionally fermented foods and beverages all over the world. Thus, many studies have examined the major role of yeast and lactic acid bacteria genera in different sorts of fermented food (meat, milk, cereals and alcoholic beverages) process (Brandt, 2007; Aidoo et al., 2006; Romano et al., 2006). Among those foods, alcoholic beverages such as "*tchoukoutou*" represent a vast diversity of products ranging from table wines, sake, cider, fruit wines, beer and distilled alcoholic products (Kayodé et al., 2007). Lactic acid bacteria, yeasts and molds may be responsible for the fermentation of "*tchoukoutou*" because, it ferment named "*kpètè-kpètè*" is dominated by lactic acid bacteria (7.8±0.16 LogUCF/g) and yeast (8.2±0.36LogUCF/g) (Kayode et al., 2006).

However, the most studied alcoholic beverages of West Africa are the beers produced from three sorghum species (*Sorghum bicolor, Sorghum vulgare* and *Sorghum guineese*) (Steinkraus, 1983; Iwuoha and Eke, 1996). These popular fermented foods and drinks are produced at local scale by small production units (women's cooperatives). It has been reported that *Lactobacillus* are the major genus of lactic acid bacteria isolated from the micro-flora of these fermented beverages (Kayodé et al., 2007; Johansson et al., 1995; Hounhouigan, 1993). That is why the lactic acid bacteria are important for food industry, and are widely used in the production process of fermented foods (Ehrman et al., 1994).

The "*kpètè-kpètè*" is reported to be obtained from a wet deposit of previous "*tchoukoutou*" keeps for approximately one day for decantation. Overall, the producers use a perforated calabash or gourd (containing, "*kpètè-kpètè*" in dried form and stones) to ferment the traditional beer "*tchoukoutou.* The fermentation is the most important step and has many advantages such as: the reduction of the risk of growth of pathogenic microorganisms by acidification of the medium, the degradation of some anti-nutritional factors (phytates, α-galactosides), the development of specific organoleptic properties by synthesis of organic acids and

aroma (Nout, 2009; Nout et al., 2003). The microbial flora of traditional beers has been wildly studied by traditional microbiological tools (Faparusi et al., 1973; Sawadogo- Lingani et al., 2007; Kayodé et al., 2007). The molecular tools can also be used for the identification of several lactic acid bacteria (Atlan et al., 2000). *Lactobacillus* is reported to be the main genus isolated from the "*tchoukoutou*" samples (Kayodé et al., 2007) but the lactic acid bacteria contained in the ferment "kpètè-kpètè" of this beer are not yet identified.

Therefore, the present study aimed at documenting the Beninese food micro-flora and to determine the lactic acid bacteria of the ferment "*kpètè-kpètè*" and their genetic diversity in the present species. Through this, the study contributed to realize the first collection of lactic acid bacteria strains with possible application in the food industry. Therefore, once identified, these bacteria could be used as starter cultures to develop food ingredient with probiotic properties.

This study mainly focuses on six (*Lactobacillus, Enterococcus, Streptococcus, Lactococcus, Leuconostoc and Pediococcus*) genera of lactic acid bacteria*.*

MATERIALS AND METHODS

Ferment samples collection

The study areas were divided by considering the large production of traditional beers in Benin. Traditional beer producers were chosen on the basis of a preliminary survey. A total of eighty-ten (90) samples (ten samples per Township) of "*kpètè-kpètè*" were collected in nine Townships (Natitingou, Tanguiéta, Boukoumbé, Parakou, N'Dali, Tchaourou, Bantè, Glazoué and Dassa). The "*kpètè-kpètè*" samples were collected in Stomacher bags and kept in coolers with ice (~4°C) and transported within 24 h to the laboratory. Once in the laboratory, the samples were immediately analyzed for physico-chemical and microbiological parameters.

Counting and isolation of lactic acid bacteria

These species of lactic bacteria contained in the 90 samples of "*pkètè-kpètè*" (a wet deposit of former "*tchoukoutou*") were counted, depending on the spices, on the MRSA, M17 or hyper-sucrose agar media according to previously described methods (Nout et al., 1989; Hounhouigan et al., 1993). The M17 agar medium (Tarzaghi agar, Difco) was used to isolate *Streptococcus* genus (37 to 45°C for 72 h), *Lactococcus* (30°C for 72 h) and *Enterococcus* (30°C for 48 to 72 h). The MRS medium agar (Man Rogosa and Sharpe, Difco) was used to isolate the genus *Lactobacillus* (30 to 45°C for 48 to 72 h) and *Pediococcus* (30°C for 48 to 72 h). The genus *Leuconostoc* was observed on the hyper-sucrose agar (Difco) at 30°C after 72 h of incubation. Thus, a volume of 0.1 mL of 4 dilutions (10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) were sown in-depth on the agar preparation. After anaerobic incubation at various temperatures, the dishes containing between 15 and 300 colonies are retained for the calculation of the number of Unit Forming Colonies (UFC) of

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lactic acid bacteria per gram of sample. All the results are expressed in Log UFC/g of ferment. After the counting of microorganisms, representative colonies were isolated, inoculated on PCA medium and incubated at 37°C for 24 h. Once isolated, the pure strains aseptically stored brain-heart infusion supplemented with 20% glycerol, incubation for 24 h anaerobically at 37°C, and then stored at -40°C.

Phenotypic identification of lactic acid bacteria

The identification of isolates of lactic acid bacteria was carried out based on the morphology of the bacteria, the Gram test and biochemical characterization tests (catalase production, capability to ferment glucose). The isolates were then identified to species level based on the results obtained from API 50CH gallery tests, biochemical tests and identification criteria reported by several authors (Klein et al., 2001; Bissonnette et al., 2000; Samelis et al., 1994, Ben Amor et al., 2007). All the following described test were performed according to the methods previously described and used by Ben Amor et al. (2007).

Catalase test

A drop of bacterial suspension was transferred in a hydrogen peroxide droplet deposited on a slide. A positive reaction (presence of catalase) results in gas evolution of air bubbles.

Gas production from glucose

The test is to assess the type of metabolism by which the carbon source is transformed to CO_2 and is used to know if the bacteria are either homolactic or heterolactic. So the production of CO_2 incubation was performed at 30°C for 24 to 72 h on glucose MRS-BCP medium (Bacilli) or on glucose M17-BCP medium (Enterococcus).

Fermentation of carbohydrates

The fermentation of carbohydrates by lactic acid bacteria isolates was determined using the API 50CH gallery (Bio Merieux, France) and by following the instructions of the manufacturer. The strains were tested twice to determine the reproducibility of the tests and the fermentation profiles were performed.

Sherman test and the thermo-resistance

Some Enterococcus was able to grow on milk medium + 0.1% methylene blue after 48 h incubation at 30°C (Samelis et al., 1994). The thermo-resistance bacteria are able to grow at 6°C. Thus, the selection of thermo-resistance species was performed into tubes containing MRS and M17 media at 60°C for 30 min. From the tubes exposed to high temperature culture a fresh culture was performed, and then incubated at 30°C for 24 h to select the strains that resist the temperature (Stiles and Holzapfe, 1997).

Growth tests

This test was carried out for cocci and bacilli, and use to classify strains as thermophilic or mesophilic. Growth was performed in the appropriate medium (MRS or M17 broth) and incubated at 10°C (Leuconostoc, Streptococcus and Lactococcus) for 5 to 7 days,

37°C (Leuconostoc), 40°C (Pediococcus, Streptococcus and Lactococcus) and 45°C (Streptococcus, Lactococcus, Lactobacillus, Pediococcus and Leuconostoc at 45°C) 24 to 48 h.

Production of arginine dihydrolase (ADH) and esculin hydrolysis

This property was detected by using M16-BCP agar (lactose, arginine and purple bromocresol) following the method describe by Thomas (1973). After 24 to 48 h of incubation at 30°C, the lactose-utilizing bacteria acidify the medium (yellow), while those using arginine re-alkalize the medium (purple color).

Growth in the presence of NaCl

The ability to grow on M17 and MRS media + different concentrations of NaCl (2% and 4%) and at various pH values (4.5 and 6.5) was observed after 2 to 3 days of incubation. The isolates were tested according to their genera as follows:

1. For Streptococcus, Lactococcus and Lactobacillus at 2%, 4% of NaCl and pH 4.5 and 6.5
2. For Leuconostoc at 6.5% of NaCl and pH 4.5, 4.8 and 6.5 and
3. For Pediococcus and Enterococcus at 4% and 6.5% of NaCl and pH 4.5, 6.5, 9.6.

It should be noted for Cocci group on MRS and M17 the adequate pH is 9.6 and Enterococcus grow in hyper-saline medium (6.5% NaCl).

Production of dextran from sucrose

The MSE agar medium composed by Mayeux et al. (1962) was used only for Leuconostoc isolates whereas the citrate utilization was evaluated on Kempler and McKay (1980) medium for isolation of Streptococcus and Lactococcus.

Production of acetoin

Clark and Lub medium was used to carry out this test (Leveau and Bouix, 1993). This medium was used for the study of two reactions: the test of methyl red (MR) and the Voges-Proskauer test (VPI and VPII).

Genotypic identification of strains to genus level

Extraction and purification of genomic DNA from Gram-positive bacteria

The genomic DNA extraction was performed using QIAamp DNA Mini kit (Qiagen, France) on a 16 h culture of strains (incubated at 30°C) in 5 ml of MRS broth following the manufacturer's instructions. The extracted DNA was stored at -20°C until used.

Amplification of 16S rDNA genes of lactic acid bacteria by PCR (Polymerase Chain Reaction)

The genes (1500 bp) encoding for the 16S rDNA of the lactic acid bacteria were determined by the polymerase chain reaction using specific primers (F and R) of 5 different species (Table 1).

Table 1. List of primer sequencesused for PCR.

Genus/ species	Primers	Anneling temperature (°C)	Seqences (5'-3')	References
Lactobacillus sp.	LbF LbR	60	GGA ATC TTC CAC AAT GGA CG CGC TTT ACG CCC AAT AAA TCC GG	Bakar et al. (2010)
Leuconostoc sp.	LnF LnR	57	GAT CCA TCT CTA GGT GAC GCC G CAC CGC TAC ACA TGG AG	Ampe et al. (1999), Savadogo et al. (2004)
Lactococcus sp	LcF LcR	53	CTT TGA GTG ATG CAA TTG CAT C CAC CGC TAC ACA TGG AG	Ampe et al. (1999), Savadogo et al. (2004)
Pediococcus spp.	PdF PdR	58	GTA AAG TGG CGT GTG TAC CTC AAGCAC CGC TAC ACA TGG AG	Heilig et al. (2002), Savadogo et al. (2004)
Streptococcus spp.	StF StR	57	AGAGTTTGATCCTGGCTCAG GTACCGTCACAGTATGAACTTTCC	Karsidani et al. (2010)
Enterococcus sp.	EnF EnR	55	TAC TGA CAA ACC ATT CAT GAT G AAC TTC GTC ACC AAC GCG AAC	Ammor et al. (2005), Jamaly et al. (2010)
Bacteriocins	BcF BcR	56	AG AGT TTG ATC CTG GCT CAG CTA CGG CTA CCT TGT TAC GA	Diop et al. (2008)

Preparation of PCR mixture and amplification

The PCR reactions were performed in a total volume of 25 µl containing 5 µl of 5x buffer, 3 µl of MgCl$_2$ (25 mM) 2 µl dNTP (10 mM), 1 µl of each primer (F and R), 0.15 µl of *Taq* polymerase (5 U /µl) and 10 µl of the DNA sample. In the negative control, 10 µl of DNA were replaced by 10 µl of sterile distilled water. The mix were then placed in a thermo-cycler (3 PRIME BASE/02/ Serial :30150, UKA) for amplifications. The initial denaturation was performed at 95°C for 2 min. This denaturation was followed by 30 cycles (denaturation at 95°C/ 45 s, variable hybridization temperature according to the primers (Table 1) for 45 s and elongation at 72°C / 1 min), and a final elongation at 72°C for 5 min.

Electrophoresis of the PCR reaction products

At the end of amplification, an aliquot of 10 µl of each amplification product was mixed with 2 µl of a loading dye. The various mixtures were migrated on 1.2% agarose gel (ethidium bromide) ¾ h at 5 V/cm. The hyper-ladder marker 100 bp (Promega, USA) was used as a molecular weight marker of DNA fragments. The gel was then photographed under ultraviolet rays (Ultraviolet radiation type T. 05X20-2A; 254 nm) with numeric camera.

Identification of lactic acid bacteria

The different results of microscopic observation, catalase and oxidase tests, the growth test and carbon sources assimilation test (API gallery) were subject to bacteria identification process using the IBIS (Intelligent Bacteria Identification system, the Netherland) software following the description of Wijtzes et al. (1997).

Factorial correspondence analysis (FCA) of genera and species vary by township

The analysis was performed with the MINITAB 16 software to show the grouping of genera and species, and to classify them. This analysis was carried out based on the two groups (G1 and G2) reported on the study previous results on physico-chemical and microbiological characteristics of "kpètè-kpètè" (N'Tcha et al., 2015). The

Table 2. Overall morphological and physiological of presumed genera of traits lactic acid bacteria.

Macromorphology	Micro morphology	Type of fermentation	T°C	Presumed genera	Number of isolates
White, round or lenticular colonies	Cocci, Diplococciand in small chains	Homo-fermentative	37-45	*Streptococci*	7
Transparent, verysmall and round colonies	Cocci, oval, in small chains	Hetero-fermentative	30	*Leuconostocs*	11
White, round colonies	Cocci, in small chains	Hetero-fermentative	30	*Enterococcus*	38
Smooth, rounded greyish colonies	Cocci in tetrads	Homo-fermentative	30	*Pediococcus*	7
Small white colonies with brown center and curved	Coiled or filamentous long rods, singly or in chains	Homo-fermentative	45	*Lactobacilli*	22
Small white colonies, round or lenticular	Small rods in chains	Homo-fermentative and Hetero-fermentative	30	*Lactobacilli*	50

T°C: Growth temperature.

first group G1 was divided into two subgroups G1A (samples of Boukoumbé, N'dali, Parakou and Tchaourou) and G1B (samples of Tanguiéta, Bantè, Glazoué and Dassa). Whereas the G2 group is characterized by the ferments having a low dry matter rate and a very high load in lactic bacteria. The ferment of this group has a low concentration of sugar.

RESULTS

Selection of isolates

A total of 209 isolates was identified from the 90 ferment samples collected. Then the study selected the bacteria that showed specific characteristics of lactic acid bacteria by taking into consideration previous work of several authors (Bekhouche and Boulahrouf, 2005; Badis et al., 2004; Axelsson, 2004; Bizzarro et al., 2000). Thus, 135 isolates were selected, all Gram-positive, catalase negative, immobile and non-sporulating. Microscopic observation of these isolates revealed two types of cells (coccus and rod). The cocci (diplococci in small chains) were 46.67% (63/135) of total number and rod-like cells represent 53.33% (72/135). These 135 isolates, representative of the dominant flora of the ferment, have been identified by phenotypic and genotypic methods.

Phenotypic identification of isolates

The 135 isolates were divided into 5 genera and classified by dominance order as follows: *Lactobacillus* (72 isolates, 53.33%), *Enterococcus* (38 isolates, 28.15%) *Leuconostoc* (11 isolates, 8.15%), *Streptococci* (7 isolates, 5.19 %) and *Pediococcus* (7 isolates, 5.19%). Overall morphological and physiological of presumed genera are presented in Table 2.

Carbohydrates fermentation of API 50 CH gallery by lactic acid bacteria

In this study, the fermentation of carbohydrates of 128 LAB isolates was examined. The pre-identification was completed by the study of the fermentative properties of strains using API 50CH gallery. The gallery comprises 49 sugars that were tested to identify the strains of lactic bacteria during growth. The results of this Characterization were used as criteria for selecting strains of lactic acid bacteria. Out of 135 isolates of bacteria, 128 isolates were characterized because the isolates belonging to the genus *Pediococcus* (7) were not

identified. The results obtained with the API system allowed us to group species into 10 groups. All isolates fermented glucose, fructose, maltose and mannose. In addition, some strains of the genus *lactobacillus* such as *Lb. Acidophilus* (2); *Lb. plantarum* (2) used esculin and fermented melibiose while others did not utilized esculin for instance *Lb. fermentum* (47). Others did not ferment lactose nor maltose and nor utilized esculin: *Lb. divergens* (17). As regards the cocci, the group 7 was the most dominant and was represented by the species *Enterococcus feacium* (33). This group of bacteria fermented galactose, glucose, fructose, mannose, maltose, sucrose, mannitol and sorbitol but did not ferment lactose and raffinose but utilized esculin. Groups 9 and 10 were represented by the species *Streptococcus thermophilus* (7) and *Leuconostoc mesentoroides* (11) respectively. These groups fermented the same sugars as in group 7 except that it did not use esculin.

Biochemical characteristics of identified genera and species

Lactobacillus

They are rod shaped bacteria. The results of the

Table 3. Physiological and biochemical characteristics of the genus lactobacillus strains.

Tests	Site 1 (3)	Site 2 (12)				Site 3 (13)	Site 4 (18)					Site 5 (8)	Site 6 (5)			Site7 (4)	Site 8 (8)		Site 9 (1)
CO_2 on glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 10°C	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 30°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 45°C	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
Growth at PH 4.5	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
Growth at PH 6.5	+	+	+	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2%NaCl	+	+	-	+	+	+	+	+	+	-	+	-	-	-	+	+	+	+	+
4%NaCl	-	-	+	V	-	-	-	-	-	+	-	-	+	+	+	-	+	-	+
Resistance at 60°C	+	-	+	-	-	+	+	+	-	+	-	+	+	-	+	+	+	+	+
ADH	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Esculin	+	+	+	+	+		-	-	-	-	-	-	ND	ND	ND	ND	ND	ND	ND
Lactose	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Melibiose	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Raffinose	+	+	+	-	+	+	+	-	+	+	+	-	-	+	+	+	-	+	+
Sucrose	+	-	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+

(+) Positive reaction; (-) Negative reaction; (V) variable; ADH: Production of Dihydrolase Arginin; (ND) No Determined.

77 isolates of lactobacilli have led us to classify them in 6 species. The different species were differentiated by their type of carbohydrates fermentation (Table 3). This classification was also performed based on the works of lactic acid bacteria characterization of several authors (Carr et al., 2002; Gunter et al., 1998). The results of identification of 72 isolates of *Lactobacillus* at species level are shown in Table 4. Also, on the basis of different growth temperatures, hydrolysis of arginine and the type fermentation, the genus *Lactobacillus* was subdivided into three groups:

Group of thermophile and strict homo-fermentative lactobacilli: The isolates of this group fermented exoses by producing exclusively lactate, but did not ferment pentose. The associated species were:
a) *Lb. Acidophilus* (02 isolates) possessed a typical fermentation of carbohydrates (glucose, fructose, mannitol, sucrose).
b) *Lb. fructuvorans*

Group of mesophile and facultative homo-fermentative lactobacilli: The isolates of this group fermented hexoses by producing exclusively lactate, and did not produce gas from glucose. They can ferment pentose. Three species belonging to this group were isolated: *Lb. casei* (2 isolates) typical fermentation

Group of mesophile or thermophile and strict hetero-fermentative Lactobacilli: The isolates of this group fermented hexoses into lactate, acetate and / or ethanol and CO_2. The species isolated were *Lb. fermentum*

(47 isolates and 40% fermented arabinose) and *Lb. fermentum* (2 isolates).

Enterococcus

Thirty-eight (38) isolates belonging to the genus *Enterococcus* were identified. Generally, these strains grew at 10°C in the presence of 6.5% NaCl, and showed a thermo-resistance at 60°C for 30 min. Among the selected strains, thirty-three (33) were citrate negative which bring them closer to the species *Enterococcus faecium*. Additionally, the remaining five (5) strains produced citrate and showed a positive growth in the presence of potassium tellurite. These characteristics are similar to *Enterococcus feacalis* (Table 4).

Leuconostoc

All strains of the genus *Leuconostoc* grew at 37°C, and were resistant to heat treatment of 60°C/30 min (Table 5). These strains also produced dextran. The 11 isolates of *Leuconostoc* species were associated with *Ln. mesenteroides*. The heterogeneity in the fermentation of sugars was observed for the isolates of this species to a very high degree.

Streptococcus

The 7 isolates identified from the genus *Streptococcus* was *Streptococcus thermophilus*. The results obtained with these strains are highly variable (Table 6). These 7 strains were able to grow rapidly at pH 6.5, and slowly at

Table 4. Physiological and biochemical characteristics of strains of the genus Enterococcus.

Tests		Site I (6)		Site 2 (4)	Site 3 (3)		Site 4 (2)	Site 5 (6)		Site 6 (6)	Site 7 (5)		Site 8 (4)	Site 9 (3)	
Growth at:	10°C	-	+	+	+	+	+	V	+	V	+	+	+	+	+
	45°C	+	+	+	+	-	+	+	+	+	+	-	+	+	-
	pH 4.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	pH 6.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	pH 9.6	-	-	-	-	+	-	+	-	-	-	+	-	-	+
	6.5% NaCl	+	+	+	+	-	+	+	+	+	+	-	+	+	-
	9.6% NaCl	+	+	+	V	+	+	+	+	+	+	+	+	+	+
	Sherman bleu	+	-	-	-	+	-	-	-	-	V	+	V	+	+
Resistance at 60°C		+	+	+	V	V	+	+	+	+	+	V	+	+	V
Acetoine		+	+	+	+	-	+	-	+	-	+	-	+	+	-
Citrate		+	+	+	+	-	+	V	+	+	V	-	V	+	-
ADH		-	-	+	-	-	-	-	-	-	-	-	-	+	-
CO$_2$ on citrate		-	-	-	-	-	-	-	-	-	-	-	-	-	-
Esculin		+	+	+	+	-	+	-	+	-	-	-	-	-	-
Lactose		+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucose		+	-	+	+	+	+	+	-	+	+	+	+	+	+
Mannitol		+	+	+	+	+	+	+	+	+	+	+	+	+	+

(+) Positive reaction; (-) Negative reaction; (V) variable; ADH: Production of Dihydrolase Arginine.

Table 5. Physiological and biochemical characteristics of strains of the genus *Leuconostoc.*

Tests	Site 1 (1)	Site 2 (1)	Site 3 (1)	Site 4 (1)	Site 5 (1)	Site 6 (1)	Site 7 (3)			Site 8 (1)	Site 9 (1)
Growth at 10°C	-	-	+	-	-	+	-	+	-	-	-
Growth at 37°C	+	+	+	+	+	+	+	+	+	+	+
Growth at 45°C	+	+	+	+	+	+	+	+	+	+	+
Growth at pH 9.6	+	+	+	-	-	-	+	+	+	+	+
6.5% NaCl	+	-	-	+	+	+	+	+	+	+	+
Resistance at 60°C	+	+	+	+	+	+	+	+	+	+	+
Acetoin	+	+	+	+	+	+	+	+	+	+	+
Dextran	+	+	+	+	+	+	+	+	+	+	+
Esculin	-	-	-	-	-	-	-	-	-	-	-
ADH	-	-	-	-	-	-	-	-	-	-	-
sucrose	-	+	+	+	+	+	-	+	+	+	+
fructose	+	+	+	+	+	+	+	+	+	+	+

(+) Positive reaction; (-) Negative reaction; (V) variable; ADH: production of dihydrolase arginine.

pH 4.5 and 4.8, they were thermo-resistant, did not produce acetoin and could not hydrolyze esculin, fermented differently some sugars (43% fermented lactose and galactose, 20.6% fermented melibiose, 18% fermented raffinose and 14% fermented melezitose).

Pediococcus

The 7 isolates presumed to belong to this genus showed very variable results. Among the strains belonging to this genus, 4 isolates demonstrated a positive growth at pH 5, 37°C and 45°C and did not ferment maltose. On the other hand, 3 isolates exhibit the same characteristics as the other strains, but they fermented maltose and showed negative growth at 15% NaCl.

Genotypic characterization of lactic acid bacteria

The PCR amplification using specific primers revealed the presence of the specific gene in various proportion according to the species (Figure 1). The amplification products obtained after amplification of *Lactobacillus* DNA showed bands ranging in size from 200 to 290 bp (Figure 1a).

Table 6. Physiological and biochemical characteristics of strains of the genus *Streptococcus.*

Tests	Site 1 (1)	Site 7 (2)		Site 8 (2)		Site 9 (2)	
Growth at 10°C	+	-	-	+	+	-	-
Growth at 45°C	+	+	+	+	+	+	+
Growth at PH 6.5	+	+	+	+	+	+	+
Growth at PH 9.6	+	-	+	-	+	-	+
Resistance at 60°C/ 30 min	+	+	+	+	+	+	+
6.5% NaCl	+	+	+	+	+	+	+
CO_2 on Citrate	-	-	-	-	-	-	-
Growth on milk " Sherman blue"	-	-	-	-	-	-	-
Acetoin	-	+	+	-	-	+	-
Citrate	-	-	-	-	-	-	-
ADH	+	+	-	+	-	-	-
Esculin	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+
Glucose	-	-	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+

(+) Positive reaction; (-) Negative reaction; (V) variable; ADH: Production of Dihydrolase Arginine.

a. Identification of strains of genus *Lactobacillus*

b. Identification of strains of genus *Streptococcus*

c. Identification of strains of genus *Enterococcus*

d. Identification of strains of genus *Leuconostoc*
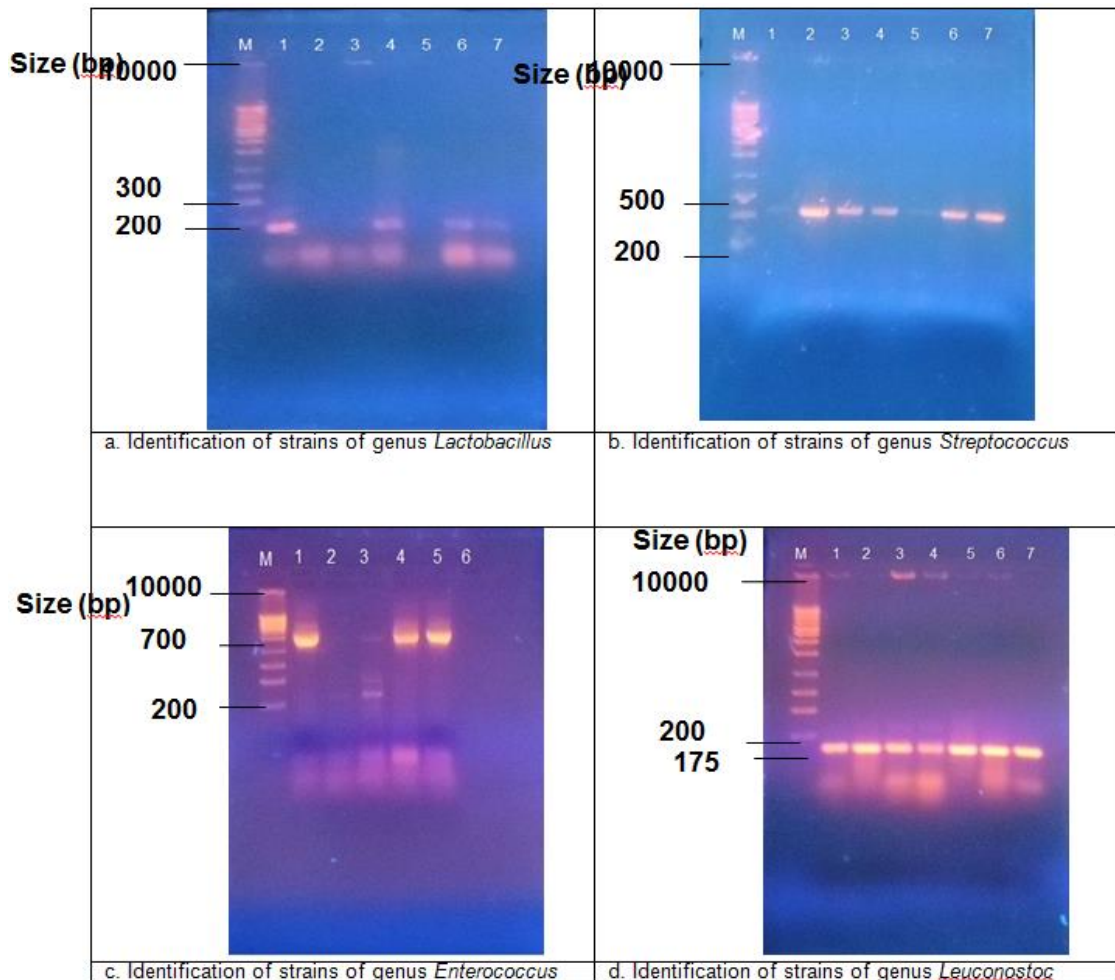
Figure 1. Identification of different strains of Lactic Bacteria using PCR at genus level.

Table 7. Overall of lactic acid bacteria identified.

Site I (Natitingou)	Site II (Boukoumbé)	Site III (Tanguiéta)
Lacobacillus fermentum (3)	*Lacobacillus fermentum* (5)	*Lacobacillus fermentum* (11)
Enterococcus faecium (5)	*Lacobacillus divergens* (5)	*Lacobacillus divergens* (2)
Enterococcus faecalis (1)	*Lacobacillus acidophilus* (2)	*Enterococcus faecium* (2)
Leuconostoc mesentoroides (1)	*Enterococcus faecium* (4)	*Enterococcus faecalis* (1)
Streptococcus thermophilus (1)	*Leuconostoc mesentoroides* (1)	*Leuconostoc mesentoroides* (1)
Site IV (Parakou)	**Site V (N'Dali)**	**Site VI (Tchaourou)**
Lacobacillus fermentum (8)	*Lacobacillus fermentum* (7)	*Lacobacillus fermentum* (1)
Lacobacillus plantarum (2)	*Lacobacillus casei* (1)	*Lacobacillus casei* (1)
Lacobacillus divergens (6)	*Enterococcus faecium* (5)	*Lacobacillus divergens* (3)
Lacobacillus fructivorans (2)	*Leuconostoc mesentoroides* (1)	*Enterococcus faecium* (5)
Enterococcus faecium (2)		*Enterococcus faecalis* (1)
Leuconostoc mesentoroides (1)		*Leuconostoc mesentoroides* (1)
Site VII (Bantè)	**Site VIII (Glazoué)**	**Site XV (Dassa)**
Lacobacillus fermentum (4)	*Lacobacillus fermentum* (8)	*Lacobacillus fermentum* (1)
Enterococcus faecium (4)	*Enterococcus faecium* (4)	*Enterococcus faecium* (4)
Enterococcus faecalis (1)	*Leuconostoc mesentoroides* (1)	*Enterococcus faecalis* (4)
Leuconostoc mesentoroides (3)	*Streptococcus thermophilus* (2)	*Leuconostoc mesentoroides* (1)
Streptococcus thermophilus (2)	-	*Streptococcus thermophilus* (2)

Table 8. Value and proportion of information concentrated on the axes.

Informations sur les axes	Value	Proportion	Cumulative
Axis1	0.2269	0.9301	0.9301
Axis 2	0.0170	0.0699	1.0000

The 733 bp bands correspond to the genus *Enterococcus* (Figure 1b), the ones of 175 to 200 bp correspond to *Leuconostoc* (Figure 1c), those of 500 to 600 bp correspond to the genus *Streptococcus* (Figure 1d), and those of 500 to 1000 bp correspond to a toxin (bacteriocin) produced by lactic acid bacteria. None gene encoding tor the *Pediococcus* were identified.

Distribution of lactic acid bacteria species isolated from ferment "kpètè-kpètè"

The results of the distribution of lactic acid bacteria according to the species are presented in Table 7. The number of species identified varied from one collection site to another. The species, *Lb. fermentum* (47) were found in all areas of the study, while *Lb. acidophilus* (2) and *Lb. fructuvorans* (2) were found only in Boukoumbé and Parakou respectively.

Factorial correspondence analysis (FCA)

Table 8 shows the values and the proportions of information concentrated on the axis. Two major

components accounted for 100% of the total variation of information. Axis 1 stands for 93.01% of the information and axis 2 account for 6.99% of the initial information. The study can therefore consider the two axes for the interpretation of results.

Correlation between the principal components and the original variables

Table 9 shows the correlations between the principal components and the original variables. The analysis of the results show that the parameters, *Lb. divergens*, *Lb. plantarum, Lb. fructivorans, Lb. casei, Lb. acidophilus, En faecalis, Ln. mesentoroides* and *St. thermophilus* were positively correlated to the first axis (PC1). *En. faecium* parameter was correlated to the axis (PC2) while *Lb. fermentum* was positively correlated to the two axes (PC1 and PC2).

Grouping of genera and species according to their similarities

Figure 2 shows the FCA with the different group of ferments according to their similarities in relation to the

Table 9. Correlation between the principal components and the initial variables.

Variable	PC1	PC2
Lactobacillus fermentum	0.417	0.583
Lactobacillus divergens	0.991	0.009
Lactobacillus plantarum	1.000	0.000
Lactobacillus fructivorans	1.000	0.000
Lactobacillus casei	1.000	0.000
Lactobacillus acidophilus	1.000	0.000
Enterococcus faecium	0.049	0.951
Enterococcus faecalis	0.994	0.006
Leuconostoc mesentoroides	0.971	0.029
Streptococcus thermophilus.	0.963	0.004
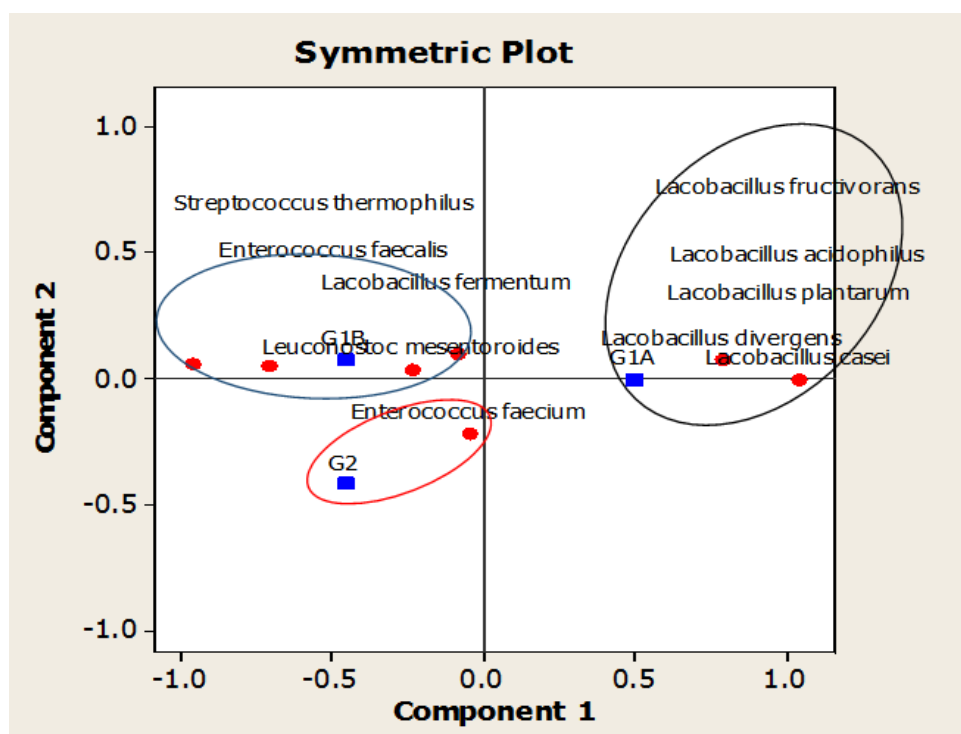
Figure 2. Grouping of lactic acid bacteria identified according to their similarity.

genera and species. The axis 1 shows a correlation between the subgroup G1A and 5 lactic acid bacteria (*Lb. divergens*, *Lb. plantarum, Lb. fructivorans, Lb. casei* and *Lb. acidophilus*). The subgroup G1B and 3 lactic acid bacteria (*En faecalis, Ln. mesentoroides, St. thermophiles*) were negative correlated to the Axis 1. The axis 2 connects ferment of group G2 and 2 spices of bacteria (*Enterococcus faecium and Lb. fermentum)*. Thus, Figure 2 shows that the ferments of the subgroup G1A is characterized by lactic acid bacteria such as *Lb. divergens, Lb. plantarum, Lb. fructivorans, Lb. casei, Lb. acidophilus.* The second subgroup (G1B) is composed of ferments from Tanguiéta, Bantè and Glazoué. These

ferments were essentially characterized by lactic acid bacteria such as *Lb. fermentum*, *En faecalis and Ln. mesentoroides St. thermophilus*. The second group (G2) includes only the ferments from Natitingou. These ferments were characterized by lactic bacteria such as *En. faecium and Lb. fermentum*. This results shows that the strains are unequally distributed in the fermentcoming from various cities.

DISCUSSION

The study of morphological, biochemical, physiological and genotypic characters of 128 isolates isolated from

the ferment "*kpètè-kpètè*" of traditional beer produced in Benin revealed 4 genera (*Lactobacillus, Leuconostoc, Enterococcus* and *Streptococcus*) and 10 different species. Their number varies from a collecting site to another. The most frequently isolated genus were *Lactobacillus* (56.25%). Five (5) species of *Lactobacillus* (*Lb. fermentum, Lb. divergens, Lb. bifermentum, Lb. fructivorans* and *Lb. casei, Lb. acidophilus*) were rod shaped and found in all the ferment samples from the nine collecting sites. The physiological and biochemical properties of species identified in "*kpètè-kpètè*" were similar to those reported by Hounhouingan et al. (1993) and kayodé et al. (2007). The predominance of the genus *Lactobacillus* was reported by kayodé et al. (2007) in their study on the characterization of lactic acid bacteria in traditional beer "tchoukoutou". The authors reported the predominance of the genus *Lactobacillus* in the traditional beer *tchoukoutou* represented by 8 species (*Lb. Divergens, Lb. fermentum, Lb. bifermentum, Lb. fructivorans, Lb. viridescens, Lb. hilgardii, Lb. kandleri, Lb. casei*). The composition of lactic acid bacteria is relative and depends on different criteria used in each study as reported by Bissonnette et al. (2000). In this study, *Lb. fermentum* (36.72%) was largely dominant and isolated from 47 samples of strains collected in the 9 sites.

These results are also similar to those of Hounhouigan (1993) in their study on composition of microbiological and physical attributes of "*mawè*," a fermented maize dough from Benin. This study results are also similar with those obtained by Vieira-Dalodé et al. (2007) during the process of "*gowé*" fermentation when they found that *Lb. fermentum* was the most predominant species of *Lactobacillus*. Muyanja et al. (2003) also enumerated the predominance of *Lb. fermentum* in "*bushera*" a Ugandan traditional fermented beverage. In Tanzania, Mugula et al. (2003) reported the same findings in the production of "*togwa*"(fermented drink). In Saudi Arabia, Gassem (2002) observed on the "*sobia*" (fermented beverage) that *Lb. fermentum* was the most dominant species throughout the fermentation process of the fermented beverage.

Leuconostoc mesenteroides isolated from "*kpètè-kpètè*" has also been isolated from *mawè* (Hounhouigan et al., 1993) and from *gowé* (Vieira-Dalodé et al., 2007). Moreover, *Lactobacillus s*p. and *Leuconoctoc* were the main microorganisms identified, respectively, during the fermentation of sorghum for the production of "*pito*" and "*burukutu*" in Nigeria (Ekundayo, 1969; Faparusi et al., 1973). Lactic acid bacteria belonging to the genera *Lactobacillus* and *Leuconostoc* were also identified during the fermentation of "*pito*" in Ghana (van der AaKühle et al., 2001). Recently, the dominant micro-flora during the transformation of "*dolo*" and "*pito*" (fermented beverage) from four production sites in Burkina Faso and Ghana were studied (Sawadogo-Lingani et al., 2007). Moreover, several other authors (Kunene et al., 2000;

Steinkraus, 1996) have shown the dominance of *Lb. plantarum* in the fermented food produced from cereal (sorghum, maize). The differences in quantity and quality noticed in our study are probably related to the difference of the physico-chemical composition of these ferments. In general, specific species of lactic acid bacteria become dominant in any particular ecological niche and based on the physicochemical niche diversity, exhibit metabolic diversity (Ali et al., 2012)

Among the identified lactobacilli, most belongs to the group of thermo-bacterium because they grew at 45°C (*Lb. fermentum, Lb. acidophilus, Lb. casei*) (Robinson, 2002; Bedi et al., 2005). Other lactic acid bacteria were also identified in "*kpètè-kpètè*": *St. thermophiles, En. faecium and En. feacalis.* These species have been reported in several cereal fermentation (Muyanja et al., 2003; Mugula et al., 2003). The role of *En. faecium* in "*kpètè-kpètè*" may warrant further investigation despite the question of its potential pathogenetic. The occurrence of *St. thermophilus* in "*kpètè-kpètè*" samples; may indicate different fermentation conditions. The growth of these species has been shown to be favoured by high temperatures and they have been isolated from food materials fermented at higher temperatures (Muyanja et al., 2003). *St. thermophilus* was also found to be effective in acidifying wet maize slurry, but it is usually not acid tolerant and is quickly inhibited by low pH values.

The microscopic observations and the phenotypic characterization were associated with the API 50CH system to better identify the strains. However, these identification methods do not always provide a sufficient basis for reliable identification of *Lactobacillus* as reported by other authors (Nigatu, 2000; De Angelis et al., 2001; Muyana et al., 2003). Therefore, these identification methods have been supplemented by genotypic characterization through the PCR technique, which is more reliable instrument of identification.

The factorial correspondence analysis (FCA) allowed us to group the genera and species identified in the ferments of different townships in our study. Diversity in the distribution of genera and species in ferments was observed. The analysis of the results showed that there was a highly significant difference between dry matters and highly significant between charges in lactic acid bacteria. This significant difference between these parameters could explain the distribution of genera and species of ferments from different townships. As a matter of fact, in metabolic reactions, there is use of the dry matter and water production by lactic acid bacteria (Hounhouigan et al., 1993). . On the other hand, the ferments characterized by high dry matter values promote the growth of genera such as *Lactobacillus* and S*treptococcus*. These results are in agreement with the studies of several authors (Greppi et al. 2013; Sefa Dedeh et al. 1999) who reported that the distribution frequency of microbial species isolated from African

beers vary according to locality and ingredients for brewing.

Fermentation is the only way to preserve the traditional beer "tchoukoutou" for three days. To improve this traditional fermentation, controlled fermentation using starter culture is very important in the production of this beer well consumed in Benin. Starter cultures have important industrial features. Our study contributes to find new strains of lactic acid bacteria in traditional products. It is essential to continue research and study the probiotic properties of lactic acid bacteria for their use in the manufacture of foods for nutrition of domestic animals and fish and humans. The differences in quantity and quality of species noticed in this study are probably related to the difference of the physico-chemical composition and the process use to product these ferments.

Furthermore, these results have indicated that several different species of lactic acid bacteria can be contained in the ferment "*kpètè-kpètè*".

Conclusion

In general, the morphological, physiological, biochemical and genotypic analysis showed a variety of lactic acid bacteria's genera, and species isolated from the ferment of Beninese traditional beer "*tchoukoutou*". The results of the identification tests showed 128 strains which demonstrated various distributions from the "*Kpètè-kpètè*". Therefore, there is a need for investigation into the selection of the most suitable strains that induce the best control of "*tchoukoutou*" fermentation. The starch-fermenting strains might be important in the development of the starter cultures and for it use in the development of small-scale commercial production of "*kpètè-kpètè*". The potential use of strains as starter cultures will depend on various parameters such as probiotics properties.

Conflict of interests

The authors declare that they have no conflict of interests

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Full Length Research Paper

Tannase production by *Aspergillus* spp. UCP1284 using cashew bagasse under solid state fermentation

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Production of tannase by *Aspergillus* species UCP1284 was studied using solid state fermentation and cashew bagasse as substrate. Amount of 35 strains of fungi species isolated from soil of Caatinga were used for qualitative selection of strains with potential for production of tannase. Through the selected fungi, a complex study was achieved about the influence of the variables: substrate amount, initial moisture content and tannic acid concentration on the production of tannase by solid state fermentation, using a factorial design (2^3). The maximum activity was 12.26 U/g of dry substrate obtained at the time of 48 hours using 10.0 g of substrate with initial moisture content of 40% and using 2.0% of tannic acid. This first design for enzyme production demonstrated the influence of the studied parameters, mainly, substrate amount on the enzyme yield, and its relevance in the process for the subsequent optimization protocol.

Key words: Tannase, *Aspergillus* species, Caatinga, cashew bagasse, solid fermentation.

INTRODUCTION

Tannin acyl hydrolase (CE 3.1.1.20) commonly known as tannase, catalyses ester bonds hydrolysis of hydrolysable tannins. The reaction produces glucose and gallic ácid as final products (Noha et al., 2014). Tannins are secondary metabolites of plants. Beside vegetable source, tannase can be obtained from animal source. However, the most important source is microbiological, since the enzymes obtained that way are more stable than others produced by alternative sources (Selwal and Selwal, 2011; Singh et al., 2011). Among microorganisms used to produce

tannase, others researchers highlight the importance of filamentous fungi, yeasts and bacteria (Renovato et al., 2011). According to Aguilar et al. (2007), most of tannase producer microorganisms are from fungus of genus *Penicillium* and *Aspergillus.*

Tannase applications are diversified among food, pharmaceuticals and rations industries. Other uses of tannase might include leather tanning, instant tea manufacturing and clarifying agent for juices, beers, some wines and sodas that have coffee as component (Selwal et al., 2011).

There's ongoing search for new sources to produce tannase, with higher catalytic stability and lower production cost (Batra and Saxena, 2005). Solid State Fermentation (SSF) is one of the alternatives to lower cost production, which could be defined as a bioprocess held on absence or near absence of substrate free water (Viniegra-González et al., 2003). However, the substrate must contain enough moisture for microbian growth (Thomas et al., 2013). This process has as one of advantages, the high concentrated enzyme production using agro industrial residues (Manjit et al., 2008).

The cashew (*Anacardium occidentale* L.) belongs to Anacardiadeae family. This fruit is native from South America and is cultivated on America (North, South and Central), Africa and Asia. In Brazil, cashew farming is highly concentrated on Northeastern region mainly on Ceará, Piauí and Rio Grande do Norte states. These locations are responsible for 97% of internal production (Carneiro et al., 2012).

In Northeastern Brazil, cashew's agro industries occupy an important place on economic and social context. Cashew's nut is the main product for exportation. However, the pseudo fruit is commercialized as sweet creams, beverages and dehydrated forms. Also, they are consumed *in natura*. Paiva et al. (2000) estimate that juice production is the most representative usage of the fruit.

Accordingly, FAO (2012) has estimated that the world production of cashew was 2.001.301 t. Brazil was responsible for 90.19% of the total, which demonstrates the importance of the country against the world market of these agro products. Fonteles et al. (2016) report that the cashew bagasse is the residue generated peduncle processing. Despite being rich in nutrients, bagasse is treated as waste. However, this disposal could be used as raw material in various industrial processes such as the production of ethanol as a substrate for solid fermentation in order to produce the enzymes.

It is estimated that 20 to 25% of cashew's pseudo fruit turns to bagasse or residual fiber, being used on animal feeding or simply being discarded, which causes damages to environment (Abreu et al., 2013). Wrong disposal of agro industrial residues might represent one of the main causes of environmental pollution and also an important loss of biomass, which could be used to produce different high-valued compounds such as enzymes.

Ernest et al. (2003) reports that after antibiotics, enzymes are the main product explores by biotechnological industries, once they are used on a large scale. Filamentous fungi have an important role on enzyme production, because of their fast growth on many substrates, easy handling and the ability to produce a lot of biotechnological applicable metabolites.

The aim of this study was to evaluate the production of tannase by *Aspergillus* species UCP1284 in solid-state fermentation (SSF) using cashew bagasse (*A. occidentale* L.) as a substrate and to evaluate the influence of variables on the enzyme production.

MATERIALS AND METHODS

Microorganisms

The 35 strains of filamentous fungi among the genera *Aspergillus*, *Penicillium* and some species of the group of Zygomycota were part of the Project Network North East of filamentous fungi in soils of Caatinga and Amazonia (RENNORFUN) collection and were kindly provided by Profª. Drª. Galba Maria de Campos Takaki. The culture medium used for the maintenance of the fungi was Malt Extract Agar and for the sporulation of the culture Potato Dextrose Agar (PDA) or Czapek were used.

Chemical reagents

The reagents tannic acid and methanolic rhodanina were purchased from Sigma (St. Louis, Mo, USA). Sodium phosphate and sodium hydroxide were purchased from Merck (Darmstad, Germany). All the other reagents were in analytical grade.

Screening of tannase producing fungi

The species of fungi were subjected to a screening in solid medium, in order to select strains with higher potential for tannic acid degradation. For selection, fragments of the culture with seven days of growth in malt extract Agar (Klich, 2002) were transferred to the center of the culture medium proposed by Murugan et al. (2007) containing agar Czapek Dox minimal medium using as substrate 0.5% tannic acid solution (in sterile Millipore filter, 0.22 µm) as the only one carbon source. The plates were incubated on Biochemical Oxygen Demand (BOD TECNAL TE-401) at 30°C for 72 h. The potential for tannic acid degradation were evidenced by the formation of halo around the colony. The potential was determined according to the methodology described by Teather and Wood (1982) and adapted to the enzyme studied. The Tannase Index (TI) was determined and expressed by the ratio of the diameter of the halo of degradation (mm) and the diameter growth of the colony (mm).The fungi that exhibited potential for tannase index equal to or greater than 1.8 mm and showed no potential for mycotoxin production were selected for solid state fermentation.

Table 1. Variables levels used in factorial design (2^3) for tannase production by Solid state fermentation (SSF).

Variable	Levels		
	Lower (-1)	Central (0)	High (+1)
Sa (g)[a]	5.0	7.5	10.0
Im (%)[b]	40	50	60
Ta (%)[c]	0	1	2

[a]Sa: Substrate amount (g); [b]Im: initial moisture (%), [c] Ta: Tannic acid.

Detection of mycotoxins

The fluorescence technique described by Lin and Dianese (1976) using the coconut agar (MAC) was used. Fungi were centrally peaked in Petri dishes containing the MAC medium with pH adjusted to 6.9 and incubated in the dark at 30°C for six days. The potential for mycotoxin production was verified by observing the presence of a halo of bluish violet fluorescent color on the reverse of the colony, when exposed to long (365 nm) wave ultraviolet light in the darkroom.

Substrate fermentation

The agro industrial substrate cashew used in the fermentation was kindly provided by Natural Pulp, which was washed and kept for an hour in a solution of sodium hypochlorite 2%, then washed and dried at 65°C to constant weight and then stored in sealed plastic containers.

Solid state fermentation (SSF)

For tannase production, the bagasse cashew was used as a substrate with a particle size between 3.0 and 8.0 mm to provide improved absorption and porosity to facilitate transport of oxygen as well as nutrients during SSF (Spier et al., 2008). The fermentation was performed in 250 ml Erlenmeyer flasks sterilized, containing 5 g of cashew substrate, initially sterilized at 60°C for 120 min and then in the ultraviolet (UV) light for 120 min. The inoculum was prepared by suspending the spores present on the malt extract agar plates in sodium phosphate buffer (10 mM, pH 5.5). The number of spores was determined in a Neubauer counting chamber and the inoculum of 10^7 spores per gram and nutrient solution containing 0.5% yeast extract and 1% dextrose in sodium phosphate buffer (10 mM, pH 5.5) containing 0.5% of tannic acid, was inoculated in the substrate used for SSF. The initial moisture of 40% the substrate was determined in accordance with the standards of the Institute Adolfo Lutz (2005). The flasks were incubated at 30°C in Biochemical Oxygen Demand (BOD) (TECNAL TE401). The fermentation was carried out for 48 h.

Experimental design to tannase production

In order to evaluate the amount of substrate influence over, initial moisture content and the concentration of tannic acid was performed full factorial design (2^3) (Table 1). All statistical analyses were carried out using *Statistica* 8.0 software (2008). The SSF utilized for planning is described in earlier.

Extraction of enzyme

The time of fermentation for the experiment was 96 h. The contents of the flasks were harvested at regular intervals (24 h). A mass of 3.0 g of the fermented mixture was mixed with 18 ml of sodium phosphate buffer (10 mM, pH 5.5). After maceration, extraction was performed with filter paper (Whatman no. 1) under vacuum. The extract was clarified by filtration and centrifugation at 2000 rpm for 10 min. The supernatant was used as an enzymatic extract for subsequent analytical determinations.

Tannase activity

The tannase activity was determined according to the methodology proposed by Sharma et al. (2000) and modified by Ordonez et al. (2011). The activity was performed using 100 μl of the enzyme extract and 100 μl of tannic acid solution (0.3 mM) in sodium phosphate buffer (10 mM, pH 5.5), incubated for 30 min at 30°C. Then were added 300 μl of solution of methanolic rhodanina (0.667% w/v) and 100 μl of sodium hydroxide (500 mM) for sample dilution was added 900 μl of distilled water, after incubation for 10 min at 30°C and the color was measured at 520 nm. One unit of enzyme activity was defined as the amount of enzyme required to catalyze the production of 1 μmol of gallic acid per minute on these conditions. The enzyme activity was expressed in units per gram of substrate in dry basis (gds).

RESULTS AND DISCUSSION

From the 35 fungal cultures isolated from Caatinga's soil, 64.57% were acknowledged as potential producers of tannase, while 31.42% did not grow (Table 2). The fungi growth absence in all Zygomycota species, in medium containing tannic acid, this could be due to sensibility or incapacity to use such substance as nutritive font. On similar screening, Pinto et al. (2001) worked with 30 species of *Aspergillus.* The authors report that the direct measurement of colony diameter is a good capacity index of degradation of tannic acid as sole source of carbon. After 72 h of growth, the best results attained by degradation index were from *Aspergillus* spp. UCP1284, UCP1290 and *Penicillium* species UCP1285. Darah et al. (2011) report that *Aspergillus*, *Penicillium*, *Paecilomyces* and *Rhizopus* species are widely studied for tannase production, obtained a good enzyme activity. In our study, the presence of halo was observed using 0.5% (w/v) of tannic acid. This value is similar to others published by literature (Purohit et al., 2006; Murugan, 2007; Ordoñez et al., 2011).

In 42.85% of cultures grown in MAC medium screening to detect mycotoxin showed production potential, while 57.14% were producers. Yazdani et al. (2010) state that HPLC and TLC tests are the most precise for mycotoxin detection. Although, other tests, as used in the present study, can be used as a preliminary detection indicator of mycotoxin. *Aspergillius* spp. UCP1284 produced the best solid state fermentation result (6.77 U/mL), for fungal

Table 2. Result of screening for tannase potential on solid medium and potential producers of mycotoxins.

Access number[1]	Culture	Øh	Øc	Ti	Access number[1]	Culture	Øh	Øc	Ti
UCP1266	*Lichtheimia hyalospora*	Nc	-	-	UCP1284	*Aspergillus* spp.	9	5	1.8
UCP1267	*Lichtheimia hyalospora*	Nc	-	-	UCP1285	*Penicillium* spp.	10	4	2.5
UCP1268*	*Aspergillus* spp.	15	10	1.50	UCP1286*	*Penicillium* spp.	14	11	1.27
UCP1269*	*Aspergillus* spp.	13	9	1.44	UCP1287*	*Aspergillus* spp.	12	7	1.71
UCP1270*	*Aspergillus* spp.	23	10	2.30	UCP1288*	*Penicillium* spp.	13	9	1.44
UCP1271	*Aspergillus* spp.	12	10	1.25	UCP1289	*Penicillium* spp.	11	8	1.37
UCP1272*	*Aspergillus* spp.	11	7	1.57	UCP1290	*Aspergillus* spp.	19	10	2.00
UCP1273*	*Aspergillus* spp.	10	4	2.50	UCP 1291	*Penicillium* spp.	11	9	1.22
UCP1274*	*Aspergillus* spp.	22	17	1.30	UCP 1292	*Penicillium* spp.	10	8	1.25
UCP1275	*Aspergillus* spp.	Nc	-	-	UCP 1293	*Fennellomyces heterothallicus*	Nc	-	-
UCP1276	*Aspergillus* spp.	17	12	1.41	UCP 1294*	*Apophysomyces elegans*	Nc	-	-
UCP1277*	*Aspergillus* spp.	12	7	1.71	UCP 1295	*Rhizopus arthizus var. arthizus*	Nc	-	-
UCP1278*	*Aspergillus* spp.	11	6	1.83	UCP 1296	*Rhizopus microsporus var. chinensis*	Nc	-	-
UCP1279	*Aspergillus* spp.	23	18	1.27	UCP 1297	*Mucor prayagensis*	Nc	-	-
UCP1280	*Aspergillus* spp.	21	15	1.40	UCP 1298	*Lichtheimia corymbifera*	Nc	-	-
UCP1281	*Aspergillus* spp.	21	12	1.75	UCP 1299*	*Cunninghamella echinulata var. achinulata*	Nc	-	-
UCP1282*	*Aspergillus* spp.	12	8	1.50	UCP 1300	*Rhizopus stolonifer*	Nc	-	-
UCP1283*	*Aspergillus* spp.	15	12	1.25	-	-	-	-	-

[1]Access number in the Cultures Collection of Environmental Science Nucleus, Catholic University of Pernambuco; [2](Ti): Tannase index = ratio between the (Øh) ring diameter (mm)/(Øc) of the colony diameter (mm). (Nc) = did not grow (*) potential mycotoxin producer.

selection. In fermentation using experimental design, 12.26 (U/gds) maximum of tannase was achieved after 48 h of fermentation, using 10 g of cashew substrate, 40% of initial moisture and 2% of tannic acid (Table 3).

In a study using apple cashew bagasse as substrate for solid state fermentation, Rodrigues et al. (2008) evaluated the effects of inoculum concentration, temperature and supplementation of the medium using other carbon sources, in the tannase production. The previous authors worked with *Aspergillus oryzae* and obtained a maximum

activity of 4.63 (U/gds) after 48 h of fermentation using 10^7 (spores/g) of inoculum, 30°C of temperature, 40% of initial moisture, 1% (p/v) of saccharose supplementation, 2.5% tannic acid addition and 40 g of substrate.

Some authors reported different tannase production time. Chhokar et al. (2010) reported that maximum production of tannase was achieved after 48 h of fermentation using *Aspergillus heteromorphus* MTCC 8818. However, Selwal et al. (2011) observed maximum production only after 98 h using *Penicillium*

atramentosum. Some works from literature related that the tannase production time ranges between 24 and 120 h and depend of microorganism and substrate used, which corroborate with our results.

According to Banerjee and Pati (2007), the tannase is produced during the first phase of the growth process, wherein the tannic acid does not penetrate cell membranes due to its high molecular weight; however, the microorganisms producing of tannase are able to hydrolyze tannic acid in the gallic acid and glucose. The available

Table 3. Experimental design results 2^3 for tannase production at 96 h of SSF by *Aspergillus* spp. SIS19.

Run	Sa (g)[a]	Im (%)[b]	Ta (%)[d]	Tannase (U/gds)			
				24 h	48 h	72 h	96 h
1	5.0	40	0.0	0.63	1.12	7.18	4.24
2	10.0	40	0.0	0.53	5.94	7.07	5.71
3	5.0	60	0.0	0.48	5.59	5.68	4.10
4	10.0	60	0.0	0.40	6.32	6.16	4.39
5	5.0	40	2.0	1.84	6.40	8.68	5.65
6	10.0	40	2.0	1.02	12.26	8.09	4.39
7	5.0	60	2.0	2.22	10.00	9.48	4.76
8	10.0	60	2.0	1.23	10.86	7.15	3.64
9[(c)]	7.5	50	1.0	2.38	6.58	7.35	5.78
10[(c)]	7.5	50	1.0	1.82	7.06	9.30	4.52
11[(c)]	7.5	50	1.0	1.14	5.79	6.50	5.99
12[(c)]	7.5	50	1.0	1.20	7.42	6.38	5.31

For the tannase cultivation time is given in hours. [a]As: Substrate amount (g), [b]Im: initial mousture (%), [d]Ta: tannic acid and (C): central points.
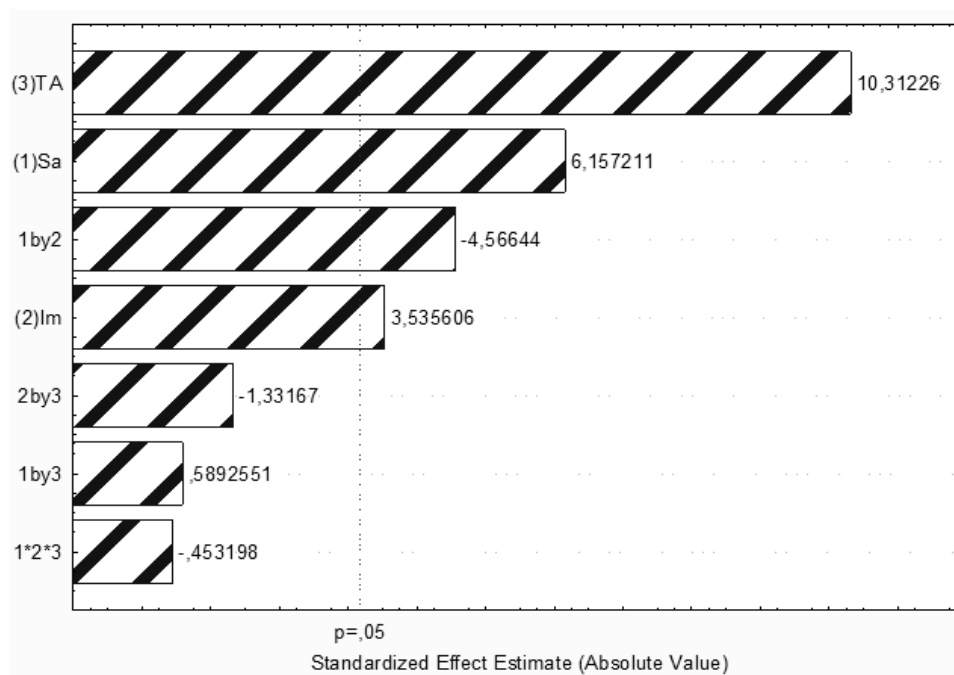
Figure 1. Pareto chart of main effects, with the response variable tannase activity (U/gds) at 48 h of SSF, using factorial design (2^3). The meanings of the symbols sorted in the figure are: (1) the quantity of substrate (g), (2) the initial moisture (%), (3) Tannic acid (%).

glucose is assimilated when glucose concentration falls and the gallic acid is also consumed in parallel. These concentrations might vary according to the species of microorganism and the type of substrate used.

The variable that most influenced positively tannase production was tannic acid concentration. Thus, higher tannase activities were obtained when higher concentrations of tannic acid were used. The initial moisture and substrate amount were also influenced significantly, both in isolated and combined variables as shown in Figure 1.

Several studies report inductive effects of different

tannic acid concentrations for tannase maximum production. Chhokar et al. (2010) reports a yield of 19.29% when purified derived from 1% concentration of tannic acid while using *A. heteromorphus* MTCC 8818. Raaman et al. (2010) used a tannic acid concentration of 1.5% (89 U/mL) along with *Paecilomyces variotii*. Abdel-Nabey et al. (2011) report production using 2% (p/v) of tannic acid as inductor. These same authors worked with *A. oryzae* and *Aspergillus japonicus* and achieved 23.7 and 14.6 U/mL, respectively. Higher values than 2.5% were reported by Madeira Jr. et al. (2011), whom obtained maximum production (2.292 U/g) using 6% of tannic acid. Even though tannase production might occur on tannic acid absence, some species of *Aspergillus* can tolerate higher concentrations than 20% without showing any deleterious effect neither over growth nor for enzyme production (Cruz-Hernández et al., 2006).

Prommajak et al. (2014) report using cashew as sole substrate, with different types of microorganisms and processing conditions, it can be obtain a wide biomolecular spectrum with pectinase and tannase enzymes amongst them.

The results obtained with this study show promising potential for *Aspergillus* spp. UCP1284, isolated from Caatinga's soil, to produce tannase using cashew bagasse as substrate. This current work also shows ideal circumstances to further studies for tannase production optimization.

Conflict of Interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

A study of high level aminoglycoside resistant enterococci

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Enterococci are a common cause of nosocomial infection and prevalence of antibiotic resistance among them is increasing. This study aimed to identify the prevalence of high level aminoglycoside resistant enterococci at Alexandria Main University Hospital. A total of 133 enterococci strains isolated from clinical specimens were all subjected to Bauer Kirby disc diffusion to detect antibiotic susceptibility pattern. High level aminoglycoside resistance (HLAR) and vancomycin resistance were confirmed by minimum inhibitory concentration (MIC). The HLAR enterococci were further identified by API 20 STREP to species level and nitrocefin test was used to detect beta lactamase production. Furthermore, polymerase chain reaction (PCR) for detection of gentamycin resistance was done to all HLGR enterococcal strains and for detection of vancomycin resistance genes. Among the 133 enterococcal isolates, 47 (35.3%) were found to be HLAR (31 *Enterococcus faecalis*, 13 *Enterococcus faecium* and 3 *Enterococcus avium*). They were all negative for beta lactamase production, 78.7% were erythromycin resistant, 63.8% resistant to doxycyclines, 51.06% to chloramphenicol, 46.8% to penicillin, 42.5% to rifampicin, and 40.4% to ampicillin. All HLAR enterococcal isolates were sensitive to Teigycyciln and Linezolid except one strain was resistant to linezolid. Urinary enterococcal isolates were also found to be 88.4, 84.6, 80.7 and 15.3% resistant to ciprofloxacin, levofloxacin, norfloxacin, and nitrofurantoin, respectively. Regarding PCR, all HLGR strains had Aac 6$'$-Ie-aph (2$''$)-Ia gene except for 2 strains. It was found also that 3 HLAR enterococcal strains were vancomycin resistant, all of which were *E. faecium* with Van A genotype. HLAR enterococci constituted 35.3% from the total enterococci isolated during the period of study denoting the importance of these isolates as nosocomial pathogens. This situation obligates the clinical microbiologist to try to identify the most useful active antibiotic for treatment. On the other hand, physicians should use antibiotics appropriately and comply with the infection-control policies in an effort to prevent further spread of high level aminoglycoside resistant enterococci.

Key words: Alexandria Egypt, enterococci, high level aminoglycoside resistance, aminoglycosides, gentamycin, antibiotic resistance, vancomycin.

INTRODUCTION

Enterococci have constituted a unique taxonomic entity since the mid-1980s when results of DNA–DNA hybridization experiments suggested their separation into the new bacterial genus, *Enterococcus* species from the former genus *Streptococcus* species (Werner, 2013).

It has emerged as a super nosocomial infecting pathogen not only due to their inherent resistance to multiple antimicrobial agents (as, clindamycin, cephalosporins

and aminoglycosides), but also because they have the capacity to acquire and disseminate determinants of antibiotic resistance (as vancomycin resistance gene clusters). Moreover, the increasing number of predisposed patients who are hospitalized and are immunosuppressed, catheterized and receiving multiple antimicrobial agents has associated this organism with hospital acquired infections. (Arias and Murray, 2012).

A common regimen for treatment of serious enterococcal infections such as septicemia and endocarditis is the synergistic combination of cell wall inhibitors as penicillin, ampicillin or vancomycin with aminoglycosides such as streptomycin or gentamycin (Levison and Mallela, 2000).

Unfortunately, this synergy is lost in enterococci exhibiting high level aminoglycoside resistance (HLAR) due to production of aminoglycoside modifying enzymes which inactivate aminoglycoside by adenylation and phosphorylation or through ribosomally mediated resistance (Gaindo et al., 2005). Making accurate detection of HLAR enterococci and rapid implementation of antibiogram policy an important issue. Also, identification at the species level of enterococci isolated from clinical specimens is considered necessary, as is quantitative evaluation of their resistance to penicillin, ampicillin, vancomycin, teicoplanin and high-level resistance to gentamicin and streptomycin (Facklam et al., 1999).

High level gentamicin resistance (minimum inhibitory concentration [MIC]≥500 μg/ml) in enterococci is predominantly mediated by aac (6')-Ie-aph(2'')-Ia, which encodes the bifunctional aminoglycoside modifying enzyme (AME) AAC(6')-APH(2''). Recently, newer AME genes such as aph(2'')-Ib, aph(2'')-Ic and aph(2'')-Id have been detected as also conferring gentamicin resistance in enterococci (Padmasini et al., 2014).

The aim of the present work was to study the antibiotic susceptibility pattern of HLAR enterococci among enterococcal isolates in Alexandria Main University Hospital.

MATERIALS AND METHODS

The study was carried out on 133 enterococcal strains that were isolated from different clinical samples referred to routine microbiology laboratory over a period of six months.

Strains that were suspected to be enterococci from their colonial morphology on blood agar were further subjected to Gram staining, Catalase test (negative) and growth on bile Esculin agar (grew as black colonies with black halo) (Wade, 1997).

Those strains were subjected to antimicrobial susceptibility testing by the Bauer Kirby method as recommended by CLSI (2014), including testing sensitivity to discs of gentamycin (120 μg)

and streptomycin (300 μg) to identify those strains of enterococci that possessed high level aminoglycoside resistance (HLAR). Also, strains proved to be vancomycin (30 μg) resistant (diameter zone ≤14 mm) and teicoplanin (30 μg) resistant (diameter zone ≤10 mm) by Bauer Kirby technique were subjected to motility testing using soft agar to exclude *Enterococcus gallinarum* or *Enterococcus cassiflavus* which are the only motile enterococci which have intrinsic vancomycin resistance mechanism (CLSI, 2014).

All HLAR were further studied by identifying their species level using analytical profile index API 20 STREP according to the manufacturer instructions (Biomerioux, Marcy L'Etoile France) and they were also tested for beta lactamase production using nitrocefin discs (Oxoid).

MIC broth micro dilution method

All strains which were previously identified as HLGR by disc diffusion (zone diameters were 6 mm) for gentamycin (120 μg), were confirmed by MIC using broth microdilution to gentamycin. The results were read for turbidity, any growth at 512 μg was considered HLGR enterococci. MIC was also done for vancomycin and teicoplanin resistant strains among HLAR strains using the CLSI recommended breakpoints shown in Table 1, *Enterococcus faecalis* ATCC 29212 was used as a negative control (CLSI, 2014).

PCR

PCR was done for detecting gentamycin resistance genes and Van A and Van B resistant enterococcal genotypes among HLAR isolates using the primers shown in Table 2 (Padmasini et al., 2014; Biendo et al., 2010).

DNA extraction

DNA extraction was done by suspending 3 to 5 colonies of enterococci grown overnight on blood agar in 25 ul of a 0.25% sodium dodecyl sulfate 0.05 N NaOH solution and boiled for 15 min. Then, 200 μl of H_2O was added to the mixture, then centrifugation at 4000 rpm for 1 min was done and DNA was obtained from the supernatant for PCR reactions (Mounir, 2011).

PCR for detection of HLAR

PCR was carried out in 2 separate reaction tubes one for Aac (6/)-Ie-aph (2//)-Ia gene and the second for the other 3 genes (Aph (2//)-Ib, Aph (2//)-Ic, Aph (2//)-Id) as multiplex PCR according to Padmasini et al. (2014) method with the following amplification conditions: initial denaturation (95°C for 5 min), followed by 32 cycles each of: Denaturation (95°C for 1 min), annealing (58°C for 1 min), extension (72°C for 1 min), final extension (72°C for 5 min) (Padmasini et al., 2014).

Conventional PCR for identification of vancomycin resistance genes done according to Biendo method

The amplification of DNA was done by the following cycling

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Table 1. CLSI MIC interpretative criteria break points (ug/ml) to Vancomycin and Teicoplanin.

Parameter	Resistant	Intermediate	Sensitive
Vancomycin	≥32	16-8	≤4
Teicoplanin	≥32	16	≤8

Table 2. Oligonucleotide primers used in PCR assay for detection of HLAR and Vancomycin resistant genotype *Enterococci.*

Gene	Forward	Reverse	Product size (bp)
Aac (6/)-Ie-aph (2//)-Ia	CAGGAATTTATCGAAAATGGTAGAAAAG	CACAATCGACTAAAGAGTACCAATC	369
Aph (2//)-Ib	CTTGGACGCTGAGATATATGAGCAC	GTTTGTAGCAATTCAGAAACACCCTT	867
Aph (2//)-Ic	CCACAATGATAATGACTCAGTTCCC	CCACAGCTTCCGATAGCAAGAG	444
Aph (2//)-Id	GTGGTTTTTACAGGAATGCCATC	CCCTCTTCATACCAATCCATATAACC	641
Van A	GGG-AAA-ACG-ACA-ATT-GC	GTA-CAA-TGC-GGC-CGT-TA	732
Van B	ATG-GGA-AGC-CGA-TAG-TC	GAT-TTC-GTT-CTT-CGA-CC	635

program that consisted of initial denaturation (94°C for 2 min). Thirty cycles each consists of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min (Biendo et al., 2010).

Statistical analysis used in this study

Qualitative data were described using number and percent. Comparison between different groups regarding categorical variables was tested using Chi-square test. Significance test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

RESULTS AND DISCUSSION

Since early 1970s, enterococci were considered as nosocomial pathogens which coincided with increased expression of antimicrobial resistance by members of the genus and this contributed to extensive administration and misuse of antimicrobial agents (Dadfarma et al., 2013).

In our study, a total of 133 (6.3%) enterococcal strains were isolated from 2100 clinical specimens during our 6 months study period, with the highest rate of isolation being from urine; 86 strains (64.6%), followed by pus 21 strains (15.7%), blood 15 (11.2%), then sputum 10 (7.5%) and only 1 (2.1%) strain was isolated from peritoneal aspirate. These results were comparable to others, who found that maximum number of enterococci isolates were from urine samples. Which is consistent with enterococci being one of the leading causes of UTI and associated with the increased usage of in dwelling urinary catheter in our hospital (Preeti et al., 2013; Adhikari, 2010).

Antimicrobial susceptibility pattern and HLAR among our isolates were detected using disc diffusion method according to CLSI guidelines; where 47 strains (35.3%)

were HLAR and the remaining 86 (64.6%) strains were non-HLAR among which 72, 43, 41.9, 26.7, 20, 6.9, 6.9 and 3.4% were resistant to erythromycin, penicillin, rifampicin, doxycycline's, chloramphenicol, vancomycin, teicoplanin and linezolid. Regarding HLAR enterococci (47 strains), the highest resistance was also to erythromycin (78.7%) followed by doxycycline's (63.8%), chloramphenicol (51.06%), penicillin (46.8%), rifampicin (42.5%) and ampicillin (40.4%). The resistance to vancomycin and teicoplanin was 6.3% for each and only 2.1% were resistant to linezolid. Doxycycline, chloramphenicol, and ampicillin resistance were significantly higher among HLAR enterococci than non HLAR enterococci with P value 0.021, 0.030 and 0.001, respectively (Table 3). As mentioned earlier, the greatest percentage of resistance was recorded for Erythromycin (74.4%), this was in agreement with Mounir et al. (2011), while Jain et al. (2011) reported that 100% of their enterococcal isolates were erythromycin resistant, which may be attributed to frequent use of Macrolides for empirical treatment of many infections .

In the current study, 6.8% of our enterococcal isolates were glycopeptide resistant, which although is higher than some rates reported in literature (Mounir et al., 2011; Asha Peter et al., 2013), but it is still relatively reassuring that 93% of our isolates are glycopeptide susceptible. As this situation is contrary to the situation in most hospitals in the USA (Perlada et al., 1997) and Europe (Schouten et al., 2000) where high prevalence of vancomycin resistance reached >20% in Ireland, Greece, Portugal as reported by The European Antimicrobial Resistance Surveillance System (EARSS).

Linezolid and tigecycline are the alternative option for treatment of vancomycin resistant enterococci (VRE) (Tsai et al., 2012). In this study, the resistance pattern to linezolid among our isolates whether HLAR or non HLAR was very low and none of them were tigecycline resistant.

Table 3. Resistance pattern to *Enterococci* isolated in the study.

Parameter	Non HLAR (86)	HLAR (47)	Total (%)
Gentamicin (120 µg)	0	47 (100)	47 (35.3)
Sterptomycin (200 µg)	0	36 (76.6)	36 (27.1)
Erythromycin	62 (72.09)	37 (78.7)	99 (74.4)
Penicillin	37 (43.02)	22 (46.8)	59 (44.4)
Rifampicin	36 (41.9)	20 (42.5)	56 (42.1)
Doxycycline's	23 (26.7)	30 (63.8)	53 (39.8)
Chloramphenicol	18 (20.9)	24 (51.06)	42 (31.6)
Ampicillin	6 (6.98)	19 (40.4)	25 (18.7)
Vancomycin	6 (6.98)	3 (6.3)	9 (6.8)
Teicoplanin	6 (6.98)	3 (6.3)	9 (6.8)
Linezolid	3 (3.49)	1 (2.1)	4 (3.01)
Teigycyciln	0	0	0
Total	86 (64.6)	47 (35.3)	133 (100)

Table 4. Urinary isolates resistance pattern of the Enterococci to the 4 antibiotics used in UTI.

Parameter	Non HLAR (%)	HLAR (%)	Total (%)
Ciprofloxacin	32 (53.3)	23 (88.4)	55 (63.9)
Levofloxacin	31 (51.6)	22 (84.6)	53 (61.6)
Norfloxacin	28 (46.7)	21 (80.7)	49 (56.9)
Nitrofurantoin	7 (11.6)	4 (15.3)	11 (12.7)
Total	60 (69.7)	26 (55.3)	86 (100)

No substantial difference in our result and the results of multiple studies that reported no or minimal resistance among their isolates (Vaibhav et al., 2013; Asha Peter et al., 2013; Sieńko et al., 2014). So tigecycline and linezolid are up till now the drugs of choice for infections caused by VRE. However, the emergence of any linezolid and or tigecycline resistant enterococci is an alarming problem in the treatment of VRE infections.

As regard urinary antibiotic resistance in the present study, 63.9, 61.6 and 56.9% of the urinary enterococcal isolates were resistant to ciprofloxacin, levofloxacin and norfloxacin, respectively, while 91.7% were sensitive to nitrofurantoin as shown in Table 4. This high resistance rate to ciprofloxacin can be attributed to its frequent use in empirical treatment of UTIs (Preeti et al., 2013; Vaibhav et al., 2013). Our high percentage of sensitive urinary enterococci to nitrofurantoin was in agreement with Preeti et al. (2013) who reported that 88.5% of urinary enterococcal isolates were sensitive to nitrofurantoin. Lower percentage was reported by Butcu et al. (2011) who had 60% of their urinary enterococcal isolates sensitive to nitrofurantoin. Looking at the 26 HLAR urinary enterococcal isolates we found that The percentage of resistance to ciprofloxacin, levofloxacin and norfloxacin were significantly higher than non HLAR enterococci with P value 0.013, 0.016 and 0.021, and one of our HLAR urinary isolates was vancomycin resistant and nitrofurantoin sensitive, putting this together with the

high resistance rate among our enterococci to the previously mentioned antibiotics, a consideration should be made to discourage the irrational use of nitrofurantoin and keep it as a possible drug of choice for the treatment of resistant urinary enterococcal isolates (Butt et al., 2004).

Multidrug resistant (MDR) strains were 84.2% (112) of the 133 enterococcal isolates, including all 47 HLAR enterococci and 65 were non HLAR enterococci. Multidrug resistance was also reported by Jain et al. (2011) as 71% of his enterococcal isolates were multidrug resistant. On the other hand, Dadfarma et al. (2013) reported that 45.7% were MDR and 31.7% among them were HLGR.

We further studied our 47 (35.3%) HLAR *Enterococcus* isolates; they were all resistant to gentamicin and among them 27.07% showed combined resistance to both high level gentamycin and streptomycin. These results were confirmed by performing MIC testing and all 47 strains were found resistant to gentamycin concentration of up to 512 µg/ml, making HLGR testing an accurate marker for detecting HLAR enterococci (Ira et al., 2013; Bhatt et al., 2015).

The species distribution among our HLAR enterococci was 65.9% *E. faecalis*, 27.6% *Enterococcus faecium* and 6.3% *Enterococcus avium*. This was on the contrary to some reports indicating that HLAR is a more common problem among *E. faecium* isolates (Abamecha et al.,
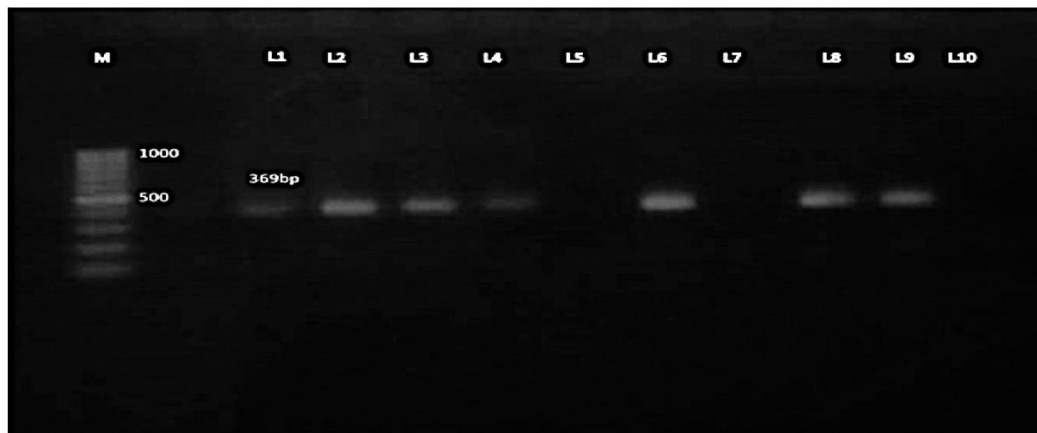
Figure 1. Lanes 1, 2, 3, 4, 6, 8,9 show the presence of Aac (6/)-Ie-aph (2//)-Ia (369 bp).

2015; Bhatt et al., 2015), while others stated that HLR was equally distributed among both species (Fernandes and Dhanashree, 2013). However, species identification has gained much importance because of the naturally occurring differences in the susceptibility of these species (Arias and Murray, 2012).

Among HLAR enterococci, aminoglycoside modifying enzymes genes (*Aac (6/)-Ie-aph (2//)-Ia*) was detected in 45 (95.7%) of HLAR enterococci (Figure 1); constituting 96.5% of *E. faecalis* and 91.6% of *E. faecium*. While only 2 (4.3%) were found to have *Aph (2//)-Ic* gene; constituting 3.4% of *E. faecalis* and 8.3% of *E. faecium*. On the other hand, *Aph (2//)-Ib* and *Aph (2//)-Id* were not detected in any isolates. This was in agreement with Wanxiang et al. (2015) and Li et al. (2015) who stated that 94.4% of HLAR enterococci were *Aac (6/)-Ie-aph (2//)-Ia* positive and in contrast to our results he detected *Aph (2//)-Id* in 1.3% of his HLAR enterococcal isolates and none of their strains were positive to *Aph (2//)-Ic* or *Aph (2//)-Ib*. Padmasini et al. (2014) stated that *Aac (6/)-Ie-aph (2//)-Ia* found in 68.4% of their HLAR enterococcal isolates, while none of other genes were detected and attributed. The HLAR among the rest of enterococcal isolates to other mechanisms or other genes not discovered till now.

VRE among HLAR enterococci constituted 3 of the 47 strains. They were all *E. faecium* constituting 23.07% (3 out of the 13 *E. feacium* strains). This high distribution of VRE among *E. faecium* may be attributed to Inc. 18 plasmid which is a broad spectrum plasmid that helps enterococci to get vancomycin resistance gene from vancomycin resistant *Staphylococci* (Zhu et al., 2008). In contrast to many studies (Abdulhakim et al., 2014; Hasani et al., 2012; Ira et al., 2013), Adhikari et al. (2010) did not report any VRE.

In this study all the VRE among the HLAR enterococci were confirmed by MIC and found not to be only resistant to vancomycin and teicoplanin (≥32 µg/ml), but also showed high level Vancomycin and Teicoplanin resistance (256 µg/ml) and were all proved to be Van A genotype explaining the presence of high level resistance to both vancomycin and teicoplanin. These results were in agreement with other multiple studies (Vaibhav et al., 2013; Lee et al., 2013). On the other hand Ira et al. (2013) reported that 96.9% were Van A genotype and were *E. faecalis* except one isolate which was *Enterococcus gallinuram* in combination with intrinsic Van C genotype, 2 isolates were Van B, both were *Enterococcus muntidii*.

Glycopeptide resistance in enterococci is one of the most important challenges. VRE takes place among the important nosocomial pathogens, in that the treatment options are limited, it easily spreads in the hospital setting through contaminated hands and surfaces and it is likely to transfer vancomycin resistance to other pathogens. As VRE is known to spread in the hospital setting. Centers for Disease Control and Prevention (1995) suggests that aggressive infection control be implemented and that hospital staff conform to the isolation precautions in order to control and prevent VRE infection.

None of the HLAR *Enterococcus* isolates were beta lactamase producers by nitrocefene test as was the situation with Mounir et al. (2011) and Asha Peter et al. (2013), while Jain et al. (2011) found only one out of 66 HLAR enterococcal isolate to be Beta lactamase producer. This explains that the resistance to beta lactam antibiotics in our HLGR enterococci is not due to beta lactamase enzyme and may be attributed to accumulation of point mutations in the penicillin binding region of PBP5.

To conclude, the current study highlighted the importance of HLAR enterococci as nosocomial pathogens in our setting. Detecting HLAR is an important task; it should be adopted as a part of the routine microbiology work. Prevention of growing resistance to linezolid and tigecycline among vancomycin resistant enterococcal isolates should be our rational in fighting antibiotic resistant enterococci. This could be achieved by

careful monitoring of their resistance pattern and adherence to an antibiotic policy created by the infection control team. Another point to be emphasized is the importance of nitrofurantoin as a therapeutic option for resistant urinary enterococcal infections.

Conflict of interests

The authors have not declared any conflict of interests.

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