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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Table of Content: Volume 9 Number 36, 9 September, 2015

ARTICLES

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Full Length Research Paper

Nosocomial infections in post-operative wounds due to *Staphylococcus aureus* and *Pseudomonas aeruginosa* in Benue State Nigeria

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Three hundred (300) post-operative wound swab specimens were aseptically collected from four hospitals and investigated. The four hospitals were Federal Medical Centre, Makurdi (FMCM), General Hospital, Gboko (GHG), General Hospital, Otukpo (GHO) and General Hospital North Bank, Makurdi (GHNBM). The swabs were cultured and organisms identified according to standard procedures. A prevalence rate of bacterial isolates (56.7%) was obtained from the post-operative wound sites investigated. *Pseudomonas aeruginosa* was the most encountered pathogen with 20.3% prevalence rate followed by *Staphylococcus aureus* (13.0%), while 8.3% accounted for co-infection of both organisms. Other organisms encountered included *Klebsiella* spp. (4.0%), *Escherichia coli* (3.3%), atypical coliform (2.7%), and *Proteus* spp. (2.3%). *Enterococcus faecalis* and *Streptococcus pyogenes* had the least prevalent rate of 1.3% each. Statistically, Chi square analysis showed that there was no significant difference in the number of isolates from FMCM, GHQ, GHO and GHNBM and in the occurrence of both organisms in relation to sex (p>0.05). The incidence of *P. aeruginosa* was highest (38.4%) at Federal Medical Centre, Makurdi, compared with other collection points investigated while that of *Staphylococcus aureus* was highest (37.5%) at FMCM compared with all other collection points' investigated. Antibiogram studies revealed that *P. aeruginosa* was most susceptible to levoxin to the magnitude of 98.4%. While *P. aeruginosa* was resistant to ampicillin, tetracycline and streptomycin, *S. aureus* was only resistant to tetracycline. The findings have revealed that nosocomial wound infections remain a menace in medical management of wounds.

Key words: Nosocomial, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, antibiotics, post-operative wounds, prevalence.

INTRODUCTION

Nosocomial infection is an infection acquired in a hospital by a patient who was admitted for a reason other than that infection (Wenzel, 2011). These infections are acquired in the hospital but appear after discharge or still

when on admission. The organisms that cause most hospital acquired infections are common in the general population and are relatively harmless. The most common are bacteria (*Staphylococcus aureus*, coagulase -negative S*taphylococci*, *Enterococci,* and *Enterobacteria*) including commensal bacteria, which are part of the normal flora, and pathogenic bacteria, which come from an exogenous source. Viruses including Hepatitis B and C, Respiratory Syncytial Virus, rotaviruses, and enteroviruses may also be transmitted nosocomially. During times of prolonged antibiotic treatment and severe immunosuppression, fungi such as *Candida albicans, Aspergillus* spp*., Cryptococcus neoformans* and *Cryptosporidium* including other opportunistic organisms can cause infections (Shittu et al., 2012). The organisms can be transferred from one patient to another (cross-infection). They can be part of a patient's own flora (endogenous infection), or they can be transferred from an inanimate object or from a substance recently contaminated by another human source (environmental transfer). Factors that increase a patient's susceptibility to nosocomial infections include age (e.g. the elderly), decreased immunity, underlying disease, therapeutic and diagnostic interventions (Mangram et al., 2011).

A number of studies in Nigeria have shown that nosocomial infections in post- operative wounds are endemic in parts of the country (Shittu et al*.,* 2012; Kolmos et al., 2013). Akinjogula et al. (2010) reported that *S. aureus* was the leading etiologic agent of post-operative wound infection in Calabar and Uyo cities of Nigeria.

In a similar study in Benin City Nigeria, it showed that *Proteus* species were the leading etiologic agents in post-operative wound infections and *P. aeruginosa* was the prevalent agent in parts of South Eastern Nigeria (Shittu et al, 2012). Haghi et al. (2010) reported that *S. aureus* was the leading etiologic agent of post-operative wound infections in India, Thailand and Japan. They also found out that *P. aeruginosa* was more prevalent among microorganisms isolated from post-operative wounds in some parts of Jordan.

Data collected from this work will be used to establish the sanitary condition of hospitals where surgical operations are carried out. It will also establish the prevalent microorganisms involved in nosocomial infections. Antibiotic susceptibility test carried out will determine the drug of choice in the treatment of post-operative wound infections. In addition, the knowledge of these infections will help physicians to give adequate treatment when such infections occur and also advise on its prevention.

This study has become necessary to ascertain bacteria implicated in wound infections which delay the normal healing process.

MATERIALS AND METHODS

Study population and area

Patients with post-operative wounds infections were targeted for this study. Three hundred (300) post-operative wounds swabs were collected from this population which comprised ninety-nine (99) from FMCM, seventy from GHG, sixty eight (68) from GHO, and sixty three (63) from GHNBM. Approval was obtained from ethical clearance committees and the Chief Medical Director of each hospital for all the samples used for the study. Confidentiality was maintained in accordance with standard medical practice.

Sample collection and processing

Sterile swab sticks were used to collect pus from the surgical sites of subjects under aseptic conditions. The samples were properly labeled and immediately conveyed to the laboratory for processing. Standard microbiological procedures for handling and transporting of specimens as enunciated by Cheesbrough (2002) were followed.

Cultivation, isolation and identification

All the swabs collected for bacteriological investigations were treated according to the methods of Isenberg et al. (2011). MacConkey, blood and chocolate agars (Oxoid, England) were prepared following the manufacturer's instructions and allowed to solidify. The samples were inoculated onto the agar plates and incubated at 37°C for 24 h. Incubation period was extended to 48 h if there was no bacterial growth within 24 h. Investigations such as characteristics, Gram stain and biochemical reactions of the organisms were carried out in line with standard operating procedures. Identification and biochemical testing of isolates were carried out following standard procedures (Cheesbrough, 2012).

Antimicrobial susceptibility test

Kirby-Bauer disc diffusion susceptibility technique as documented by Isenberg et al. (2011) was adopted for the susceptibility assay. Only *P. aeruginosa* and *S. aureus* isolates obtained were used for this assay. In this technique, a well dried agar plate was seeded with appropriate inoculum. Filter paper discs impregnated with various antibiotics were placed at specific locations on the seeded agar plate. The plates were incubated at 37°C for 18 hrafter which susceptibility to antimicrobial agents was measured in millimeter as zones of inhibition, around the antibiotic discs.

RESULTS

With respect to all the hospitals where samples were processed, 170 (56.7%) bacteria were isolated, with *P. aeruginosa* having the highest 61 (20.3%) followed by *S. aureus* 39(13.0%), *E. faecalis* and *S. pyogenes* the least

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Table 1. Prevalence of bacterial isolates in 300 post-operative wounds examined in Benue State.

Organism	Total number isolated	%
Pseudomonas aeruginosa	61	20.3
Staphylococcus aureus	39	13.0
Klebsiella species	12	4.0
Escherichia coli	10	3.3
Atypical *coliform*	8	2.7
Proteus species	7	2.3
Enterococcus faecalis	4	1.3
Streptococcus pyogenes	4	1.3
Co-infection (*S. aureus* and *P. aeruginosa*)	25	8.3
Total	170	56.7

Table 2. Prevalence of *pseudomonas aeruginosa* and *staphylococcus aureus* in wounds according to type of surgical operation.

Types of operation	Number examined	Number (%)	
		P. aeruginosa	***S. aureus***
Appendicetomy	122	36 (12.0)	24 (8.0)
Caesarean section(c/s)	69	19 (6.3)	15 (5.0)
Herniotomy	63	17 (5.7)	12 (4.0)
Amputation	14	6 (2.0)	5 (1.7)
Cystostomy	11	4 (1.3)	2 (0.7)
Leparatomy	7	2 (0.7)	3 (1.0)
Mastectomy	6	1 (0.3)	2 (0.7)
Prostatectomy	3	1 (0.3)	1 (0.3)
Osteotomy	2	0 (0)	0 (0)
Colostomy	1	0 (0)	0 (0)
Gastrectomy	1	0 (0)	0 (0)
Thyroidectomy	1	0 (0)	0 (0)
Total	300	86 (28.7)	64 (21.3)

4(1.3%) respectively while co-infection of *P. aeruginosa* and *S. aureus* was 25 (8.3%) (Table 1).

Table 2 reveals that appendicetomy has 12and 8% of *P. aeruginosa* and *S. aureus* respectively according to type of surgery while Prostatectomy the least with prevalence of 0.3% for both *pseudomonas* aeruginosa and *S. aureus*. Coinfection was highest in appendicetomy (3.7%) and lowest in amputation (0.3%), mastectomy (0.3%) and prostatectomy (0.3%) (Table 3).

Among the health facilities investigated, FMC has the highest incidence of *P. aeruginosa* (11.0%) with GHO the least (4.7%) (Table 4). Similarly, *S. aureus* has 8.0% prevalence in FMCM while both GHO and GHNBM were lowest (4.0%). The rate of co infection in the various hospitals was more at FMCM (3.0%) with General hospitals Otukpo coming last (1.3%) (Table 5).

Table 6 indicated the number of males and females infected with *P. aeruginosa* as 15.7 and 13.0% respectively. In contrast, the rates of *S. aureus* infection in males were 9.7% and females 11.6%. Table 7 shows the distribution of *P. aeruginosa* and *S. aureus* infections according to age group with both organisms showing higher occurrence of infections in the young people compare to the elderly. Tables 8 and 9 also show the occurrence of co-infections of both organisms in age and sex of patients respectively with female having a higher prevalence of 4.6% and male 3.7%. Table 10 gave the distribution of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in various hospitals in relation to age of patients with FMCM having the highest prevalence of both organisms when compared to the other hospitals.

DISCUSSION

The results of this study show that the prevalence of *P. aeruginosa* (20.3%) and *S. aureus* (13.0%) post-operative wound infections differed. This finding agrees

Table 3. Co-Infections of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in different types of surgical operation.

Types of operation	Number examined	Number (%) of co-infection
Appendicetomy	122	11(3.7)
Caesarean section (c/s)	69	5(1.7)
Herniotomy	63	4(1.3)
Amputation	14	1(0.3)
Cystostomy	11	0(0)
Leparatomy	7	2(0.7)
Mastectomy	6	1(0.3)
Prostatectomy	3	1(0.3)
Osteotomy	2	0(0)
Colostomy	1	0(0)
Gastrectomy	1	0(0)
Thyroidectomy	1	0(0)
Total	300	25(8.3)

Table 4. Prevalence of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in relation to health facilities.

Organism	Frequency (%)				
	FMCM	GHG	GHO	GHNBM	Total
*P. aeruginosa**	33(11.0)	24(8.0)	14(4.7)	15(5.0)	86(28.7)
*S. aureus***	24(8.0)	16(5.3)	12(4.0)	12(4.0)	64(21.3)

*χ^2 = 5.028 (p>0.05); **χ^2 = 1.344 (p>0.05).

Table 5. Prevalence OF *Pseudomonas aeruginosa* and *Staphylococcus aureus* CO-infection in relation to health facilities.

Parameter	FMCM	GHG	GHO	GHNBM	Total
Number examined	99	70	68	63	300
Number co-infected (%)	9 (3.0)	7 (2.3)	4 (1.3)	5 (1.7)	25 (8.3)

χ^2= 0.8773 (p>0.05)

Table 6. Prevalence of *pseudomonas aeruginosa* and *Staphylococcus aureus* according to sex of patients.

Sex	Number examined	Number (%)	
		*P. aeruginosa**	*S. aureus***
Male	156	47 (15.7)	29 (9.7)
Female	144	39 (13.0)	35 (11.6)
Total	300	86 (28.7)	64 (21.3)

*χ^2= 0.3395(p>0.05); **χ^2= 1.458(p>0.05).

with those of Dantas et al. (2013) (18.5%) in Karacchi city of Pakistan, Akinjogunla et al. (2010) (19.7%) in Calabar, and Anjum et al. 2010 (14.3%) in Eastern Nigeria. The rates of *Pseudomonas aeruginosa* and *Staphylococcus*

Table 7. Prevalence of *Pseudomonas aeruginosa* and *Staphylococcus aureus* infections according to age group.

Age group	Number examined	Number %	
		***P. aeruginosa**	***S. aureus***
10-19	74	24(8.0)	14(4.7)
20-29	76	19(6.3)	16(5.3)
30-39	64	12(4.0)	14(4.7)
40-49	27	7(2.3)	7(2.3)
50-59	25	13(4.3)	5(1.7)
≥ 60	34	11(3.7)	8(2.7)
Total	300	86(28.7)	64(21.3)

*X^2 = 0.5040; (p>0.05); **χ^2 =1.110; (p>0.05).

Table 8. Co-infection of *Pseudomonas aeruginosa* and *Staphylococcus aureus* among age group.

Age group	Number examined	Number co-infected (%)
10-19	74	6(2.0)
20-29	76	7(2.3)
30-39	64	3(1.0)
40-49	27	2(0.7)
50-59	25	3(1.0)
≥ 60	34	4(1.3)
Total	300	25(8.3)

χ^2 = 2.865; (p>0.05)

Table 9. Co-infection of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in relation to sex of patients.

Sex	Number examined	Number co-infected (%)
Male	156	11 (3.7)
Female	144	14 (4.6)
Total	300	25 (8.3)

χ^2= 0.6993; (p>0.05)

aureus wound infections reported in their results fall within the range obtained in this study. The rate of infection obtained in this study were however higher than (10.5%) reported by Joshi et al. (2011) in Benin City and (8.6%) reported by Shittu et al. (2012) in south west Nigeria. The disparity in infection rate could be attributed to differences in geographical location and possible differences in hygienic practices. Other scientists have obtained increasing prevalence of *P. aeruginosa* and *S. aureus* in post-operative wound infections especially in recent years. It is thus clear that the prevalence of *P. aeruginosa* and *S. aureus* obtained in this study is in agreement with what is obtained in other hospitals in Nigeria.

The microbial analysis revealed that *P. aeruginosa* and *S. aureus* were the leading etiologic agents of post-operative infection in this study. Similar results were obtained by et al. (1992) in Bombay town of India, Konno (2011) and Akinjogunla et al. (2010). The virulence of the microorganisms may be responsible for their high infection rates as suggested by Coffin et al. (2011).

The rate of *P. aeruginosa* and *S. aureus* were higher at Federal Medical Centre Makurdi than in other hospitals. In Federal Medical Centre, patients on admission stay long in the overcrowded wards, and are therefore exposed to cross infections

The prevalent rate of *P. aeruginosa* was higher than *S. aureus* in all the hospitals and this finding agrees with

Table 10. Distribution of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in relation to age of patients in various hospitals.

Age group	FMCM			GHB			GHO			GHNBM		
	No. exam.	No. (%) *P.aeru.**	No. (%) *S.aur.***	No. exam.	No. (%) *P.aeru**	No. (%) *S.aur***	No. exam.	No. (%) *P.aeru**	No. (%) *S.aur***	No. exam.	No. (%) *P.aeru.**	No. (%) *S.aur.***
10-19	23	8 (2.7)	5 (1.7)	15	6 (2.0)	4 (1.2)	18	5 (1.7)	2 (0.7)	18	5 (1.7)	3 (1.0)
20-29	22	7 (2.3)	5 (1.7)	19	7 (2.3)	5 (1.7)	19	2 (0.7)	3 (1.0)	16	3 (1.0)	3 (1.0)
30-39	18	6 (2.0)	6 (2.0)	15	3 (1.0)	3 (1.0)	17	2 (0.7)	3 (1.0)	14	2 (0.7)	2 (0.7)
40-49	9	1 (0.3)	2 (0.7)	5	1 (0.3)	1 (0.3)	6	2 (0.7)	2 (0.7)	7	2 (0.7)	2 (0.7)
50-59	11	5 (1.7)	2 (0.7)	6	4 (1.2)	1 (0.3)	4	2 (0.7)	1 (0.3)	4	1 (0.3)	1 (0.3)
60 and above	16	6 (2.0)	4 (1.2)	10	3 (1.0)	2 (0.7)	4	1 (0.3)	1 (0.3)	4	1 (0.3)	1 (0.3)
Total	99	33 (11.0)	24 (8.0)	70	24 (8.0)	16 (5.3)	68	14 (4.7)	12 (4.0)	63	15 (5.0)	12 (4.0)

*χ^2=10.525 (p>0.05); **χ^2=4.933 (p>0-05). FMCM = Federal Medical Centre Makurdi; GHG = General Hospital Gboko; GHO = General Hospital Otukpo; GHNBM= General Hospital North Bank Makurdi. *P.aeru =Pseudomonas aeruginosa*; *S.aur* = *Staphylococcus aureus*; No. = Number; exam. = examined

the reports of Joshi et al. (2011) and Cheadle W (2010). In another study by Prinsloo et al. (2010) and Burke (2012), *P. aeruginosa* was reported to be responsible for most nosocomial infections. This could be as a result of its ability to grow in disinfectants, sinks, water and other materials in the hospitals. It is also possible that patients may have developed immunity to *S. aureus* infection but this assumption contradicts the report of Johnson et al. (2013) where patients were more infected with *S. aureus* than *P. aerginosa* infection.

Incidence of *P. aeruginosa* was higher in males (except in General hospital Gboko) than females who were more infected mostly with *S. aureus* (except in General hospital Otukpo). This result is consistent with the reports of Kolmos et al. (2013) and Dulworth and Pyenson (2012), but contrary to that of Church et al. (2010) in which females were more infected with *P. aeruginosa*. It is possible that there are differences in hygienic practice of both males and females including the hospitals environment.

Patients within the age groups 10-19 years, 50-59 years and ≥60 years are at the highest risk of infections. Maltezou et al. (2012) in southern Uganda had reported that the age groups 10-19 years and ≥50 years were the most infected. According to Joshi et al. (2011) in south east Nigeria, children less than 13 years old were infected with post-operative wound infections whcich is also common in other parts of the world. Our results also agree with findings of Dantas et al. (2013) that infections were more common among the young and debilitated elderly people. From this study also, the age group 30-39 years had the least rate of infection in most of the hospitals. This may be due to good hygienic practices and avoidance of cross-infections exhibited by these groups of patients.

The rate of co-infection of *P. aeruginosa* and *S. aureus* according to health facility, gender and age differs but was not statistically significant. This result implies that co-infection is not influenced by these factors.

Chi square analysis at 99% confidence limit did not show any significant difference in the number of organisms isolated from the four hospitals.

The susceptibility rate of *P. aeruginosa* and *S. aureus* isolates to the eight antibiotics tested *in vitro* were relatively low compared to the sensitivity pattern to different anti pseudomonal and staphylococcal drugs reported worldwide (Haghi et al., 2010). In this study, *P. aeruginosa* isolates was highly susceptible *to* levoxin (97.7%) followed by ciprocin (81.4%) and norbactin (70.9%) (Table 11), while *S.aureus* isolates was also highly susceptible to levoxin (98.4%), ciprocin (93.8%), and norbactin (81.3%) (Table 12). Other drugs showed very low percentage of susceptibility. The non-hygienic measures in hospitals, the ability of some bacteria to grow in hospital materials or indiscriminate use of antibiotics, fake drugs, and self-prescription among patients are favourable conditions which overtime encourages the development of antibiotic resistant bacteria.

The isolates were completely resistant to three of the antibiotics (ampicillin, tetracycline and streptomycin) tested *in vitro*, which is much higher compared to a Belgian study (Prinsloo et al., 2010) but lower than the Turkish study where one third of the isolates were multidrug resistant. This could be due to misuse of these drugs without running sensitivity tests thereby resulting to development of resistant organisms.

The prevalence and sensitivity of *P. aeruginosa* and *S. aureus* often varies between communities, hospitals in the same community and among different patient populations in the same hospital (Kolmos et al., 2013). Faced with these variations, the physician in clinical practice has the responsibility of making clinical judgments, and should have access to recent data on the prevalence and antimicrobial resistance pattern of commonly encountered pathogens. It is therefore important to institute a system for the surveillance of antimicrobial resistance that will involve the clinical collection of

Table 11. Susceptibility pattern of *Pseudomonas aeruginosa* isolates to common antibiotics.

Antibiotics	No. of isolates sensitive (%)	No. of isolates resistant (%)
Ampicillin	0(0)	86(100)
Gentamycin	34(39.5)	52(60.5)
Colistin	47(54.7)	39(45.3)
Streptomycin	0(0)	86(100)
Tetracycline	0(0)	86(100)
Levoxin	84(97.7)	2(2.3)
Norbactin	61(70.9)	25(29.1)
Ciprocin	71(81.4)	16(18.6)

The concentration of each antibiotic was 10µg. The number of isolates tested against each antibiotic was 86.

Table 12. Susceptibility pattern of *staphylococcus aureus* isolates to common antibiotics.

Antibiotics	No. of isolates sensitive (%)	No. of isolates resistant (%)
Ampicillin	10(15.6)	54(84.4)
Gentamycin	31(48.4)	33(51.6)
Colistin	43(67.2)	21(32.8)
Streptomycin	25(39.1)	39(60.9)
Tetracycline	0(0)	64(100)
Levoxin	63(98.4)	1(1.6)
Norbactin	52(81.3)	12(18.8)
Ciprocin	60(93.8)	4(6.0)

The concentration of each antibiotic was 10 µg. The number of isolates tested against each antibiotic was 64.

microbiological data. Shittu et al. (2012) found that patients were the sources of bacteria in all cases of wound infection and that increase in post-operative infections was due to high penicillin resistant carrier rate in hospital personnel and patients as a result of widespread use of Penicillin. However, Kolmos et al. (2013) reported that cleaners and patients were the major source of wound contamination.

The high incidence of *P. aeruginosa* and *S. aureus* may be related to indiscriminate use of antibiotics without laboratory diagnosis and antibiotic sensitivity report. This single factor could eliminate the normal flora and provide a non-competitive environment for *P. aeruginosa* and *Staphylococcus aureus* to occur. The resistance of the organism to antimicrobial agents, nutritional versatility and the difficulties encountered in maintaining proper hygienic standards especially among personnel involved in wound dressing and general care of patients may have contributed to the high rate of *P. aeruginosa* and *S. aureus* infections.

Conflict of interest

The authors have not declared any conflict of interests.

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Full Length Research Paper

Isolation and identification of lactic acid and non-acid lactic bacteria from *"dèguè"* of Western Africa traditional fermented millet-based food

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Déguè **is a traditional fermented millet-based food which is consumed in Burkina Faso and other countries of West Africa. A total of 125 strains of bacteria were selected from 16 samples of *déguè*. Isolates were studied by determination of morphological and biochemical characteristics. Among the 125 strains of lactic acid bacteria selected, 68 strains were identified as *Lactobacillus* and 57 strains as *Lactococcus.* The representative species of the *Lactobacillus* were: *Lactobacillus plantarum* (25.6%), *Lactobacillus delbrueckii subsp delbrueckii* (11.2%), *Lactobacillus acidophilus* (7.2%), *Lactobacillus brevis* (4.8%), *Lactobacillus buchneri* (8%). *Lactobacillus cellobiosus* (4%), *Lactobacillus pentosus* (2.4%), *Lactobacillus crispatus* (1.6%), *Lactobacillus fermentum* (1.6%), *Lactobacillus curvatus* (0.8%), *Lactobacillus paracasei subsp paracasei* (0.8%). Among the 57 strains of lactic acid coccus isolated predominated *Pediococcus damnosus* strains (14.4%), followed by *Lactococcus lactis subsp lactis* (6.4%), *Pediococcus pentosaceus* (3.2%), *Pediococcus* sp. (3.2%), *Leuconostoc lactis* (1.6%), *Leuconostoc mesenteroides subsp mesenteroides* (0.8%), *Pediococcus acidilactici* (0.8%), *Lactococcus curvatus* (0.8%) and *Tetragenococcus halophilus* (0.8%). Many Gram negative bacteria were also isolated as coliforms and proteolic strains that can play a negative contribution on the quality of *dèguè.***

Key words: Fermented millet, *dèguè*, *Lactobacillus* sp., *Enterobacteria.*

INTRODUCTION

The finger millet (*Eleusine corocana*) is an important food crop in arid and semi-arid regions of the world. In Burkina Faso Finger millet production represents a third of the total consumption of food cereals. Pearl millet *Pennisetum glaucum* is native to the tropical region of western Africa where it is meeting wide all the cultivated and wild varieties (Hama et al., 2009).

These crops are used in production of many traditional fermented products in African and Asian countries (Kumar et al., 2010). Africa is a source of production of

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traditional fermented foods and is perhaps the continent with the richest variety of lactic acid fermented foods (Franz et al., 2014).

Some varieties of pearl millet-based foods are produced and consumed in Burkina Faso like: cooked doughs (*tô, déguè*), steamed products (couscous), porridges (*binsalga*), fermented food and alcoholic beverages (*dolo*) (Hama et al., 2009). *Déguè,* made from pearl millet flour is consumed in most of the important cities in Burkina Faso: 32% in Ouagadougou and 25% in Bobo-Dioulasso (Hama et al., 2009).

During fermentation, microorganisms contribute to the development of characteristic properties such as taste, aroma, visual appearance, texture, shelf life and safety. The microbiota of fermented foods is dominated by lactic acid bacteria (LAB), which contribute to their nutritional and sanitary qualities (Nout and Motarjemi, 1997).

Lactic acid bacteria play an important role in traditional fermented foods consumed in different countries (Oguntoyinbo and Narbad, 2012).

The lactic acid fermentation is a process which not only improves the organoleptic and hygienic quality but also the nutritional quality in food; it allows especially the good preservation (Ampe et al., 1999). Other microorganisms as *Enterobacteriaceae*, yeasts and mould were isolated in a lot of based-cereals fermented foods and can influence their quality (Ashenahi, 1994).

LAB may have probiotic characteristics (Lei and Jakobsen, 2004). These bacteria first have to be selected for their ability to survive passage through the gastrointestinal tract.

Probiotic functions may also be associated with other functions that are of interest for nutrition. This is of particular interest for at-risk popula-tions such as pregnant women and young children in developing countries. For instance, the amylase activity of some LAB helps increase the energy content of gruels for the complementary feeding of young children through partial hydrolysis of starch in the food matrix (Songre-Ouattara et al., 2008) but also helps sustain the growth of the microbiota of starchy foods (Tou et al., 2006). Other functions for example, folate and riboflavin synthesis may improve the quality of the food matrix and may be beneficial for the host.

Folate deficiency can lead to neural tube defects, early spontaneous abortion, and megaloblastic anemia, while riboflavin deficiencies can result in growth failure, inflammation of the skin, or vision deterioration (Rohner et al., 2007).

LAB capable of producing B vitamins could be used for fortification of cereal-based foods (Iyer and Tomar, 2009) and as probiotics (Rossi et al., 2011). In this way, bacteria that combine different functional characteristics could be useful for developing improved or new foods made from local raw materials that target specific nutritional needs and health issues.

Many studies have focused on the phenotypic diversity of the LAB in the tropical fermented foods but few works were made on the *dèguè*, which plays nevertheless an important role in the food in the Burkina Faso.

The objective of this work was to isolate, characterize and identify some bacteria occurring in the *déguè* samples. This information could contribute to the better knowledge of the microbiota of this kind of food, and perhaps the development of starter cultures with predictable characteristics for use in small-scale and commercial production of *déguè*

MATERIALS AND METHODS

Sampling

Sixteen samples of « *déguè* » olded from one to three days were obtained from local households of Ouagadougou (Burkina-Faso). Samples were carried out in an ice box for microbial analysis in the Laboratory of Microbiology and Biotechnology (Department of Biochemistry and Microbiology, University of Ouagadougou).

Isolation of lactic acid bacteria (LAB)

The 10^{-1} dilution was made by diluting 10 g of each *dèguè* sample in 90 ml of sterile peptone saline water (10 g of peptone, 5 g of NaCl and 1000 ml of water). Further 10-fold serial dilution, ranging from 10^{-2} to 10^{-7} was done.

LAB were isolated in two media: Man, Rogosa and Sharpe (MRS) and Rogosa agar. Spreed-plated MRS and ROGOSA were incubated anaerobically using BBL Gas Pak plus Anaerobic System, Beckon Dickinson Microbiology System (Cockeysville, MD, USA) at 30°C for 48 h. Unit forming colonies were randomly picked from plates at higher dilution 10^{-6} and transferred into 10 ml in test tubes with sterile MRS broth. Pure cultures were made. The isolates were Gram-stained and tested for catalase and oxydase reaction (Harrigan and McCance, 1990). Presumptive LAB were selected based on the morphology, Gram reaction and the catalase test.

Isolation of total coliform (non-LAB)

Coliforms were isolated using the same dilutions, plated on Plate Count agar and Violet Red Bile Lactose Agar, and cultivated for 24-48 h at 37°C.

Characterization and identification of isolated LAB and non-LAB

The carbohydrate fermentation profiles of LAB isolates were investigated using API 50CH strips and API CHL medium according to manufacturer's instructions (API system, Bio-Merieux, France).

Phenotypical identification of non-LAB was done by the API 20 E gallery. Strains were designated to species using APILAB PLUS (Version 3.33, Bio-Merieux) and standard taxonomic descriptions.

RESULTS AND DISCUSSION

Morphological and biochemical characteristics of the LAB strains

All 125 selected isolates were Gram-positive, non -motile,

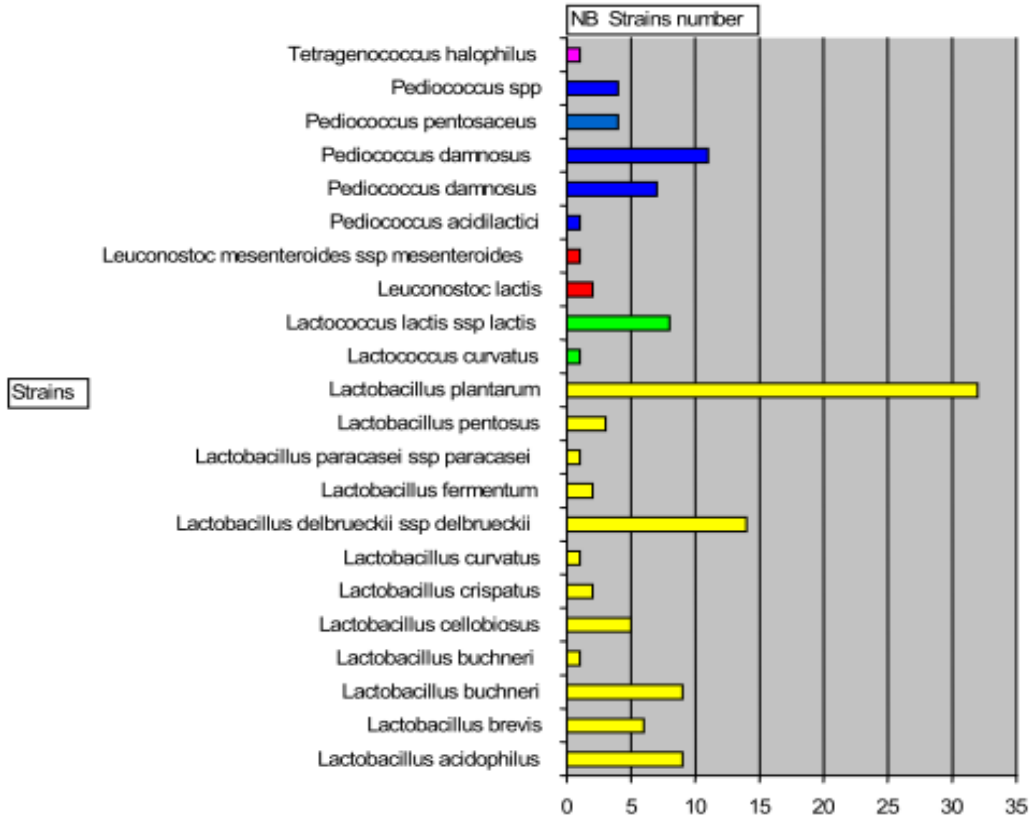

Figure 1. Identified LAB from *dèguè* by API 50.

catalase-oxydase negative and non-spore forming bacteria, they occurred in short rods and in cocci, singly, in pairs or short chains. These characteristics according to Holzapfel (1997) and Bergey's Manual of Systematic Bacteriology indicate lactic acid bacteria. The results are shown in Figure 1.

The LAB isolated were composed of five genera of *Lactobacillus* (68 % from 125 isolates), *Pediococcus* (21.6 %), Lactococcus (7.2 %), *Leuconostoc* (2.4 %), *Tetragenococcus* (0.8 %). *Lactobacillus* sp. was the predominated genera among the isolates.

The following species are involved in the fermentation of one kind of *dèguè*: *Lactobacillus acidophilus* (7.2%), *L. brevis* (4.8%), *L. buchneri (*8%), *L. cellobiosus* (4%), *L. crispatus* (1.6%), *L. curvatus* (0.8%), *L. delbrueckii subsp delbrueckii* (11.2%), *L. paracasei subsp paracasei* (0.8%), *L. fermentum* (1.6%), *L. pentosus* (2.4%), *L. plantarum* (25.6%), *L. curvatus* (0.8%), *L. lactis subsp lactis* (6.4%), *L. lactis* (1.6%), *Leuconostoc mesenteroides subsp. mesenteroides* (0.8%), *Pediococcus acidilactici* (0.8%), *Pediococcus damnosus* (14.4%), *Pediococcus pentosaceus* (3.2%), *Pediococcus spp* (3.2%) and *Tetragenococcus halophilus* (0.8%).

The species *L. plantarum* was the strain isolated in higher number (25.6%), from *dèguè* and is frequently isolated from traditional fermented foods made from cereals (Muyanja et al., 2003; Hama et al., 2009). Other species isolated were: *Pediococcus damnosus* (14.4%), *L. delbrueckii subsp delbrueckii* (11.2%), *L. buchneri (*8%), *L. acidophilus* (7.2%), *L. lactis subsp lactis* (6.4%), *L. brevis* (4.8%), *L. cellobiosus* (4%), *P. pentosaceus* (3.2%), *Pediococcus spp.* (3.2%), *L. pentosus* (2.4%), *L. crispatus* (1.6%), *L. fermentum* (1.6%), *L. lactis* (1.6%), *L. curvatus* (0.8%), *L. paracasei subsp paracasei* (0.8%), *L. curvatus* (0.8%), *L. mesenteroides subsp mesenteroides* (0.8%), *P. acidilactici* (0.8%) and *T. halophilus* (0.8%).

Many of these species were isolated from other fermented foods as *bushera* (an Ugandan traditional fermented drink made from *Sorghum*), *ben-saalga* (a traditional porridge of Burkina) Cassava, *fufu* and *ogi*, fermented doughs made from corn, etc. (Corsetti et al., 2003; Miambi et al., 2003; Tou et al., 2006).

L. plantarum (25.6%) was more frequently isolated than other species of LAB in household *dèguè*.

It has been observed that the presence of *L. plantarum* in the cheese (Cameros) from goat's milk decreased the number of fecal coliforms and other *enterobacteria* in the final product. Indeed LAB starters can contribute to reducing spoilage problems encountered in this domestic fermentation.

Using Api 50 CHL galleries allowed us to find certain strains capable to ferment some complex sugars. For

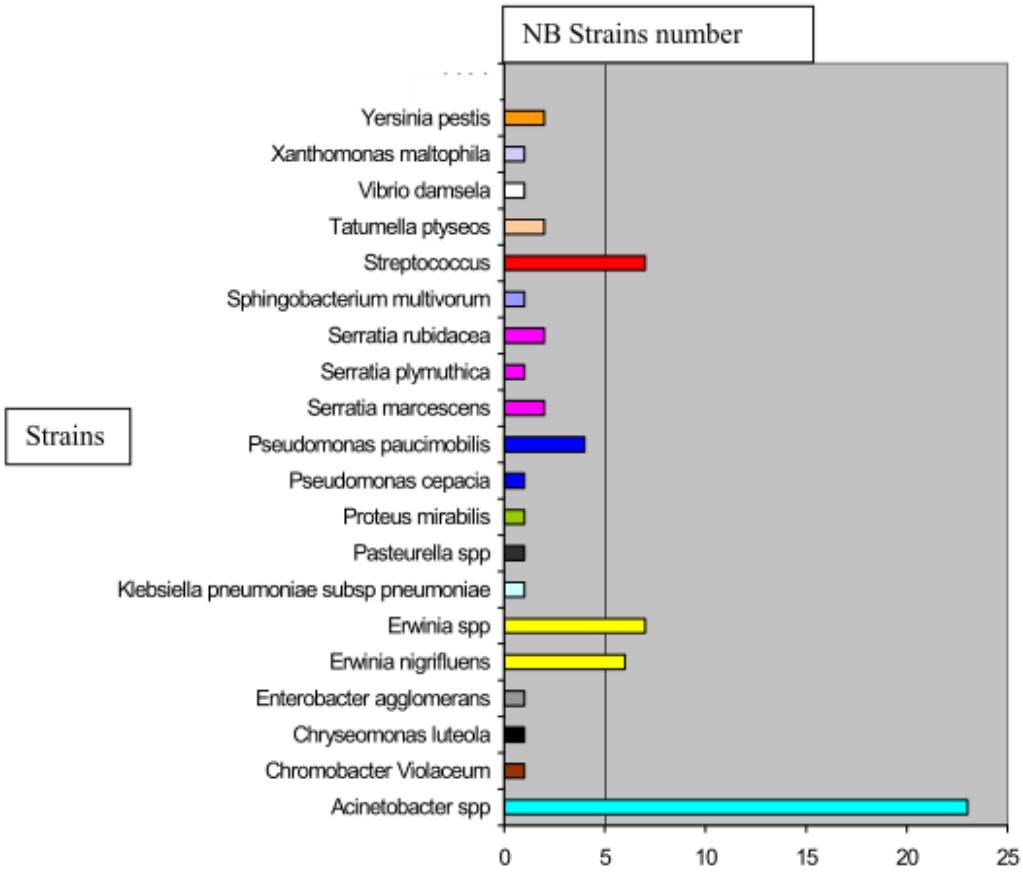

Figure 2. Identified non-LAB by API 20 E.

example, 46 amylolytic lactic acid bacteria *L. plantarum* (21 strains), *L. delbrueckii ssp delbrueckii* (5), *L. lactis ssp lactis* (4), *L. cellobiosus* (3), *L. acidophilus* (3), *L. pentosus* (3), *L. buchneri* (3), *L. fermentum* (2) and *L. crispatus* (2). Amylolytic LAB contributes in pH reduction in the medium inhibiting the growth of some pathogenic microorganisms such some faecal coliforms.

Certain strains isolated presented a potential to ferment raffinose by hydolysing α-galactosidic bonds: *L. pentosus* (2 isolates), *L. buchneri* (8 isolates), and *L. plantarum* (26 isolates). Indeed, raffinose is oligosaccharide that typically occurs in legumes and cereals, and cause flatulence, diarrhoea and indigestion in humans.

Phenotypical identification of non-LAB by the API 20 E gallery

The results of non-LAB are shown in Figure 2. On the total of non-LAB isolates of the majority (39.39%) of the strains belong to the family of *Enterobacteriacaeae.* The following species were identified: *Erwinia spp.* (10.07%), *Erwinia nigrifluens* (10.07%), *Serratia marcescens* (3.03%), *Serratia rubidacea* (3.03%), *Serratia plymuthica* (1.51%), *Tatumella ptyseos,* (3.03%), *Enterobacter agglomerans* (1.51%), *Proteus mirabilis* (1.51%), *Klebsiella pneumonia subsp pneumoniae* (1.51%).

The fecal streptococci isolated from the *dèguè* and identified by APILAB PLUS represent 10.60% of non-LAB isolates. The presence of fecal streptococci shows fecal contamination origin of samples. That can be explained by the lack adequate hygiene.

Other strains of non-LAB were identified: 31.81% of isolates are represented by *Acinetobacter* genus. *Pseudomonas* represents 7.57% of isolates; two species were identified: *Pseudomonas paucimobilis* (6.06%) and *Pseudomonas cepacia* (1.51%).

Acinetobacter and *Pseudomonas* are characterized by significant proteolytic and lipolytic activities. The proteolysis leads to the formation of free amino acids then to decarboxylation or desamination reactions. The volatile amines and ammonia formed are responsible of unpleasant odors and savors in food. Lipolysis leads to the release of free fatty acids modifying the gustatory properties and leading to rancid taste.

The following species were also isolated: *Flavimonas oryzihabitans* (1.51%), *Vibrio damsela* (1.51%), *Chromobacter violaceum* (1.51%), *Xanthomonas*

maltophilia (1.51%), *Pasteurella* spp. (1.51%), *Sphingobacterium multivorum* (1.51%) and *Chryseomonas luteola* (1.51 %). The presence of *E. coli* and other coliforms in the samples indicate that manipulations of these foods were not made in good sanitary conditions.

Conclusion

This study brings out that *dèguè* contain several microorganisms, lactic acid bacteria and non-lactic acid bacteria. *L. plantarum* was the lactic acid bacteria species isolated in higher numbers. Many proteolitic species was observed and could influence the quality of the product.

Author's contributions

The present study was carried out in collaboration with all authors. All authors participated in drafting and revising the manuscript. They also read and approved the final manuscript.

Conflict of interests

The authors did not declare any conflict of interest.

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African Journal of Microbiology Research

Full Length Research Paper

In vitro evaluation of antifungal activity and interactive effect of *Anadenanthera colubrina* (Benth)

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***Anadenanthera colubrina* (Benth) Brenan, a plant known in the Northeastern Region of Brazil as angico, is widely used in traditional folk medicine to treat respiratory and inflammatory diseases. This study aimed to evaluate the antifungal activity, determine the minimum inhibitory concentration (MIC), the minimum fungicidal concentration and the fungal kinetics (death curve) in addition to the interactive effect of the dry extract of angico in association with the antifungals fluconazole and nystatin against yeasts of the genus *Candida*. The dry extract was obtained by rotoevaporation. Tests for evaluation of antifungal activity, determination of the MIC and the MFC as well as the evaluation of the interactive effect with conventional antifungal were done by disk diffusion and microdilution technique. For the evaluation of the angico's effect on fungal growth, death curve was utilized. The results show the angico's antifungal potential in all of the strains tested, having MIC of 1.0 mg/mL. It was observed that the fungal kinetics of 2x MIC, MIC and ½ MIC had similar effects; 6 h was their best time after incubation. There was fungistatic activity reduction (2 log 10 UFC/mL) from the initial inoculum of 1.0 mg/mL. Interactive effect was not observed when used in association with nystatin, but showed synergistic effect when used with fluconazole. In these data, one can see that angico is a species rich in biological activity; being promising species, the isolation and detection of its bioactive compounds is necessary.**

Key words: Angico, *Candida albicans*, natural product.

INTRODUCTION

The use of natural products by mankind is as ancient as his own history. During the evolution process that occurred on Earth, the vegetable kingdom always filled an important place, as it is used for food and therapeutic

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purposes (Arruda et al., 2006). From individual and community observation of nature, with the utilization of animals and plants with medicinal purposes, the primitive man tried out and used several biologically active species. That followed him since prehistory and evolved throughout the years to compose the primitive man's medicine (Coutinho et al., 2004). Folk medicine is a tradition, where individuals pass information to each other, throughout several generations. This shared knowledge, over time, is the main cause of the diversity in folk medicine (Mahmood et al., 2013). Since 4000 a.C., several historical records were found related to the use of plants for treatment of diseases. The first medical record kept on a Pennsylvania museum dated back to 2100 a.C. and includes a collection of 30 different formulae of medicines with vegetal, animal or mineral components (Duarte, 2006). In 1875, a manuscript found by Georg Ebers in Egypt, named "Ebers Papirus" (1500 a.C.), contains 811 prescriptions and 700 drugs. In China, the first text about medicinal plants (500 a.C.) reports names, doses and guidelines on the use of plants for disease treatments. Some of those plants are still used, such as Ginseng (*Panax*spp), *Ephedra* spp., *Cassia* spp. and *Rheum palmatum L.*, as sources to the pharmaceutical industry (Arruda et al., 2006). Nowadays, approximately 80% of the population in least developed and developing countries rely on plants as a first option to their primary health needs; also, the use of plants as a source of medicines prevails in developing countries as an alternative solution to their health problems (Pilla et al., 2006).

The use of herbal medicines in Brazil is a strong economic alternative in relation to allopathic medicines. It is as a result of the indigenous culture's influence, African traditions and European culture brought by the colonizers (Almeida, 2000). With precarious economic conditions, as most times were allied to the large usage of medicinal plants, a wide commerce of those plants emerged in several brazilian regions. In some of these regions, this commerce is the main financial support to a lot of families, which allows them to continue living in their community and moving to bigger cities (Alves et al., 2007; Almeida et al., 2009).

A great extent of drugs applied in therapeutics come directly or indirectly from natural sources, especially from medicinal plants, that remain an important source to obtain drugs (Carvalho et al., 2007). Angico is a big tree; it has a winding and medial stem and its bark varies from smooth and light to rugged and dark. It is found mainly in high and well drained grounds. It flourishes between September and November with almost no leaves; while the fruit matures from August to September (Lorenzi and Matos, 2002). In folk medicine, angico, when prepared as syrups, is used for cough treatments, pertussis and bronchitis. The maceration of its bark is used for the treatment of inflammation and leukorrhea. When it is prepared with alcohol or cachaça, it is used on external wounds, having hemostatic and healing effects (Matos, 1997; Palmeira et al., 2010). The tear of its bark releases a resin used to treat skin infections (Mors et al., 2000). However, its fruit is considered poisonous, making it impossible to use it for folk medicine (Agra, 1996).

The antimicrobial activity of its plant is, probably, caused by the presence of flavonoids, tannins and terpenes in its leves and fruits. The flavonoids are complex with the bacterial cell wall, resulting in the rupture of the cell wall (Reginatto et al., 2001). The occurrence of fungal infections, dermatomycosis in particular in recent years, has shown a significant increase. In the meantime, this fact may be related to improvements in clinical and laboratory diagnosis, and the increased survival of patients immunocompromised beyond the use of immunosuppressive drugs, which in some cases are misused and may favor installation of microorganisms (Fenner et al., 2006). The fungi that cause these infections, usually, are the dermatophytes (*Epidermophyton, Microsporum, Trichophyton*) and yeasts; among them are *Cryptococcus neoformans* and *Candida albicans*. There are many *Candida* genus species that are able to colonize the skin and human mucosal surfaces (Hassan et al., 2009). This genus is composed of microorganisms, usually opportunistic; however they may cause local or systemic infections on a predisposed person. They affect immunocompromised patients frequently, mainly those going through long antibiotic therapy, chemotheray or even newborns (Samaranayake and Hanes, 2011).

Candida genus is also related to several cases of invasive infections, hence previous colonization of the skin and oral, intestinal and vaginal mucosa by these kind of species is considered an important factor for developing invasive infections; also it is a growing concern in brazilian and worldwide hospitals (Holzheimer and Dralle, 2002). It is relevant to mention that invasive fungal infections are related, among other factors, to high morbidity and mortality rates, difficulties to diagnose some diseases, resistance to antimicrobials and increase of hospitalization time and costs. It is also important to mention that different fungal species may infect humans, animals and plants (Zacchino, 2001). Among the most common antifungals, it is important to emphasize amphotericin B, which is considered as the main drug for treating most fungal infections. However, its use is restricted due to some side effects, like its nephrotoxicity (Zardo and Mezzari, 2004; Lópes-Medrano et al., 2005); nystatin, which presents similar spectrum and mechanisms of action to amphotericin B. However, it is highly toxic when used as injectable formulations (Martinez, 2006). Fluconazole has advantage over amphotericin B for presenting excellent gastrointestinal absorption and distribution in the body (Boucher et al., 2004; Bicanic and Harrison, 2014). The objective of this article was to evaluate the *in vitro* biological activity of angico's dry extract and to determine its fungal activity

through assessing concentrations of minimum inhibitory (MIC), minimum fungicide (MFC), the fungal kinetics (FC) and its interaction with synthetic antifungals like *Candida albicans* ATCC® 76485 and ambulatorial lineages of *C. albicans*.

MATERIALS AND METHODS

Anadenanthera colubrina (Benth) Brenan belongs to the Fabaceae family and Mimosoideae subfamily; it is commonly known as angico; black, red, yellow and white angico; bravo, do campo, rajado, fava, jacaré, rosa, do mato, arapiraca, brincos de sagui, cambuí ferro, curupaí, guarapiraca, angico de casca, paricá, cebil and angico de cortume. It is known by the nutritional value of its seeds, proteins, carbohydrates and oils. It is also possible to detect other phytochemicals like lectins, protease and amylase inhibitors, toxins and secondary metabolites (Silva Filho et al., 2013).

Preparation of plant material

This plant was collected in September 2012 in the semi-arid region of Paraíba state, at Serra do Bodocongó located in Queimadas city (7º 22' 25" S, 35º 59' 32"W), at the same region of Borborema and micro region of the West Cariri. *A. colubrina* (Vell.) Brenan specimen, also known as *A. colubrina* (Benth) Brenan, is found at Herbário Manuel de Arruda Câmara (ACAM), located in the Universidade Estadual da Paraíba (UEPB), campus I, Campina Grande, Paraíba (nº 667/ACAM).

An hydroalcoholic extract was obtained from the plant's bark, with 80% alcohol, through maceration technique for 48 h. 10 mg of the plant and 25 ml of solvent proportion were used. Then, it was put in a rotaevaporator and, after, lyophilized.

Microorganisms used and inoculum preparation

For the antimicrobial activity screening, eight clinical strains of *C. albicans* were used (LM 11; LM 94; LM 15; LM 520; LM 14; LM 70; LM 17; LM 410), belonging to the Laboratório de Micologia da Universidade Federal da Paraíba, and a reference strain, *C. albicans* ATCC 76485. The isolated preparations were kept and stored in Ágar Sabouraud Dextrose (Difco®). For testing of the interactive effect the ATCC strain was only used. For the inoculum preparation, isolated colonies of new cultures (24 h) were selected, and with the aid of a inoculation loop, they were transferred to a tube containig 5 ml of NaCl; 0.85%. They were homogenized, comparing its turbidity with a 0.5 tube of the McFarland scale (1.5 x 10^8 UFC/mL).

Antifungal used

The selection of the antifungal discs: fluconazole (25 µg) and nystatin (100 UI) (Cefar®) was based on its use in human clinical medicine.

Determining the antimicrobial activity and the minimum inhibitory concentration (MIC)

Sterile microplates were used containing 96 wells with flat bottoms, where in each well was poured 0.1 ml of Sabouraud Dextrose (Difco®) broth. The plant extract was diluted in 40% alcohol (16 mg/mL-double concentration) and transferred to the first well. Serial dilutions were then performed to obtain concentrations between 8 and 0.015 mg/mL. Floconazole was used as the positive control and 40% alcohol was used as the negative control. Also sterility controls were performed from the culture medium and angico extract. Cell viability was observed from the inoculum (CLSI, 2008). The plates were incubated at 35/37°C for 24/48h, and the experiments were peformed in triplicate. Fungal viability was detected by adding 20 µL of resazurin (0.01%) in aqueous solution. The plates were reincubated at 35°C for 2 h, and in those wells where fungal growth occurred the resazurin changed to pink. MIC was defined as the lowest concentration of antibacterial agents that inhibited visible growth, as indicated by resazurin staining. The minimum fungicidal concentration was defined as one that prevented the growth of the microorganism, being revealed after sowing.

Fungal kinetic

For this test, the fungal inoculum containing about 10^6 CFU/mL, of Sabouraud Dextrose (Difco®) broth was standardized. The angico's extract was used at three different concentrations: 2x MIC, MIC and 1/2 MIC, 2, 1 mg/mL and 0.5 mg/ml respectively. The loss of cell viability was noticed through the decrease of CFU/ml number, at intervals of 0, 2, 4, 6h, 8h, 10h and 24 h of exposure. An aliquot of 10 uL of test tubes containing the solutions was withdrawn and uniformly seeded on the surface of Petri dishes containing Sabouraud Dextrose Agar. The plates were incubated for 48h at 37°C. The curves were constructed by plotting the mean colony count (log10UFC / mL) versus time of incubation (hours).

Angico's interaction with synthetic antifungals

The analysis of the angico's lyophilized extract interference over effectiveness of antifungal was performed by disk diffusion. The fluconazole discs (25 µg, Cefar®) and nystatin (100 U.I. Cefar®) were soaked with the extract in the following concentrations: 8, 4, 2, 1 and 0.5 mg/mL. It was regarded as interactive effect when there was a change in diameter of the inhibition zones (halo) of microbial growth after this process and, as synergistic interactive effect, if the diameter of the inhibition zones is formed by combining the test product (P). The antifungal (AF) showed an increase of ≥ 2mm when compared to the inhibition zones formed by the AF tested alone. If the inhibition zone formed by the reciprocal activity (AF + P) showed a smaller diameter than the one formed by the AF isolated activity, an antagonistic effect was considered (Cleeland and Squires, 1991; Oliveira et al., 2006). These tests were performed in triplicate and the test's results were obtained by the average of the inhibition zones formed.

RESULTS

All the tested lineages showed sensitivity to nystatin and fluconazole. The inhibition formed opposite nystatin ranged between 23 and 27 mm (mean and SD 24.518 mm ± 0.939) while the inhibition halos in front of fluconazole ranged between 25 and 30 mm (mean and SD 27.592 mm ± 1.494). It was also noticed that the *A. colubrina* extract was active to these tested lineages; it presented halos of 8 mm.

Figure 1 shows the MIC and the minimum fungicide concentration (MFC) of the lineages of tested yeasts, by microdilution. There were the same results in all of them

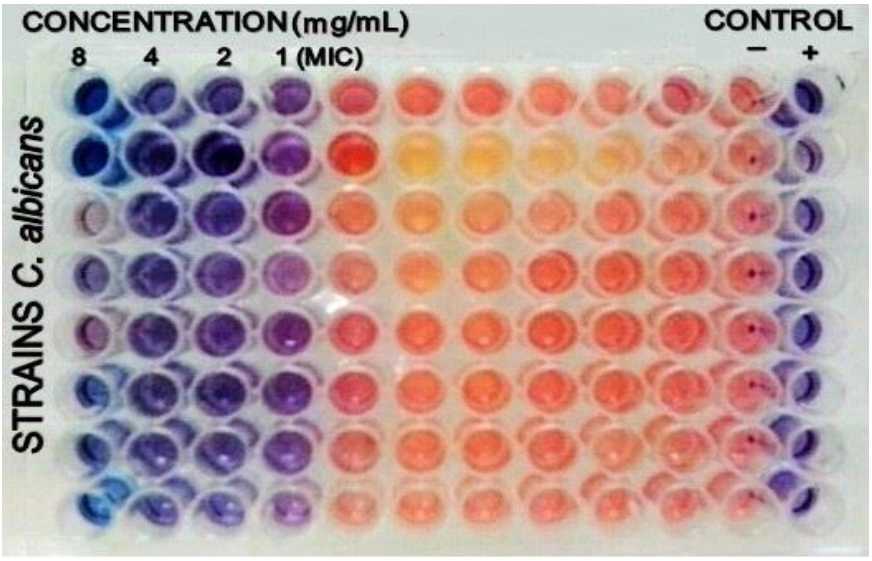

Figure 1. Determining the antifungal activity of the *Candida albicans* strains to the angico's extract.

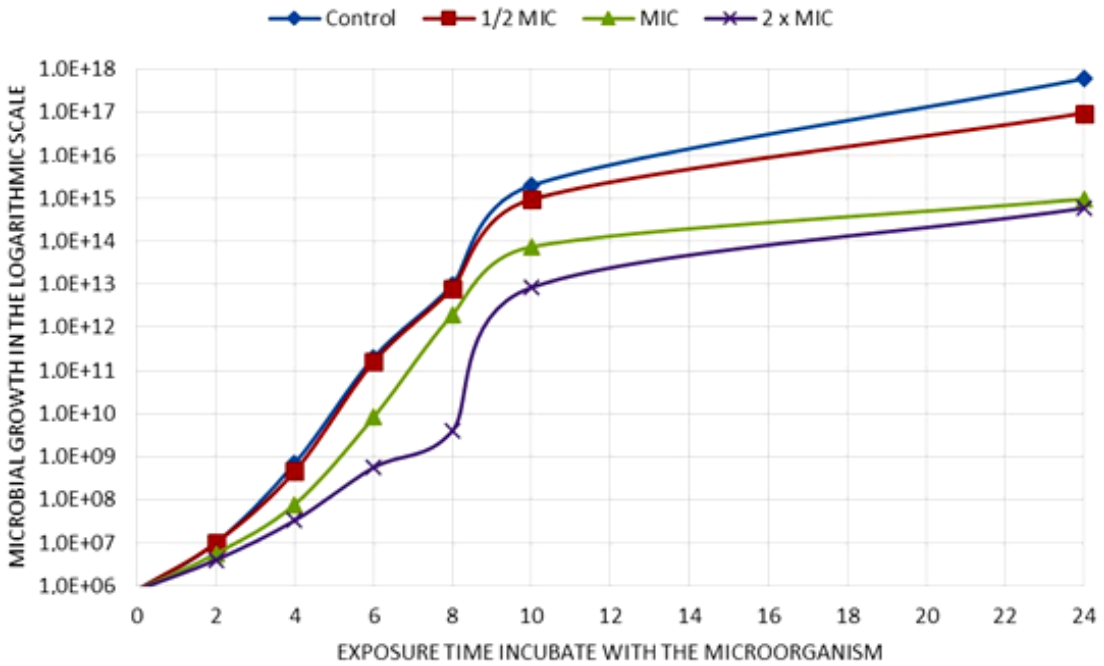

Figure 2. Effect of angico extract on fungal growth kinetics of *Candida albicans* ATCC® 76485. (Control SD± 2.27E+17; ½ MIC SD±3.58E+16; MIC SD±3.59E+14; 2x MIC SD±2.22E+14).

(1.0 and 2.0 mg/ml, respectively). MFC was equivalent to 2x MIC. The change of colours (from blue to pink) in the wells of the microtiter plate, after the addition of resazurin solution shows the decrease of this pigment and indicates the microbial viability. This means that in the wells that changed colours, the concentration of the product was not able to eliminate the yeasts. In the wells with change in colours, the resazurin was not decreased, showing the microbial infeasibility (Rolón et al., 2006). According to the obtained results, it was considered that were similarities in determining the MIC and MFC in the tested lineages.

Figure 2 shows the results of mean values of the angico's solution over *Candida albicans* ATCC® 76485

Table 1. Angico's combination with nystatin and fluconazole antifungals by disk diffusion over the *C. albicans* ATCC® 76485 strain.

Antifungals tested	Medium diameters of the growth inhibition zones (mm)					
	Antifungal isolated	Angico's combination with antifungals				
		8 mg/mL	4 mg/mL	2 mg/mL	1 mg/mL	0.5 mg/mL
Nystatin (100 U.I.)	25±1.414	27±1.224	27±1.871	27±1.224	27±1.414	27±1.702
Fluconazole (25 µg/mL)	30±1.632	30±0.707	30±1.414	30±2.121	30±0.707	30±2.449

strain. This shows the number of viable cells through the colony forming units (CFU/ml). When this microorganism was placed in the angico's extract at 2x MIC (2 mg/mL), MIC (1 mg/ml) and ½ MIC (0.5 mg/mL), there was a decrease in the cell multiplication rate in the first hours of its exposure to the growth control without adding the angico. This evidence is the highest rate of reduction of fungal growth 6 h after incubation.

The interactive effects evaluation of the angico and antifungal combination was performed using only *C. albicans* ATCC® 76485 strain. There was the presence of a synergistic effect with nystatin, proven by the increase of 2 mm on the diameter of the inhibition zone compared to the halo of the nystatin when tested alone. In relation to the effect of the angico and fluconazole combination, it was considered neutral because no changes in the inhibition zones were observed in none of the added angico concentrations (Table 1).

Analysis of basic descriptive statistics was performed to determine the average, minimum, maximum and standard deviation of each evaluation (antimicrobial activity, interactive effect and fungal kinetics). These parameters were separatly evaluated using the Microsoft Excel 2010 and were regarded as the percentage ratio between the standard deviation and arithmetic mean of the tests, under 10%.

DISCUSSION

Medicinal plants have been a rich source for obtaining molecules used therapeutically, since several isolated substances of plants continue to the source of medicines (Foglio et al., 2006; Rocha et al., 2013). The discovery of new drugs that originated from plants led to the isolation of many substances that, still nowadays, are clinicaly used as prototypes for the synthesis of new drugs.

The search for new products and/or drug combinations with antifungal activity is due to the increase of fungal infections worldwide, associated with various stages of immunodeficiency, found mainly in pacients with HIV or immunosuppressive therapies. It increases the number of antifungal prescriptions and favors the appearance of resistant strains, occurring due to the high interaction between the microorganism that causes the infection and antifungal administered (Rivera et al., 2013).

In this study resistant strain to fluconazole was found. The data are compatible with those reported by Castro and Lima (2011). However, there are discrepancies between the accounts of some authors that inform a high number of *Candida* sp. strains resistant to this antifungal (Marr et al., 2000). Other authors indicate that the resistence to fluconazole in *C. albicans* is around 3%, with slight regional variations (Wang et al., 2004; Quintero, 2010).

It is known that the resistant phenomenon is complex and multifactorial. Its mechanisms vary and have several different influences: direct inactivation of the molecule, reduced drug concentration, chemical structure, efflux pumps, as well as physiological changes (Pontón and Quindós, 2006).

The method of microdilution in plates is accurate and practical and it can be used to test microbial sensibility, simultaneously to different drugs. It can easily be used, on a large scale, by laboratories with few technological features. Other techniques such as disk diffusion, E-test, colorimetric methods are used; however, the broth dilution method is considered the standard practice due to its good reproducibility (Koga-Ito et al., 2008).

The results are significant, since the fungistatic activity of the angico's solution over the *C. albicans* was well characterized after 6 h of incubation, with decrease of 3 log 10 CFU/ml when compared to the inoculum of control. Somehow the angico reduced cell multiplication; however, the mode of action needs to be clarified. As Jones et al. (2002) and Shelburne et al. (2004) stated, the fungal kinetics of a product is considered substantially satisfactory if there is decrease in the values of the inoculum tested, compared to the initial inoculum of control; if there are equal or superior numbers of 2 and 3 log 10 CFU/ml, at incubation time of 24 h or less. These lesser degrees of cell death are considered as fungistatic effect. Therefore, according to these definitions, angico has fungistatic effect on the tested strains.

The angico's fungistatic *in vitro* effect observed in this study confirms reports by other authors (Moura et al., 2012), that noted the presence of antioxidant and fungistatic effects on an experimental diet based on angico.

The combined effect of angico extract with antifungals shows the synergistic effect exerted by the extract on interaction with fluconazole in *C. albicans*. According to

Bird et al. (2010), the efficacy of a crude extract may be due to the interplay between the different active constituents that may be present in the extract leading to better activity and/or decrease in potential toxicity of some individual constituents. In spite of that, plant derived antimicrobials are less potent, and plants fight infections successfully. Hence, it becomes apparent that plants adopt a synergistic mechanism between their compounds (Wagner and Ulrich-Merzenich, 2009).

Considering the resistance of yeasts belonging to the genus *Candida* to the main antifungal currently used, it is possible to assert that the search for new chemicals, especially from plants, is very important, as the development of new studies about the effect that these antifungal may cause when used with other products.

The proof of the angico's antibiotic potential *in vitro* and the possibility of this products use on the prevention and treatment of fungal infectious diseases caused by *C. albicans* suggest that toxicological and clinical studies are needed in order to safely determine the possible use of these products as medicine.

Conflict of interests

The authors did not declare any conflict of interest.

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