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Full Length Research Paper

Sero-prevalence of camel brucellosis (*Camelus dromedarius*) and phenotypic characteristics of *Brucella melitensis* biovar 3 in Shalateen City, Red Sea Governorate, Egypt

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The objective of this study was to estimate the sero-prevalence of brucellosis in camels in Shalateen city, Red sea Governorate. A total of 801 Sera were collected from apparently healthy dromedary camels from 2014 to 2015 spring. Sera were consequently serologically tested and confirmed using Rose Bengal plate test (RBPT), buffer acidified plate antigen test (BAPAT) and complement fixation test (CFT). 103 (12.90%), 93 (11.60%), and 92 (11.50%) were positive for RBPT, BABAT and CFT, respectively. Young camels were more sero-positive than old one (13.30 vs.10.80%). In addition, females were more sero-positive than males (19.10 vs. 7.10%). Moreover, *Brucella melitensis* biovar 3 was isolated from stomach content of aborted camel fetus. Statistically, the apparent prevalence (AP) was estimated to be 11.50%, while true prevalence (TP) was 13.60% (95% CI: 11.20 to 16%; P < 0.05). There was non statistical significant association between different age groups, while a highly significant difference were detected between seasons and genders. This study documented a high prevalence of camel brucellosis in the area of study and there is a need for planning and implementation of joint programs by stakeholders in prevention and control of the disease as well as raising public awareness in decreasing the distribution of the disease.

Key words: Camel brucellosis, Egypt, serological tests, sero-prevalence, Shalateen.

INTRODUCTION

Camel brucellosis is an insidious disease, since it hardly provokes any clinical signs (Musa and Shigidi, 2001). The disease is caused by *Brucella abortus* (*B. abortus*), *Brucella melitensis* (*B. melitensis*) and *Brucella ovis* (*B. ovis*) affecting mainly the dromedary camels (Seifert, 1996). In camels, the manifestation of the disease is mild or even asymptomatic with abortion if compared to cattle. So it may silently affect the reproductive performance of camels through low herd fertility and relatively low milk production (Gwida et al., 2012).

The disease can also have an impact on export and import of animals constraining livestock trade (Radostits et al., 2006). However, information about economic losses due to camel brucellosis is scarce. Although camels are not the primary host of *Brucella*, *B. abortus* and *B. melitensis* isolated from milk, aborted fetus, lymph nodes and vaginal swabs (Radwan et al., 1992; Gameel et al., 1993; Agab et al., 1994; Abou-Eisha, 2000; Hamdy and Amin, 2002; El-Gohary et al., 2016; El-Diasty et al., 2016; El-Hady et al., 2016). Disease transmission depends on *Brucella* spp. being prevalent in contact animals (Musa et al., 2008).

Brucellosis may spread from camels to humans, either through direct contact or via raw milk consumption especially in Arabian and African Countries (Cooper, 1991; Al-Juboori and Baker, 2012). The uncontrolled movement of camels from *Brucella* infected areas to *Brucella* free areas is consider the major obstacles in brucellosis eradication program (Radostits et al., 2006). Most of the reports addressed the seroprevalence of brucellosis in camels; this is not surprising due to the relative ease by which samples can be obtained and handled. The complement fixation test (CFT), is a recomended test for international trade as required by the World Organisation for Animal Health (OIE).

Serological diagnosis of brucellosis depend mainly on detection of IgG_1 immunoglobulin because most of cross reactive bacteria share the IgM antibody with *Brucella* species, also IgG_2 and IgA were inconstant and small in amount so, trails was made to eliminate IgM and to detect IgG_1 (Radostitis et al., 2006). Serological tests used for diagnosis of brucellosis in cattle may also be adequate for diagnosis of brucellosis in camels. However there is no validation for brucellosis serological test for camel sera done (Gwida et al., 2012). *B. melitensis* biovar 3 were isolated from camel stomach contents and swabs of lungs, livers, spleens of aborted fetuses and infected joint (Al-Majali et al., 2008; Musa et al., 2008).

Finally, it must be kept in mind that the serodiagnosis of brucellosis is additionally impaired by the allegedly strong cross-reactivity between *Brucella spp.* and *Yersinia enterocolitica* O:9 and other gram-negative bacteria (Emmerzaal et al., 2002). Therefore, the present study was aimed to determine the seroprevalence of brucellosis in dromedary camels imported from Sudan at Shalateen quarantine in Egypt.

MATERIALS AND METHODS

Study area

Shalateen is a town north of the Halayeb Triangle, Egypt. It is located 520 km south of Hurghada and serves as the administrative center of all Egyptian territory up to the border between Egypt and Sudan including the villages of Abu Ramad, 125 km to the southeast; Halayeb, 165 km to the southeast; Ras Hadarba 200 km to the southeast. Ras Hadarba or Cape Hadarba lies on the shores of the Red Sea to the southeast of the city of Halayeb and to the east of mount Hadarba from which it takes its name.

The village of Ras Hadarba lies on north of the borders between Egypt and Sudan which run along the 22°N parallel of latitude; Marsa Hameera, 40 km to the north; and Abrak, 90 km to the west. The first three towns (Abu Ramad, Halayeb and Ras Hadarba) are located within the disputed Halayeb Triangle. In Egypt, the number of camels was estimated to be 120.000 heads (SADS, 2009). About half of the camels in Egypt are present in the Shalateen area (Mahran, 2004).

Study design and samples size estimation

A cross-sectional study was designed and adopted in this survey participating with camel owners. It was carried out from spring 2014 to 2015 in Shalateen quarantine. The sample sizes for animals for serological studies, serum samples from camels for molecular studies, were calculated by the formula of multistage random sampling (Thrusfield, 2005).

Each animal was examined clinically and information on different aspects of age, gender, date of sampling, and history of abortions was also recorded. Samples from camels were screened serologically for the presence of *Brucella*.

Sample collection

Blood samples

Blood samples were taken from examined animals; about 10 mL of jugular vein blood were collected in sterile silicon coated vacuum tubes 'vacutainers' (catalogue no. 02-683-60, Becton Dickinson, 38241 Meylan, Cedex, France), identified, kept in a slant position in the shade for about 2 h for complete clotting and transferred on ice packs to the laboratory avoiding shaking.

Samples were kept overnight at 4°C to allow separation of serum, centrifuged at 1000 g for 10 min to obtain amber clear serum. Sera were kept at -20°C each in 2 aliquots in sterile Bijou bottles until examined. Sera were screened for *B. abortus* antibodies by RBPT, BAPAT and CFT and positive sera were kept for further serological diagnosis.

Tissue samples

Mesenteric, retropharyngeal and supramammary lymph nodes of suspected camels were sampled at postmortem examination. Fetal stomach contents were collected carefully by heating the outer surface of the abomasum by heated spatula, sterile syringe was then introduce from the sterile point to obtain some of the fetal stomach contents.

Serological tests

All collected sera were initially screened by RBPT using RBPT antigen according to Alton et al. (1988) and OIE (2012). Antigens

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Table 1. Seroprevalence of camel brucellosis in Shalateen city as determined by BAPAT, RBPT and CFT in relation to season.

Season	Camels examined	BAPAT		RBPT		CFT	
		Pos.	%	Pos.	%	Pos.	%
Spring 2014	145	18	12.40	17	11.70	17	11.70
Summer 2014	49	2	4.10	2	4.10	2	4.10
Autumn 2014	233	40	17.20	36	15.50	36	15.50
Winter 2015	96	3	3.130	3	3.130	3	3.13
Spring 2015	278	40	14.40	35	12.60	34	12.20
Total	801	103	12.90	93	11.60	92	11.50

Pos. = Number of animals positive for brucellosis; P<0.05: significant differences between different seasons.

for BAPAT and RBPT were obtained from Veterinary Sera and Vaccine Research Institute (VSVRI), Abbassiya, Cairo 11517, Egypt. Antigen for CFT was kindly supplied by the National Veterinary Services Laboratories (NVSL), Ames, IA 50010, USA. In CFT, titers of 1/4 were regarded as suspicious, while titers of 1/8+ or above were considered as positive.

Sera that tested positive to RBPT and BAPAT were further tested using CFT for confirmation and standard *B. abortus* antigen S99 (CVL, New Haw Weybridge, and Surry KT15 3NB, UK). Preparation of the reagent was evaluated by titration and performed according to protocols recommended by World Organization for Animal Health (OIE, 2004). Sera with strong reaction, more than 75% fixation of complement (3+) at a dilution of 1:5 or at least with 50% fixation of complement (2+) at a dilution of 1:10 and above were classified as positive and lack of fixation/complete hemolysis was considered as negative.

Bacteriological examination

Swabs from stomach contents of two aborted feti, also, samples of fetal membranes and uterine discharges of two aborted cows were taken under complete aseptic condition for culture of *Brucella spp.* This was performed according to the recommendations of the FAO/WHO Expert Committee on Brucellosis (Alton et al., 1988; OIE, 2012) using direct culture on *Brucella* Agar Media containing *Brucella* selective antibiotics (Oxoid, England).

The plates were examined for *Brucella* colonies. The suspected colonies were identified and typing on the base of colonial morphology, urease, CO_2 requirement, susceptibility to *Brucella* phages, growth in the presence of thionin and basic fuchsin dyes (1:25000, 1:500000, 1:100000), production of H2S, and antigenic are characteristics using specific antisera (A, M, R).

Data analysis

Descriptive and analytic statistics were computed using software SPSS®Version20. The degree of association was computed using odds ratio (OR) signified by 95% confidence intervals (Thrusfield, 2005). True Prevalence was estimated according to Rogan and Gladen (1978) from the following equation:

$$\text{True prevalence (TP)} = \frac{\text{Apparent prevalence} + \text{combined specificity of RBPT and CFT-1}}{\text{Combined sensitivity of RBPT and CFT} + \text{combined specificity of RBPT and CFT-1}}$$

RESULTS

Seroprevalence

Eight hundred and one (801) camels were examined for brucellosis in Shalateen Quarantine from spring 2014 to 2015. 103 (12.90), 93 (11.60%) and 92 (11.50%) were positive for RBPT, BABAT and CFT, respectively (Table 1). These results reveal that the apparent prevalence (AP) was estimated as 11.50% by CFT, while TP was estimated as 13.60% (95%; CI: 11.20 to 16%). Among the total 103 camels positive for the disease in Shalateen quarantine, 42 (16.90%) were at 1 to 2 years old, 33 (11.20%) at 2 to 4 years old and 28 (10.80%) at the breeding age (Table 2).

By CFT, brucellosis-infected camels were observed in 36 (17.10%) out of the 507 examined male camels while 56 (19%) out of the 294 examined female camels were positive for brucellosis. There was no significant difference between different age groups while a highly significant difference was detected between different sexes (P<0.05) (Table 3).

Bacterial isolation

A smear from one fetal stomach contents showed partially acid fast organisms. *B. melitensis* biovar 3 was isolated from stomach content of this aborted fetus; the morphological, cultural, biochemical and serological identification of the isolated *Brucella* strain. One *Brucella* isolates could be recovered from the stomach content of one aborted foetus by culture on artificial media, followed by isolates identification by its morphology and growth characteristics of the colonies and biochemical tests.

This isolate was typed as *B. melitensis* biovar 3 based

Table 2. Seroprevalence of camel brucellosis in Shalateen city as determined by BAPAT, RB PT and CFT in relation to different age group.

Age	Camels examined	BAPAT		RBPT		CFT	
		Pos.	%	Pos.	%	Pos.	%
1- 2 years	248	42	16.90	33	13.30	33	13.30
2-4 years	294	33	11.20	32	10.80	31	10.60
≥ 4years	259	28	10.80	28	10.80	28	10.80
Total	801	103	12.90	93	11.60	92	11.50

Pos. = Number of animals positive for brucellosis, P> 0.05: no significant differences between different age groups.

Table 3. Sero-prevalence of camel brucellosis in Shalateen city as determined by BAPAT, RB PT and CFT in relation to sex.

Sex	Age	Number of examined animals	CFT		Total number	Total pos.	
			Pos.	%		Number	%
Male	1- 2 years	168	10	6	507	36	7.1
	2-4 years	177	9	5.10			
	≥ 4years	162	17	10.50			
Female	1- 2 years	80	23	28.80	294	56	19
	2-4 years	117	22	18.80			
	≥ 4years	97	11	11.30			

Pos. = Number of animals positive for brucellosis, P<0.05: significant differences between the two sexes.

on as if it does not required CO_2 for growth, negative for H_2S production, grow in the presence of thionin and basic fuchsin dye (1:250000 and 1:500000), urease positive after 20 h, phage (Izatnagar) lyses and agglutinated only with A and M monospecific antisera (Table 4).

DISCUSSION

During the last few years, camel brucellosis has been a subject for many researches in many countries of the world especially those rearing racing camels such as the Arabian Gulf countries as well as other countries where camels constitute an important part of their livestock in many African and Asian countries (Yasmin and Remya, 2011).

Serological investigation still has played a dominant role in diagnosis of the disease (Konstantinidis et al., 2007). BAPAT, RBPT and CFT were used as screening for diagnosis of brucellosis (Morgan et al., 1969; Hunter and Allen, 1972; Farina, 1985). Moreover we used CFT as confirmatory test for the positive serum samples (OIE, 2012). In the present study, BAPAT, RBPT and CFT were used as screening and confirmatory tests for diagnosis of camel brucellosis and detection of naturally infected cases in a total of 801 dromedary camels during the period between 2014 and 2015 from Shalateen quarantine.

The overall prevalence of camel brucellosis was 12.90, 11.60 and 11.50% as determined by BAPAT, RBPT and CFT, respectively. Statistical analysis revealed that AP was estimated as 11.50%, while true prevalence (TP) was estimated as 13.60% (95% CI: 11.20 to 16%). High prevalence appears to be due to the fact that these camels were imported from Sudan which is known to have high prevalence of 12.30, 15.50 and 30.50% in 2004, 2005 and 2006, respectively as recorded by (Omer et al., 2007); 23.80% (Musa et al., 2008) and 37.5% (Omer et al., 2010). These studies attributed insufficient preventive measures, the lack of adequate control programs and uncontrolled animal transportation across "open" borders. Chi square analysis for comparison between seasonal occurrences of *Brucella* infection revealed high significant differences between different seasons (P ≤ 0.05). The prevalence was being high in spring and autumn (Abdel-Raouf and El-Naggar, 1964; Shalash, 1965; Musa and Abusineina, 1978; Mares, 1954).

In Egypt, the sero-prevalence of camel brucellosis has been reported by different authors at different localities using different tests. The present results were higher than that recorded by Abdel Moghney (2004) (9.26%), Al-Gaabary and Mourad (2004) (6.75%) and El-Boshy et al. (2009) (7.35%). However, this results is in agreement with those of Hamada et al. (1963) (10.29%), Ahmed and Nada (1993) (11.6%) and El-Sawally et al. (1996) (11.3%),

Table 4. Phenotypic characteristics of Brucella isolates (*Brucella melitensis* biovar 3) recovered from stomach content of aborted fetus of she-camel.

Strain source		CO$_2$ requirement	H$_2$S production	Urease	Growth on dyes					Lysis by macrophage				MS			Conclusion
					Thionin			Basic fuchsin		Tb		Iz1 RTD	R/C RTD	A	M	R	
					a	b	c	b	c	RTD	RTD 10^4						
Field strain	One Stomach content	-	-	+ in 20 h	-	+	+	+	+	-	-	+	-	+	+	-	Brucella melitensis biovar 3
Reference strains	*Brucella melitensis Ether*	-	-	+in 18-24 h	-	+	+	+	+	-		-	+	-	+	+	Brucella melitensis biovar 3
	B. abortus 544	-	+	+in 2 h	-	-	-	+	+	+	+	+	-	+	-	-	Brucella abortus 1
	B. suis 1330	-	+++	++ in <15 min	+	+	+	-	-	-	+	+	-	+	-	-	Brucella suis 1

RTD: Routine test dilution a: 1:25000 b: 1:500000 c: 1:100000 Tb: Tbilisi IZ1: Izatnagar R/C: R/C: Rough Brucella. B: Brucella MS=Monospecific sera.

respectively. The differences in sero-prevalence observed from the previous researchers, might be due to differences in herd size, camel origin, tests used, management conditions, and the presence or absence of infectious foci, such as *Brucella*-infected herds, which could spread the disease among contact herds.

The RBPT detected 93 (11.6%) reactors lower than BAPAT which detects 103 (12.9%) reactors, this variation on the incidence of positive reactors may be attributed to the difference in the acidity of their antigen as reported by Davis (1971) and Corbel (1973). The acidic pH of the RBPT antigen (3.65±0.05) inhibits more amount of IgM fraction (Alton et al., 1988). The test is an excellent screening test but may be oversensitive for diagnosis in individual animal particularly vaccinated animals (Wolrld Health Organisation, 2006).

IgG$_1$ was the main immunoglobulin measured by the CFT with a possible cause that IgM is denatured during the test (MacMillan, 1990). CFT was only measured IgG$_1$ while IgG$_2$ and IgA do not fix complement (Curtain, 1971; Cho and Ingram, 1972). The results from the CFT may be adversely affected by IgG$_2$ interference (prozone effect) and by anti-complementary activity (Plackett and Alton, 1975). The CFT should be used only as a confirmatory test (Al-Dahouk et al., 2003).

All examined camels were clinically normal at the time of sampling. Prevalence of brucellosis in apparently healthy camels indicates that many infected camels might be silent carriers for brucellosis and their products may pose a serious health problem for consumers (Abu Damir et al., 1989; Bekele, 2004). Non pregnant camels experimentally infected with *B. abortus* had no clinical manifestations and only negligible pathological changes were found (Abu Damir et al., 1989). On the contrary, individual cases of abortion, fetal death, mummification, delayed sexual maturity, infertility, stillbirth, mastitis, orchitis and joint disease might be encountered in naturally infected camels with *B. abortus* (Higgins, 1986; Obeid et al., 1996; Musa and Shigidi, 2001).

The prevalence of camel brucellosis according to their age was determined. In young camels (less than 2 years old), 42 (16.9%) and 33 (13.3%) were positive for BAPAT and RBPT, respectively, and 33 (13.3%) samples were confirmed as positive reactor for CFT, while in the adult mature camels (2 to 4 years old), 33 (11.2%) and 32 (10.8%) were positive for BAPAT and RBPT, respectively, and 31 (10.6%) samples were confirmed as positive reactors for CFT. In

addition, the examined adult mature camels at the breeding age (more than 4 years old) were positive for BAPAT, RBPT and CFT (28, 10.8 and 10.8%, respectively). Chi square analysis for comparison between occurrences of *Brucella* infection at different age groups revealed that there is no significant difference between different age groups, which suggests that all ages of camels were susceptible to brucellosis.

Brucellosis can affect camels at an early life probably through sucking and persisted into adulthood. This is confirmed by highly significant infection rate in she-camels in this study. Also younger animals may be infected through transmission from adults during the long journey from Sudan to Shalateen quarantine through contact with other herds around source of water. The result is supported by those of Higgins (1986) who reported that young camels under 11 month were resistant to brucellosis because sex hormones and erythritol tend to increase by age and sexual maturity.

Sero-prevalence of camel brucellosis according to their sex was recorded. In male camels examined, 36 (7.1%) were positive for CFT while in she-camel examined, 56 (19%) were positive for CFT. Chi square analysis for comparison between occurrence of *Brucella* infection by

different tests and sexes revealed that there is a high significant difference between male and females. These results may be associated with the effect of erythritol (Smith et al., 1962). Reduction of immunity in females during lactation, pregnancy and other reproductive stress may also contribute to higher prevalence in female camels (Gyles and Prescott, 2004). These results agreed with Bekele (2004) and Hadush et al. (2013) from Ethiopia, Yagoub et al. (1990) and Agab et al. (1994) from Sudan, and Ajogi and Adamu (1998) and Junaidu et al. (2006) from Nigeria. On the other hand, others results shows equal distribution between both sexes (Abu-Damir et al., 1989; Abbas et al., 1987).

In the present study, our trials to isolate the organism from the stomach content of one aborted fetus has been successful and the morphological, cultural, biochemical and serological identification of the isolated *Brucella* strain revealed isolation of *B. melitinsis* biovar 3. This biovar of *B. melitinsis* was previously identified and considered as the prevalent type in Egypt in different animals as recorded by (Sayour, 2004; Hoda et al., 2006; Khoudair and Sarfenaze, 2007; El-Diasty, 2009; Rehab, 2011; Abdel Hamid, 2012; Menshawy, 2013; Affi et al., 2015). Originally *B. melitensis* affects mainly sheep and goat. Such inter-species transmission situation may be the outcome of close contact between sheep, goats and camels (Musa et al., 2008).

This may explain the occurrence of this biotype in camels in the current study which consider the most dominant biotype of *Brucella* isolated from both animals and human in Egypt as reported by (Mohamed and Eisa, 2004; Soliman, 2006; El-Diasty, 2009; El-Sayed et al., 2011; Abdel Hamid, 2012; Afifi et al., 2015, El-Diasty et al., 2016, El-Hady et al., 2016).

The isolation of *Brucella* from lymph nodes failed, and this may have occurred if the number of viable organisms in the examined samples is low or contaminated with other bacteria which may prevent *Brucella* growth (Seleem et al., 2010). The specificity of serological tests cannot usually be determined by bacteriological isolation because some animals that yield negative culture results are in fact infected (Alton et al., 1975; Poster et al., 2010).

Conclusion

It is concluded that brucellosis is present at a level of 11.6% (as determined by CFT) among the examined camels in Shalateen city. A combination of several serological tests such as BAPAT and RBPT, followed by a confirmatory test of high specificity such as CFT can be used for diagnosis of brucellosis. One isolate of *Brucella* are typed as *B. melitensis* biovar 3. This is represented as zoonotic threat to the public health.

Routine screening of animals for brucellosis is crucial that may help to detect positive cases and reduce the risk

of transmission of the disease. Effective implementation of control measures including test and culling of the infected animals, quarantine and movement controls may prevent the spread of infection. Applications of hygienic measures which help in the control of brucellosis in camels imported from Sudan are considered as the main source of infection and contamination of environment in Egypt. The present data highlights the need for further research, including the isolation and characterization of the causative agents, reliable epidemiological studies, implement a transparency policy and effective control measures in Egypt.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of crude toxins of *Ustilaginoidea virens* on rice seed germination

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The plant pathogen *Ustilaginoidea virens* is known to generate secondary metabolite ustiloxins, which are poisonous to humans and domestic animals and to affect plant cells during the growth process. In this study, rice false smut was collected from different locations in Sichuan province and the strains isolated. The crude toxins of *U. virens* were extracted from the liquid culture medium of isolated strains to determine the effects on rice seed germination. Our results, as expected, show that the crude toxins had inhibitory effects on the growth of rice germ and radicle. Moreover, these inhibitory effects were maintained even when the toxin was diluted up to 100 times. Crude toxins caused higher inhibition in the radicle than in the germ. Inhibitory effects of crude toxins on rice germination varied significantly depending on the location of where the isolated strains were extracted. This study also showed that inhibition of rice seed germination by crude toxins was different depending on the type of rice used, which corresponded with the field disease resistance evaluation.

Key words: *Ustilaginoidea virens*, crude toxins, inhibitory effect, significant difference.

INTRODUCTION

Ustilaginoidea virens (Cooke) (Takahashi, 1896) (teleomorph: *Villosiclava virens*) is a causative agent of the false smut of rice (Tanaka et al., 2008). The pathogen initiates infection through rice floral organs in the rice booting stage and forms white spikelet balls, which are called smut balls. The smut balls change their color to yellow, then orange, and finally to greenish black (Fu et al., 2015a; Hu et al., 2014; Tang et al., 2013). False smut not only results in severe yield losses but also contaminates rice grains and straw, which poses a risk of human or animal poisoning (Li et al., 1995; Shan et al.,

2012). Previous studies report that *U. virens* produces two types of mycotoxins, named ustiloxins and ustilaginoidins. Ustiloxins belong to the cyclopeptides containing a 13-membered core structure. Six ustiloxins have been identified and named as ustiloxins A, B, C, D, E and F (Zhou et al., 2012; Lu et al., 2014). Ustiloxins is an inhibitor of cell growth, especially by inhibiting microtubule assembly and cell skeleton formation (Koiso et al., 1994). Ustilaginoidins are naphthalene pyrone derivatives, which have shown weak antitumor cytotoxicity in human epidermoid carcinoma (Koyama et al., 1998).

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Table 1. Isolates of conidiospore used in this study.

Isolate	Locality	Longitude	Latitude	Sample number	Isolates number
UvLZ	Luzhou	105.39	28.91	13	11
UvYA	Yaan	102.97	29.97	8	7
UvPJ	Pujiang	103.29	30.2	10	5
UvXD	Xindu	104.13	30.82	13	6
UvNC	Nanchong	103.47	30.42	5	4
UvYB	Yibing	104.56	29.77	5	5
UvMY	Mianyang	104.73	31.48	8	8
UvZZ	Zizhong	104.85	29.81	10	9
UvQL	Qionglai	103.47	30.42	9	7
UvMZ	Mianzhu	104.19	31.32	12	10
UvJT	Jintang	104.32	30.88	9	8
UvPX	Pixian	103.86	30.8	8	8
UvMY	Miyi	101.41	26.42	5	4
UvWJ	Wenjiang	103.81	30.97	13	10

When livestock was fed with the rice grains and forage contaminated by the rice false smut pathogen, they showed a great variety of symptoms such as poor growth, diarrhea, abortion, and hemorrhage (Nakamura et al., 1994; Luduena et al., 1994). Additionally, ustiloxins, acting as phytotoxins, have a wide range of biological activities in plants, such as growth inhibition of the plumule and the radicle during seed germination of maize and wheat and abnormal swelling of seeding roots (Kois et al., 1994; Tian and Tao, 2000).

Ustiloxin is the main toxin component that has been isolated and identified from rice false smut balls and mycelia of the *U. virens* (Shan et al., 2013; Fu et al., 2015b). In previous studies, Chen et al. (2004) suggested that the rough ustiloxins extracted from false smut balls strongly inhibited the growth of plant roots. In the present study, the rice seed was used with rough toxins extracted from the liquid culture medium of the pathogen. The aims of the study were to determine the toxins obtained from different isolated strains of *U. virens* at various concentrations, on seed germination of different rice varieties.

MATERIALS AND METHODS

Fungal collection, isolation and culture conditions

Naturally infected rice spikelets showing typical false smut symptoms were collected in a field plot at Luzhou, Yaan, Pujiang, Xindu, Nanchong, Yibin, Mianyang, Zizhong, Qionglai, Mianzhu, Jintang, Pixian, Miyi, and Wenjiang, Sichuan province, China, in 2015 (Table 1). The samples were stored in dry envelopes at room temperature.

The yellowish false smut balls, which were covered with a mass of chlamydospores, were surface-sterilized with 0.1% mercuric chloride solution for 30 s and rinsed four times with sterile water. The treated samples were resuspended into a conidiospores suspension and diluted to 10^3 ml^{-1}. The spore suspension was poured onto potato sucrose agar (PSA, which was made from a boiled extract of 300 g of peeled potatoes, 20 g of sucrose, and 10 g of agar per liter of distilled water) solid medium and subsequently incubated at 28°C in the dark. When visible colonies appeared, the colonies were transferred individually onto fresh PSA plates and incubated at 28°C in the dark (Fu et al., 2014). Each isolate was individually maintained *in vitro* containing 10 ml of a PSA solid medium at 4°C.

Preparation of crude ustiloxins

The fungus was transferred into the PS fluid medium (PS, which was made from a boiled extract of 300 g of peeled potatoes, and 20 g of sucrose) at 28°C under 150 rpm by shaking for 10 days. The nutrient solution contained toxins that had been produced. The mycelium was filtered with sterile gauze then the supernatant was harvested by centrifuging at room temperature for 8 min at 8000 rpm. The supernatant (100 ml) was concentrated under vacuum at 60°C by a rotary evaporator to a concentrated dry matter. The dry matter was dissolved in 100 ml of methanol and shaken for 2 h to fully extract, and then the supernatant was collected by centrifuging. The supernatant was concentrated under vacuum at 55°C by a rotary evaporator to a concentrated dry extract. The dry extract, which was in the form of crude ustiloxins, was dissolved in 100 ml of distilled water. The water extract was stored at 4°C until required.

Rice materials and treatment

Seven rice varieties were tested in this experiment: '9311', 'Guichao 2', 'F you 399', '2 you 838', 'Y liangyou 1', 'Jingyou 127', and 'Chuanyou 6203', respectively. The full rice seeds were chosen and surface-sterilized with 2% sodium hypochlorite solution for 90 s and rinsed three times with sterile water. Rice seeds were germinated in sterile water at 27°C for 48 h.

Bioassays of ustiloxins

For the experiment, 20 sprouted seeds of rice were placed into

each sterile Petri dish containing 15 sample solutions and covered with two layers of sterilizing filters. These treatments were placed in a biochemical incubator at 27°C for 4 days in the dark. Then, the length of the germ and radicle of each seed was measured. The crude ustiloxins was diluted into different ratios of the concentration solution: 10, 50 and 100. The length of germ and radicle of treated seeds were compared with those grown in distilled water (control group). In addition, the number of radicle of each processing was counted in this test. The experiments were repeated two times.

Statistical analysis

All of the experiments were carried out two times and the data from the two runs of each test were pooled. All of the statistical analyses of variance were performed by using SPSS statistical analysis software. The statistical comparisons performed among strains, among rice varieties and among toxin concentrations. The treatment means were separated using Fisher's protected least significant difference test (LSD) (α=0.05).

The inhibitory rate of the germ (radicle) growth (%) = [(Average length of control group germ (radicle) – Average length of treated germ (radicle) / Average legth of control group germ) × 100].

RESULTS

Isolates of *U. virens*

In this study, 128 samples of false smut balls which came from Sichuan province, China, in 2015 were collected and 102 isolates were obtained by using the suspension liquid of chlamydospores (Table 1).

Effect of different concentration of crude ustiloxins on germination of rice seeds

The crude toxins were extracted from 14 *U. virens* strains that were isolated from 14 Sichuan regions (Table 2). As shown in Table 2, the crude toxins strongly inhibited the germ and radicle, and the number of radicle of '9311' and the inhibitory rate of radicle was higher than the germ. The results show that the different concentrations had different effects on rice seed germination and that the inhibition rate, which was diluted 10 times, was significantly higher than the inhibition rate shown for rice germination that was diluted 50 times. The experiment found that the inhibitory effects of the toxin on germ and radicle growth were maintained even after being diluted 100 times but were significantly lower than 10- and 50-times dilutions.

Effect of different crude ustiloxins

In order to investigate the effect of different crude ustiloxins on the growth of rice seed, 14 toxins that were extracted from different *U. virens* strains isolated from 14 Sichuan regions were studied (Table 3). The results show that toxins extracted from UvJT6, UvNC1-1, and UvQL3-

2 showed significantly higher inhibitory rates of germ growth compared to the toxins extracted from the other 11 strains. The inhibitory rates of germ growth in these three strains were 76.13, 81.55 and 76.13%. Moreover, for UvJT6, UvNC1-1 and UvQL3-2 strains, the inhibitory rates of the radicle growth were 96.83, 97.53, and 88.47%, respectively, and the inhibitory rates of radicle number were 91.38, 91.88 and 87.96%, respectively. Both of these effects were also significantly higher than most other strains.

As shown in Table 3 and Figures 1 and 2, the crude toxins showed greater inhibition of the radicle growth and numbers, while the inhibition of the germ growth was relatively small. This outcome could be explained because the radicle came in direct contact with the toxin, and the absorption eventually led to the death of seedling.

Effect of crude toxin on the germination of different rice varieties

The results show that the toxins had a certain inhibitory effect on the germ and radicle of each rice variety (Table 4). The inhibitory rate of the radicle growth was higher than that of the germ growth, and the range of inhibitory ratio was 62.78 to 82.06 and 26.71 to 78.8%. The inhibitory action of germination of 'Guichao 2' was significantly higher than the other six varieties, and the inhibitory ratio of the radicle and germ was 82.06 and 78.8%, respectively. The inhibitory effect of '2 you 838' and 'Jingyou127' was relatively poor. In addition, the results indicate that the inhibitory effect was different based on the test varieties and that these results were similar to the disease resistance of rice varieties in the field. Hence, such rice seed varieties can be further considered to identify the mechanisms of resistance to false smut and screen the resistant materials.

DISCUSSION

The ustiloxins may exhibit a broad range of biological activities in animals and plants and can be used for medicinal and agrochemical purposes (Zhou et al., 2012; Li et al., 1995; Shan et al., 2013). It was previously reported that the toxins were mainly obtained from rice false smut (Chen et al., 2004; Fu et al., 2015b; Shan et al., 2013). But in this study, the crude toxins were extracted from the liquid diet of *U. virens* mycelium. The findings show that the crude toxins had inhibitory effects on the germ and radicle growth and that the inhibitory effects on radicle were significantly higher than for the germ. This could be because the radicle directly contracted the toxin through absorption. The results were similar to those of previous findings of Chen et al. (2004). In addition, the experiment found that the toxins maintained their inhibitory effects on the growth of the

Table 2. The different concentration of crude ustiloxins on germination of rice seeds.

Strain	Concentration ratio	Inhibitory rate of germ (%)	Inhibitory rate of radicle (%)	Inhibitory rate of radicle number (%)
UvNC1-1	100	46.41[cB]*	62.39[cA]	49.58[c]
	50	61.95[bB]	82.98[bA]	82.07[b]
	10	76.13[aB]	97.53[aA]	91.88[a]
UvJT6-1	100	29.79[c B]	49.04[cA]	57.98[c]
	50	42.64[bB]	71.3[bA]	72.55[b]
	10	81.55[aB]	96.83[aA]	91.38[a]
UvQL3-2	100	35.28[cB]	36.17[cA]	49.58[c]
	50	62.31[bB]	77.24[bA]	77.59[b]
	10	79.54[aB]	93.81[aA]	87.96[a]
UvYA1-2	100	44.97[cB]	34.69[cA]	55.74[c]
	50	58.72[bB]	74.22[bA]	71.43[b]
	10	70.57[aB]	88.47[aA]	83.75[a]
UvPJ1-1	100	34.6[cA]	28.01[cB]	56.3[c]
	50	48.56[bA]	43.94[bB]	62.75[b]
	10	74.37[aB]	92.08[aA]	82.07[a]
UvPX5-2	100	42.28[cB]	45.57[cA]	64.99[c]
	50	47.74[bB]	67.54[bA]	65.83[b]
	10	62.31[aB]	91.34[aA]	80.11[a]
UvXD5-1	100	25.23[cB]	45.57[cA]	34.73[c]
	50	35.39[bB]	62.89[bA]	49.58[b]
	10	60.34[aB]	90.1[aA]	71.99[a]
UvMY1-2	100	21.03[cB]	44.09[cA]	13.17[c]
	50	36.11[bB]	66.35[bA]	66.39[b]
	10	50.11[aB]	90.1[aA]	76.75[a]
UvWJ2-1	100	22.83[cB]	48.49[cA]	40.34[c]
	50	32.99[bB]	62.35[bA]	57.42[b]
	10	53.02[aB]	84.36[aA]	77.59[a]
UvLZ10-2	100	31.41[cB]	3.51[cA]	50.14[c]
	50	46.16[bB]	31.82[bA]	61.34[b]
	10	53.34[aB]	85.16[aA]	75.49[a]
UvYB5-2	100	13.85[cB]	68.43[cA]	0.28[c]
	50	44.15[bB]	71.3[bA]	53.22[b]
	10	58.47[aB]	82.48[aA]	71.99[a]
UvMY1-1	100	26.42[cB]	63.48[cA]	39.22[c]
	50	31.8[bB]	71.75[bA]	59.94[b]
	10	41.53[aB]	63.73[aA]	76.47[a]
UvZZ9-1	100	44.62[cB]	46.71[A]	63.87[c]
	50	47.7[bB]	49.14[bA]	71.99[b]
	10	57.43[aA]	54.38[aB]	77.79[a]
UvMZ9-2	100	24.12[cB]	49.47[cB]	52.1[c]
	50	37.44[b B]	53.71[bB]	63.87[b]
	10	49.75[a B]	58.44[aB]	70.59[a]
Control group	-	27.86 mm	20.21 mm	3.57

*Different lowercase letters indicate significant difference among toxin concentration for each strain. *Different capital letters indicate significant difference between germs and radicle.

Table 3. Effect of crude ustiloxins on germination of '9311' rice seed.

Strain	Concentration ratio	Length of germ (mm)	Inhibitory rate of germ (%)	Length of radicle (mm)	Inhibitory rate of radicle (%)	Number of radicle	Inhibitory rate of radicle number (%)
UvNC1-1	10	6.65	76.13a[b*]	0.5	97.53[a]	0.29	91.88[a]
UvJT6-1	10	5.14	81.55[a]	0.64	96.83[a]	0.31	91.38[ab]
UvQL3-2	10	5.7	79.54[a]	1.25	93.81[ab]	0.43	87.96[bc]
UvYA1-2	10	8.2	70.57[c]	2.33	88.47[cde]	0.58	83.75[ab]
UvPJ1-1	10	7.14	74.37[bc]	1.6	92.08[bc]	0.64	82.07[cde]
UvPX5-2	10	10.5	62.31[d]	1.75	91.34[bc]	0.71	80.11[def]
UvXD5-1	10	13.09	60.34[de]	2.01	90.1[cd]	1	71.99[gh]
UvMY1-2	10	13.9	50.11[g]	2	90.1[cd]	0.83	76.75[fg]
UvWJ2-1	10	13.09	53.02[fg]	3.16	84.36[de]	0.8	77.59[ef]
UvLZ10-2	10	13	53.34[g]	3	85.16[de]	0.875	75.49[fg]
UvYB5-2	10	11.57	58.47[e]	3.47	82.48[e]	1	71.99[h]
UvMY1-1	10	16.29	41.53[h]	7.33	63.73[f]	0.84	76.47[fg]
UvZZ9-1	10	11.86	57.43[ef]	12.06	54.38[g]	0.66	77.59[fg]
UvMZ9-2	10	13.4	49.75[g]	8.41	58.44[f]	1.3	63.59[i]
Control group	-	27.86	-	20.21	-	3.57	-

Figure 1. Effect of crude ustiloxins on the germ and radicle of '9311'. a. CK; b. UvZZ9-1; c. UvQL3-2; d. UvPJ1-1; e. UvWJ2-1; f. UvMY1-1; g. UvJT6-1; h. UvYA1-2.
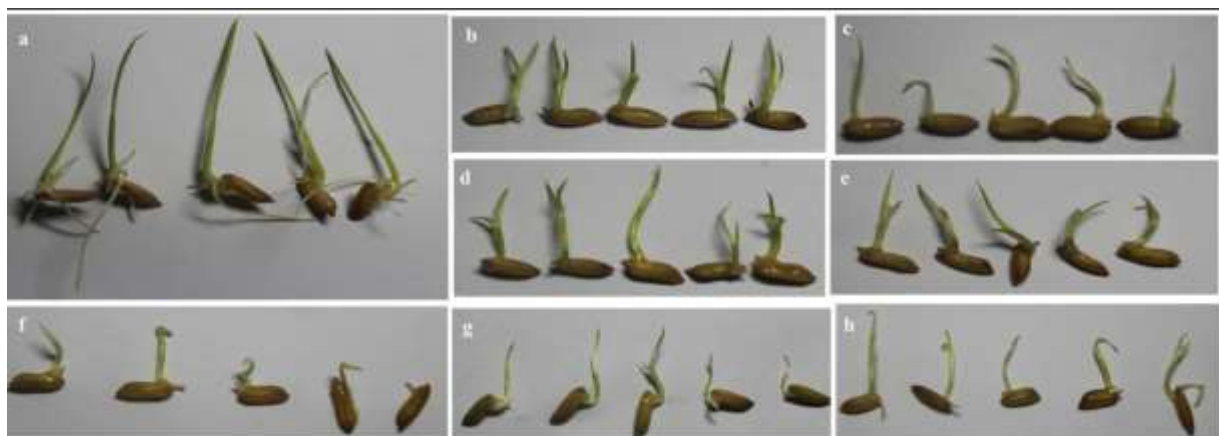
Figure 2. Effect of crude ustiloxins on the germ and radicle of '9311'. a. CK; b. UvLZ10-2; c. Uv MY1-2; d. Uv MZ9-2; e. UvYB5-2; f. UvNC1-1; g. UvPX5-2; h. UvXD5-1.

Table 4. Effect of crude toxin of UvJT6-1 on the germination of different rice varieties.

Rice varieties	Concentration ratio	Germ length (mm)	Inhibitory rate of germ (%)	Length of radicle (mm)	Inhibitory rate of radicle (%)	Number of radicle	Inhibitory rate of radicle number (%)
Guichao 2	10	5.28	78.8[aB*]	3.23	82.06[aA]	0.48	77.14[a]
9311	10	8.33	66.55[bB]	3.56	80.22[abA]	0.5	76.19[a]
F you 399	10	8.11	67.43[bB]	4.56	74.67[cdA]	0.75	64.29[b]
Y liangyou 1	10	7.89	68.31[bB]	4.8	73.33[cdA]	0.69	67.14[b]
Chuanyou 6203	10	11.18	55.1[cB]	4.23	76.5[bcA]	0.89	57.62[c]
2 you 838	10	14.86	40.32[dB]	5.31	70.5[dA]	1.24	40.95[d]
Jingyou127	10	18.25	26.71[eB]	6.7	62.78[eA]	1.56	25.71[e]

germ and radicle even after being diluted 100 times. However, this finding was not consistent with the former reports in which the low concentration of toxin stimulated the germination of rice seed (Wang et al., 1995). Because ustiloxins have inhibitory effects on rice radicle, it is possible that the inoculation by a spikelet with conidial suspension may hurt the young ear, eventually leading to death of a section or the whole spikelet. Therefore, the conidial suspension should undergo dialysis to remove toxins before inoculation.

This study utilized toxin extracts from different isolates with rice seeds. The results show that these toxins, depending on the location of the strain, had significantly different physiological effects on germ and radicle. If there is a certain direct correlation between the toxicity of ustiloxins and the pathogenicity of *U. virens*, it needs further research. To identify false smut resistant rice varieties, inoculums that can generate stronger toxicity of ustiloxins can be chosen to obtain more effective and reliable results.

For now, the resistance identification method mainly adopts artificial inoculation with spore suspensions or natural inducements in the field (Fu et al., 2015a; Yang et al., 2011). This study found that the toxin had different inhibitory actions on the germination of rice varieties and that there was significant difference among the varieties. The findings are similar to resistance identification of rice varieties in the field (Fu et al., 2015a). If this method can reduce manpower, financial resources and identification time by using the toxins as selection pressure of resistant material, it will become our focus in field research.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Lipolytic activity of fungi isolated from *Jatropha curcas* L. (physic nut) fruit rot

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Isolation, identification and screening of lipid-degrading fungal organisms causing rot of unripe, ripe and dry fruits of *Jatropha curcas* L. were carried out, and determination of the fungus showing highest lipolytic activity on the substrate analysed from three different locations within Edo State, Nigeria was done using standard methods. Five fungal isolates (*Curvularia geniculata*, *Lasiodiplodia theobromae*, *Trichoderma harzianum, Mucor* sp. and *Penicillium* sp.) were recovered from the samples. *Penicillium* sp. had the highest frequency (37.5%) while *Mucor* sp. and *Trichoderma* sp. had the lowest (25%). Fungal isolates screened on solid agar showed low extracellular lipolytic activity detected by their non-production of a distinct clear zone of inhibition. In submerged fermentation, the growth of the lipolytic fungi ranged between 0.0492 (*Mucor* sp.) and 0.1539 g (*T. harzianum*). Spectrophotometric measurement (at 600 nm) of lipase production varied with *Mucor* sp. having the highest production of lipase. There was a significant difference (p≥0.05) between growth and lipolytic activities of the fungi. The study showed that *Mucor* sp. and *L. theobromae* are lipase producers and efficient lipid degraders. The implication of this is that these fungi could reduce the oil content of *J. curcas* seed and consequently its economic value.

Key words: Fungal isolate, lipase, *Jatropha curcas,* Nigeria.

INTRODUCTION

Jatropha curcas L., commonly known as Barbados nut, purging nut or physic nut, belongs to the family, Euphorbiaceae. It is believed to have originated from Central America, most probably Mexico (Makker and Becker, 2009). The plant is a poisonous, semi-evergreen shrub or small tree, reaching a height of 6 m (20 ft) and growing in wastelands of almost any terrain or any kind of soil, even on gravel, sandy, acidic or alkaline soils with pH between 5.5 and 8.5 (Sharma et al., 2012). Plants from the same climatic zone show morphological

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differences with regard to the shape and size of the seeds and their protein and lipid content (Wolf et al., 1994). The plant is monoecious and flowers are unisexual, occasionally hermaphrodite. Ten stamens are arranged in two distinct whorls of five each in a single column in the androecium, and in close proximity to each other. In the gynoecium, the three slender styles are connate to about two-thirds of their length (Dehgan and Schutzman, 1994).

The plant is resistant to a high degree of aridity, allowing it to be grown in deserts. The seeds contain 27 to 40% oil (average: 34.4%) that can be processed to produce a high-quality biodiesel fuel, usable in a standard diesel engine (Makkar and Becker, 2009). The seeds are also a source of the highly poisonous toxalbumin and curcin (Abou-Arab and Abu-Salem, 2010). The seed cake can be used for fish or animal feed (if detoxified), biomass feedstock to power electricity plants, or as biogas or high-quality organic fertilizer. It can also be used as a bio-pesticide and for medicinal purpose (Henning, 2002).

Lipases are enzymes capable of catalysing the hydrolysis and synthesis of esters formed from glycerol and long-chain fatty acids (Svendsen, 2000; Sharma et al., 2001) and are produced by many microorganisms and higher eukaryotes (Elibol and Ozer, 2000; Kamimura et al., 2001). The ease with which enzymes could be isolated from microbes has made both bacteria and fungi predominant sources of lipase. However, fungi are the best lipase sources and are preferably used for industrial applications (Gupta and Soni, 2000; Mahadik et al., 2002).

Lipase producers have been isolated mainly from soil, or spoiled food materials that contain vegetable oil. Lipase production from a variety of bacteria, fungi and actinomycetes has been reported in several works (Kulkarni and Gadre, 2002; Maldonado et al., 2014). Lipase-producing fungi are present on a wide range of substrates in the ambient environment and these results could also provide basic data for further investigations on fungal extracellular enzymes (Griebeler et al., 2011). Among the available lipase-producing microorganisms, filamentous fungi belonging to various species of genera *Aspergillus, Rhizopus, Penicillium* and *Trichoderma* are described as prospective lipase producers (Lima et al., 2003; Kashmiri et al., 2006; Karanam and Medicherla, 2008). This study was undertaken to isolate, identify, screen and determine the most active lipase-producing fungus from *Jatropha curcas* fruit rot.

MATERIALS AND METHODS

Sample collection

Fruit samples of *J. curcas* L. (unripe, ripe and dry) used were obtained from three different locations, viz. Nigerian Institute for Oil Palm Research (NIFOR), Ugbowo and Upper Sakponba areas, in Edo State, Nigeria.

Preparation of medium and bacterial growth inhibition

Chloramphenicol at 0.02 g per 200 ml of medium was introduced into potato dextrose agar (PDA) to inhibit the growth of bacteria. Inoculation and transfer of culture were carried out on sterile inoculating bench CRC (Model HSB 60*180) after disinfection with methylated spirit.

Isolation of fungi associated with fruit rot

Small portions (including rotten and healthy) of 5 mm diameter were cut from the mesocarp with a flamed scalpel blade. These were sterilized in 0.1% mercuric chloride solution for 2 min and rinsed in three changes of sterile water, and thereafter dried with sterile tissue paper and crushed before plating in Petri dishes containing PDA medium. Inoculated Petri dishes were incubated at temperatures of 10 ± 2, 28 ± 2 and $35 \pm 2°C$ for 3 to 7 days (Malik, 1996). After the period of incubation, different colonies of fungi associated with the fruit rot were each aseptically sub-cultured using a flamed inoculation loop, into a sterile plate containing PDA. The frequencies of the various colonies were observed; distinct colonies were sub-cultured to obtain pure isolates which were then maintained on PDA slants and stored at -4°C for further study. After ascertaining the purity of cultures, the fungi were identified on the basis of cultural, macroscopic and microscopic features with the help of suitable literature (Barnett and Hunter, 1998). Culture samples were also sent to the Commonwealth Mycological Institute, England for confirmation.

Identification of fungal isolates

Mycelia from the different fungal isolates were harvested. Slides were prepared. The prepared slides were examined under bright field (BF) or differential interference contrast (DIC) illumination microscope. Additional microscopic samples were made by gently pressing a piece of transparent tape onto a colony. A Leica DM 2500 microscope with bright field, phase contrast and DIC contrast optics was used to view the slides for spore shape, hyphae and colour. A spot camera (with spot imaging software) was mounted on the microscope and used for photomicrography. A Panasonic High Definition (HD) 1920×1080 camera with 14_{MEGA}PIXEL lens was used for colony photography.

Screening of the fungal isolates for lipase production on solid agar

A plate detection method containing a chromogenic substrate (Congo red) was used to screen the isolates for lipase-producing ability. The medium used for screening has the following composition in g/L: Peptone 10; $NaCl_2$ 5; Calcium chloride 0.1; Castor oil 1 ml; agar 50; Congo red 0.5; and distilled water 1,000 ml. The sterile medium was poured into plates and allowed to solidify. The agar plates were separately spot-inoculated with the fungal isolates and incubated at room temperature ($28\pm2°C$) for 14 days. Lipolysis was determined by the appearance of a clear zone of inhibition around the spot of inoculation. The diameters of the colonies and clear zones were measured from the 2nd (24 h after inoculation) to the 14th day (Gupta et al., 2004).

Screening of the selected isolates for lipase production using submerged fermentation

All the isolates were screened for lipase production in submerged fermentation medium. This was carried out using the modified

Table 1. Effect of temperature on growth of fungi isolated from *Jatropha curcas* fruits obtained from NIFOR.

Temperature (°C)	Sample	Growth (CFU)
	Unripe	-
35	Ripe	-
	Dry	20×10^{-5}
	Unripe	
28	Ripe	1×10^{-5}
	Dry	25×10^{-5}
	Unripe	-
10	Ripe	-
	Dry	-

- = absence of growth.

Table 2. Effect of temperature on growth of fungi isolated from fruits obtained from Ugbowo.

Temperature (°C)	Sample	Growth (CFU)
	Unripe	-
35	Ripe	2×10^{-5}
	Dry	4×10^{-5}
	Unripe	30×10^{-5}
28	Ripe	27×10^{-5}
	Dry	36×10^{-5}
	Unripe	-
10	Ripe	-
	Dry	-

- = Absence of growth.

Table 3. Effect of temperature on growth of fungi isolated from *J. curcas* fruits obtained from Upper Sakponba Road.

Temperature (°C)	Sample	Growth (CFU)
	Unripe	-
35	Ripe	-
	Dry	-
	Unripe	33×10^{-5}
28	Ripe	33×10^{-5}
	Dry	30×10^{-5}
	Unripe	-
10	Ripe	-
	Dry	-

- = Absence of growth.

method of Gupta et al. (2004). The sterile basal medium was inoculated with seed cultures of *Lasiodiplodia theobromae*, *Curvularia geniculata*, *Trichoderma harzianum*, *Penicillium* sp. and *Mucor* sp. Fermentation was carried out at room temperature (28±2°C) for 3 days for fungal isolates. Lipase production was determined by spectrophotometer at 600 nm (Gupta et al., 2004).

Data analysis

Experiments were performed in triplicate and the results were analysed statistically using SPSS version 16. The treatment effects were compared and significant differences were assessed with Duncan's multiple range test at p≥0.05.

RESULTS AND DISCUSSION

Five fungi were isolated from the fruit surface of *J. curcas* as the associated agents of fruit rot disease. The fungi are *Penicillium* sp., *Lasiodiplodia theobromae*, *Curvularia geniculata*, *Mucor* sp. and *Trichoderma harzianum.* At 35°C, there was no visible growth of fungi in the unripe fruit samples (Tables 1, 2 and 3). In addition, at 10°C, no growth was seen for both the unripe, ripe and dry samples. Fungal growth was noticed mainly at 28°C in the unripe, ripe and dry samples, with the highest colony-forming unit (CFU) count of 36×10^{-5} seen in dry sample from Ugbowo. The lowest CFU (1×10^{-5}) was recorded in the NIFOR ripe samples at 28°C. Lipases are able to catalyze hydrolysis, esterification, trans-esterification and lactonization or intermolecular esterification (Gupta et al., 2011). Lipase producers have been isolated mainly from soil, or spoiled food materials that contain vegetable oil. Lipase production from a variety of bacteria, fungi and actinomycetes has been reported in several works (Sztajer et al., 1988; Kulkarni and Gadre, 2002). The fungal isolates screened in this present investigation showed low extracellular lipolytic activity in solid substrate fermentation. Decrease in extracellular lipase production can be associated with either decrease in fungal growth or inactive nature of enzyme itself (Lui et al., 1995).

Table 4 shows the microscopic and morphological descriptions of the five fungi isolates from *J. curcas* fruit rot. Screening results of fungal isolates for lipase production and growth determination using submerged fermentation are presented in Table 5. The growth (dry weight) of fungal isolates in submerged fermentation ranged from 0.0492 (*T. harzianum*) to 0.1539 g (*Mucor* sp.). At 600 nm, *Mucor* sp. had the highest absorbance (0.284) as compared to *C. geniculata* which had the lowest absorbance of 0.022. However, in the liquid state (submerged fermentation), high lipolytic activities were observed. Lipase activity was highest in *Mucor* sp. followed by *L. theobromae* and *Penicillium* sp. Minimum activity was observed in *T. harzianum* and *C. geniculata.* Growth of the fungal isolates grown in PDA and lipolytic media is shown in Table 6. There were significant differences in fungal isolates grown in PDA medium

Table 4. Microscopic and morphological description of fungal isolates.

Isolate	Microscopic description	Morphological description
Penicillium sp.	Simple or branched conidiophores with round conidia.	Dark creamy mycelia growth
Lasiodiplodia theobromae	Conidia were unicellular, hyaline, ovoid to ellipsoid, with thick wall.	Whitish mycelia growth with granulated orange colour beneath
Curvularia geniculata	Septate brown hyphae were seen and had curved conidia and elongated conidiophores	Pinkish mycelia growth
Mucor sp.	Round and slightly elongated sporangiospores with sparsely or non-septate hyphae were noticed.	Whitish fluffy mycelia growth
Trichoderma harzianum	Hyaline septate hyphae with one-celled and round conidia.	Dark green mycelia growth

Table 5. Screening of fungal isolates for lipase production and growth determination using submerged fermentation.

Fungi isolate	Lipase production (absorbance at 600 nm)	Growth (dry weight in g)
Penicillium sp.	0.063	0.0889
Mucor sp.	0.284	0.1539
Lasiodiplodia sp.	0.142	0.1182
Curvularia sp.	0.022	0.0875
Trichoderma sp.	0.022	0.0492

Table 6. Growth of the fungal isolates in PDA and lipolytic media.

Fungi isolate	Growth (PDA medium) incubation time (Days)			Growth (lipolytic medium) incubation time (Days)		
	3	7	14	3	7	14
Penicillium sp.	0.70[a]	1.77[b]	5.07[c]	1.53[a]	3.43[b]	4.10[c]
Mucor sp.	4.70[a]	8.00[b]	8.07[b]	4.10[a]	8.00[b]	8.00[b]
L. theobromae	3.83[a]	7.93[b]	8.17[c]	2.90[a]	5.87[b]	6.30[c]
C. geniculata	7.50[a]	7.83[b]	8.00[b]	5.50[a]	7.90[b]	8.00[b]
T. harzianum	2.30[a]	3.60[b]	4.70[c]	1.80[a]	3.37[b]	3.87[c]

Values are means of triplicate determinations. Values in the same row with different superscripts are significantly different with Duncan's multiple range test at $p \geq 0.05$.

between 3rd day and 14th day. Similarly, the growth of fungal isolates grown in lipolytic medium varied significantly. Previous reports by Prakash and Sivakumar (2013) have also shown that lipase activity is high in Mucor sp. in an experiment on isolation and screening of degrading enzymes from mangrove-derived fungi. The result is similar to that of Lazer and Schroder (1992) who investigated fungal lipases which degrade lipids from palm oil. Savitha et al. (2007) reported that fungal strains of different genera were isolated from various sources of which three *Aspergillus* spp. and one *Mucor* sp. were found to be positive for lipase production. In the present study, maximum biomass production was attained at the late period of fermentation (14th day) for *T. harzianum*

and *Penicillium* sp., and 7th day for the other three fungi. This, however, is in contrast with the report of Kashimiri et al. (2006) who reported that maximum biomass production by *T. viride* was observed during the early hours of fermentation. This difference might be due to the species used. There was a significant difference in lipase production by the various fungi.

The present study showed that *Mucor* sp. and *Lasiodiplodia theobromae* are lipase producers and efficient lipid degraders of *J. curcas* seeds. If the activities of these fungi are not checked, they could lead to great losses in the *J. curcas* seed oil. This oil is currently being used for production of biodiesel- an environment friendly diesel, among other uses. It is suggested that further

research on production, characterization and purification of the fungi be carried out.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Variables associated to anti-HIV, anti-rubella antibodies, syphilis reagins and enteroparasitosis in pregnant women

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The aim of this paper is to determine the prevalence of anti-HIV, anti-rubella antibodies, syphilis reagins and enteroparasitosis in pregnant women during their pre-natal monitoring at the Basic Health Units in the city of Umuarama (PR) and checking possible social-economic and behavioral associations. A cross-section study was performed in 690 pregnant women who went to have pre-natal checkup in the period from June 2012 to April 2014. From the 690 pregnant women analyzed, 78 presented enteroparasites, and from these, 69 were monoparasited and nine were polyparasited, nine presented antibodies against syphilis and two against HIV. None was reagent for IgM regarding rubella. The most prevalent parasite species was *Endolimax nana* 43/78 followed by *Entamoeba coli* 33/78 and *Giardia duodenalis* 05/78. Regarding the variables analyzed, the ones associated to enteroparasitary infections were water treated from public suppliers, fundamental level of schooling, sewage destination and public collection of garbage. For syphilis, the variables associated to infection were per capita income and age group. For HIV and rubella, no association among the variables was observed. The number of pregnant women infected in the present study was not considered high when compared to other studies.

Key words: Pregnant women, prevalence, prevention, public health.

INTRODUCTION

Women who experienced metabolic, endocrinological and immunological changes during pregnancy, as such might be at greater risk of infections (Vieira, 2008). Infections during pregnancy present a great risk for both the mother and her fetus (Vieira, 2008). During antenatal visits, pregnant individuals are examined for intestinal parasitosis, syphilis bacteria, rubella virus and human immunodeficiency virus (HIV). Of importance, the early diagnosis of these infections and early treatment can potentially minimize deleterious impacts to the newborn.

Enteroparasitosis is caused by the intestinal helminths and other protozoa, and approximately 3.5 billion people globally experienced enteroparasitosis (Belloto et al., 2011). Studies have shown that, during pregnancy, infection with intestinal parasites can results in intra-uterine growth Furthermore, enteroparasitosis can contribute anemia as well as deficiencies in iron, protein, folic acids and zinc, which can interfere with the course of pregnancy, leading to possible damages to the fetus (Macedo and Rey, 1996; Souza et al., 2002; Morales et al., 2006).

According to DATASUS (Department of Information Technology for the Brazilian Unified Health System), in Paraná (PR), from 2008 to 2012, a total of 772 cases of congenital syphilis were confirmed (REF). Such high prevalence reflects the mandatory need to examine such infection during pre-natal checks, ultimately reducing the possibility of transmitting *Treponema pallidum*, the etiologic agent of the disease to the newborn (Costa et al., 2013a). Furthermore, syphilis during pregnancy can lead to early and late complications including, fetal and neonatal death, prematurity, low birthweight, physical defects and neurologic damages (Rodrigues and Guimarães, 2004; Amorim and Melo, 2009).

Rubella, on the other hand, is a disease caused by a virus, belonging to the *Rubivirus* genus from the Togaviridae family (Costa et al., 2013b). When the fetus gets infected, high rates (up to 90%) of spontaneous abortion, stillbirth, congenital defects, vision problems, deafness, and heart and mental problems have been observed (Francisco et al., 2013). After vaccination was implemented, there was a reduction of 61.5% in the incidence of rubella, in the period from 1999 to 2001 ranging from 8.85 to 3.3 cases in every 100 thousand inhabitants (Francisco et al., 2013).

Finally, when considering HIV, 0 to 2.0% of pregnant women are carrier of the virus (Amorim and Melo, 2009). Critically, approximately 90% of the cases of HIV infection in people was acquired through vertical transmission (Carvalho and Piccinini, 2006) and therefore, the Brazilian government is dedicating itself to reduce the rate of vertical transmission of the Human Immunodeficiency Virus (Santos and Souza, 2012). When infected, the child usually presents unspecific symptoms, which includes deficiency in immunity that contributes to the child vulnerable to opportunistic infections. Neurological changes due to HIV neurotropism in a brain that is not fully developed, may lead to the delay in neuro-psychomotor development, delay in language learning, mental deficiency, hyporeflexia and pyramidal syndrome (Manfredi et al., 2011).

Vertical transmission of etiologic agents (Human Immunodeficiency Virus (HIV), *Treponema pallidum* (Syphilis) and *Togavirus* (Rubella), can take place during pregnancy and at the time of birth or during breastfeeding (Araujo et al., 2008; Amorim and Melo, 2009; Costa et al., 2013a, b). It is important to emphasize that in the case of enteroparasitosis, there is no transmission of the parasite but infected pregnant women experienced nutrients deficiencies that are necessary for the fetus (Vieira, 2008).

Considering the importance of these diseases and the damage they can cause to the newborn, it is necessary to diagnose infections during prenatal examinations. Thus, the objective of this study is to determine the prevalence of anti-HIV antibodies, anti-rubella, syphilis reagents and enteroparasitosis in pregnant women during prenatal monitoring in the Basic Health Units of the city of Umuarama (PR) and to verify possible economic and behavioral.

MATERIALS AND METHODS

Study location and sampling

The city of Umuarama is in the Northwestern region in the state of Paraná, Brazil (latitude 23°47' 55 South and longitude 53°18' 48 West), with 100676 inhabitants (IBGE, 2010).

To determine the size of the sample, an estimated number of 1,000 pregnant women/year seen at 23 Basic Health Units (UBS) in PR was used. For this study, the expected prevalence of 50, 5 error and 5% significance level (p ≤ 0.05) were used, resulting in the minimum ideal number of 278 samples.

This research included pregnant women who agreed in signing the free and informed consent term (TCLE) and who had their pre-natal in one of the 23 UBSs in Umuarama (PR), in any phase during their pregnancy. Blood and fecal collection were performed between June 2012 and May 2013, totaling 690 samples.

Laboratory examinations

Diagnostic tests were performed at the Clinical Analysis Laboratory of UNIPAR. For detection of *T. pallidum,* the samples were submitted to VDRL (Venereal Disease Research Laboratory) and in the presence of agglutination the sample was confirmed by Indirect Immunofluorescence Assay.

For detection of antibodies to rubella virus, serum samples were subjected to IgG and IgM antibody screening by chemiluminescence examination, with reagent IgM higher than 1.0 IU / mL and non-reactive IgM of less than 0.8 IU / ML, reagent IgG of greater than 20.0 IU / mL and non-reactive IgG of less than 10.0 IU / mL (ARCHITECT i2000sr®-Abbott, USA) according to the manufacturer's instructions.

For the HIV scan, a Rapid Immunochemography - HIV 1/2 3.0 strip test (Bioeasy, India) was performed according to the manufacturer's instructions and the reagent samples were confirmed by western blotting. Fecal samples were submitted to Spontaneous Sedimentation, and Centrifuge-fluctuation techniques for parasitological analysis (Hoffman et al., 1934; Faust et al., 1970).

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Table 1. Variables associated to the presence of enteroparasites in pregnant woman seen at the basic health units (UBS) in the city of Umuarama, Paraná, Brazil, 2012-2013.

Variable	Positivity Total (%)	P	OR (CI 95%)
Treated water from public supply			
No	9/39 (23.1)	0.023*	2.53 (1.07-5.85)
Yes	69/651 (10.6)	-	-
Schooling Level			
Basic	33/197 (16.8)	-	-
Secondary	40/412 (9.7)	0.011**	1.87 (1.11-3.16)
Tertiary	5/81(6.2)	0.032**	2.06 (1.08-9.30)
Public supplied sewage system			
No	39/263 (14.8)	0.030*	1.73 (1.05-2.85)
Yes	39/427(9.1)	-	-
Public collection of trash			
No	10/46 (21.7)	0.038*	2.35 (1.04-5.20)
Yes	68/644 (10.6)	-	-

p=probability; *Fisher's Exact Test; **Chi-square corrected by Yates (comparison between 1-2 and 1-3) OR=Odds Ratio; CI=Confidence Interval.

Research instrument

For the detection of variables associated to the different infections, the pregnant women were interviewed. They answered an epidemiological questionnaire containing information on their age, marital status, number of pregnancies, profession, house, level of schooling, per capita income, origin of water consumed, destination of sewage and garbage, consumption of raw fruit and vegetables.

Statistical analysis

The information in the research instrument was stored and analyzed by the EpiInfo Program (Dean, 1996). The Fisher's exact test and the Chi-Square test corrected by Yates were used to verify the association among the variables studied, and the Odds Ratio (OR) was calculated to verify the intensity of associations among the four different infections and the variables studied. For all analyses, 5% significance level (p ≤ 0.05) was used.

RESULTS

Fecal samples from 690 pregnant women were analyzed out of which, 78 (11.30%) samples were positive for eneteroparasites. Among the 78 positive samples, 69 (88.46%) presented monoparasitism and 9 (11.54%) presented polyparasitism.

The most prevalent parasite species was *Endolimax nana* 43/78 (55.13%), followed by *Entamoeba coli* 33/78 (42.31%), *Giardia duodenalis* 5/78 (6.41%), *Enterobius vermicularis* 3/78 (3.85%), *Strongyloides stercoralis* 2/78 (2.56%) and *Ascaris lumbricoides* 01/78 (1.28%).

Regarding the other diseases, 9/690 (1.3%) pregnant

women presented antibodies against syphilis bacteria and 2/690 (0.30%) against the human immunodeficiency virus (HIV). Regarding rubella, 664/690 (96.20%) presented reactivity for IgG and no sample was reacting to IgM. Here, no pregnant woman analyzed presented co-infection, that is, infection by more than one disease, among the four diseases studied herein (Enteroparasitosis, Syphilis, Rubella and HIV). The positivity of pregnant women infected with the respective diseases was not very high when compared with other studies.

Regarding the variables analyzed, the variables associated to enteroparasitary infections were water treated from public suppliers (p=0.023), basic/secondary level of schooling (p=0.011); basic/tertiary level of schooling (p=0.032), sewage destination (p=0.030), and public collection of garbage (p=0.038). For syphilis, the variables associated to the infection were per capita income (p=0.048) and age group (up to 20/21-30: p=0.010; up to 20/older than 31; p=0.022). Regarding HIV and rubella, there were no significant associations with the variables analyzed (Tables 1 and 2).

DISCUSSION

In this study 813 pregnant women were involved, seen at the Basic Health Units in the city of Umuarama (PR). From these, only 690 (84.87%) were tested for four diseases which were researched in this study (enteroparasitosis, syphilis, HIV and rubella). Such

Table 2. Variables associated to the presence of syphilis in pregnant woman seen at the basic health units (UBS) in the city of Umuarama, Paraná, Brazil, 2012-2013.

Variable	Positivity Total (%)	P	OR (CI 95%)
Age group			
Until 20 years old	7/193 (3.6)	-	-
21 -30 years old	2/361 (0.6)	0.010**	6.76 (1.28 - 47.51)
Over 31 years old	0/136	0.022**	-
Income per capita			
≤350	6/237 (2.5)	0.048*	3.87 (0.96-15.65)
>350	3/451 (0.7)		

p=probability; *Fisher's Exact Test; ** Chi-square corrected by Yates (comparison between 1-2 and 1-3) OR=Odds Ratio; CI=Confidence Interval.

information raises concerns, since these pregnant women seem to be unaware of the importance of undergoing such exams. Such unawareness can be attributed to the lack of information on these pathologies, as well as the lack of guidance from local health professionals.

Regarding intestinal parasites, 78 (11.30%) of the 690 pregnant women analyzed were positive to the copro-parasitological tests performed in this study. This result is lower than those found in other states in Brazil, such as Pernambuco (PE), where Souza et al. (2002) found 37.00% positive pregnant women in the pre-natal outpatient clinic at the Instituto Materno Infantil (IMIP) and also in Rio de Janeiro (RJ), where Macedo and Rey (1996) found a prevalence of 37.60% in the pregnant women analyzed. The results of this study are also lower when compared to other countries in Latin America, since Alba et al. (2013) in Bolivia and Acurero et al. (2008) in Venezuela detected 22.52 and 65.90% respectively, of enteroparasites in pregnant women. The prevalence number is also lower when compared to countries in other continents, such as in Nigeria, where Adedoja et al. (2010) found a prevalence of 45.70% in pregnant women infected with intestinal parasites.

Among the several species found in this study, *E. nana* (55.13%), *E. coli* (42.31%) and *G. duodenalis* (6.41%) were the three most prevalent ones. When analyzing the three most frequent species, the results differ from the studies performed in the state of Pernambuco (PE), where the most frequent species found were *Entamoeba histolytica* (13.30%), *A. lumbricoides* (12.00%) and *Trichuris trichiura* (5.40%) (Souza et al., 2002). However, in Rio de Janeiro (RJ), the species detected were *A. lumbricoides* (15.50%), *E. coli* (9.00%) and *T. trichiura* (7.80%) (Macedo and Rey, 1996) and in São Paulo (SP), *A. lumbricoides* (19.00%), *Ancilostomideos* (16.70%) and *T. trichiura* (15.90%) (Guerra et al., 1991) were the most

prevalent ones. However, the results in this study regarding the prevalence of species are similar to the work by Alba et al. (2013) in Bolivia, who detected *E. histolytica* (15.30%), *E. coli* (15.30%) and *Giardia lamblia* (3.60%), and Acurero et al. (2008) in Venezuela, who detected *Blastocystis hominis* (48.30%), *E. nana* (25.00%) and *E. coli* (13.30%).

This difference in the number of infected pregnant women and also in the prevalent parasite species in the different studies and locations demonstrates the variation in relation to the deficit in education, basic sanitation and structure, as well as environmental and climate factors that might ease the survival and dissemination of parasites (Souza et al., 2002; Porta et al., 2014).

The two most prevalent species found in this study were *E. nana* and *E. coli*; two species that are not pathogenic for humans. However, their presence is a strong indicator of low hygiene-sanitary conditions, since the human infection by these parasites and pathogenic intestinal parasites is made by fecal-oral means (Duarte, 2011).

Regarding the variables associated to the enteroparasitary infection, the level of schooling (basic/secondary p= 0.011; basic/tertiary p=0.032) has been considered significant, showing that the prevalence of intestinal parasites is associated to the schooling level. By analyzing the results obtained, it could be observed that the presence of enteroparasitosis increases according to the decrease in schooling level, since 16.8% of pregnant women only with basic schooling were infected, while 9.7% of those women who had secondary schooling were also infected. Among those with tertiary education, only 6.2% were infected. These results are similar to the work by Souza et al. (2002) in Pernambuco (PE), who also noticed a decrease in the number of infected women according to the increase in the schooling levels.

Basic sanitation was also considered as an important variable associated to the infections. In the present study, 14.8% of the pregnant women interviewed did not have treated public sewage in their homes, and 23.10% of the houses of the pregnant women did not have treated water, which corroborated with the work developed in the city of Assis (SP), where Ludwig et al. (1999) found a decrease in the prevalence of intestinal parasitosis when water and sewage (basic sanitation) were treated in the houses of the pregnant women.

The public garbage collection variable (p=0.0038) was also associated to the enteroparasitary infection, which agrees to the work by Furtado and Melo (2011), who reported on the hygiene-sanitary conditions related to the high prevalence of intestinal parasitosis, with special attention to the inefficient garbage collection as an increase factor.

In relation to syphilis, 1.30% of the pregnant women presented antibodies against *Treponema pallidum*. The disease is described in several Brazilian states with

prevalence ranging from 0.20 to 6.00% (Brazil, 2012). This disease, when not detected and treated, can reach the fetus in 70 to 100% of the cases (Costa et al., 2013a). Bastos et al. (2013), in Carapicuíba (SP), detected 0.3% cases of congenital syphilis, and, according to the Ministry of Health (Brazil, 2013), between 1998 and 2012, a total of 80,041 cases of congenital syphilis in children with less than one year old were notified at SINAN (Notification Grievance Information System).

The results in this work are pivotal for the local health secretariat, since congenital syphilis is a disease that can easily be prevented when detected early in the pre-natal period and when the appropriate treatment is followed by the positive pregnant women. Thus, it is fundamental to identify and monitor this disease throughout the entire pregnancy, reducing the number of vertical transmission.

In this study, it was possible to detect two variables associated to the infection by *T. pallidum*. The age group variable (up to 20/ 21-30: p=0.010; up to 20/ older than 31: p=0.022) corroborates with the results by Figueiró-Filho et al. (2012) in Campo Grande (MS) and Costa et al. (2013a) in the state of Ceará (CE), who also observed this variable associated to the infection. In this study the most important fact is that, 7(3.60%) pregnant women aged up to 20 years presented antibodies against the syphilis virus, followed by the 21 to 30 years old age group, with two cases (0.60%). However, in pregnant women aged over 31 years, no positive cases were detected. Such situation shows the lack of knowledge and care in relation to the prevention and treatment of the disease in less experienced pregnant women, who, even in lower number (28.00%) than those aged between 21 and 30 (52.3%), presented the highest prevalence for this disease (Lima et al., 2013).

The *per capita* income variable (p=0.048) was also associated to the syphilis infection, with6 (2.50%) infected pregnant women stating they had salary equal or lower than R$ 350.00 per month.

Lima et al. (2013), studying congenital syphilis, states that this disease is present in greater proportion in low-income families. Such situation can be explained by the lower number of pre-natal consultations, insufficiency of resources for transportation to the laboratories and basic health units (UBS), and lack of financial support, and many others. Magalhães et al. (2013) also found that 80.6% of the pregnant women studied had family income of up to four minimum salaries, concluding that syphilis, even if not being a disease that is restricted to the least privileged classes, is related to the low social-economic level and therefore, public health policies must be implemented to help pregnant women in this sense.

Regarding rubella, the present work did not find any confirmed case of the disease during pregnancy, that is, no IgM positive results were found among the interviewed women. This result is similar to those found in other research, like in Ferenzin et al. (2012), there was no report of pregnant women in the northwestern region of

Paraná, and Inagaki et al. (2009), detected 0.10% of pregnant women infected in Sergipe (SE). However, it is important to emphasize that 26 (3.80) pregnant women were not immunized (presenting antibodies) against this disease. This situation can be explained in two reasons. The first is the non-seroconversion, since this vaccine presents an efficiency of 96.6 to 97%. Despite being a good efficiency, some people do not acquire immunity after vaccination (Candeias et al., 1977), and the second is the absence of vaccine. When question concerning vaccine arise, the pregnant women who were not immunized will reply by saying "we were not sure, if we had been vaccinated against rubella before the pregnancy". Such situation has already been described by Francisco et al. (2013), reporting the absence of information on vaccine status in 9.4% women (10 to 49 years old) interviewed in the city of Campinas (SP), noting a lack of guidance by the health teams before and during immunization on which vaccine is being given and its benefit to the population.

The non-detection of rubella in this study might be associated to the prevention campaigns developed against this disease, and the immunization of women in fertile age, a low-cost action with 97% effectiveness. These campaigns are promoted due to several manifestations (hearing, cardiovascular, neurological, among others) caused by the Congenital Rubella Syndrome (CRS) which generates high direct and indirect costs caused by the disease due to the chronicity and severity of the manifestations (Francisco et al., 2013).

Regarding HIV, this paper detected 0.30% of pregnant women infected. Such result is similar to work by Ferezin et al. (2012), in the Northeastern region of Paraná (PR), and Tavares et al. (2013) in the Federal District (GO), who detected a prevalence of 0.30 and 0.33%, respectively. Nonetheless, this was lower than the results found in the works of Vieira et al. (2011) in Vitória (ES) and Machado-Filho et al. (2010) in western Amazon (AM), who reported 0.44 and 0.60%, respectively. Most HIV vertical transmissions take place at the time of birth (65.00%) and the remaining (35.00%) happens during the last weeks of pregnancy.

Breastfeeding is also expressive (7.00 to 22.00%) in the transmission of this disease (Santos and Souza, 2012). As reported by Santos and Souza (2012), the earlier the diagnosis of maternal infection the better the prophylaxis results. Therefore, it is necessary that the anti-HIV test should be offered to all pregnant women at the beginning of their pre-natal evaluation.

It is pivotal that, all pregnant women have equal access to quality pre-natal programs during their entire pregnancy, and for one month after childbirth. These specific exams against diseases that might incur in damages to the newborn, especially the ones discussed herein (HIV, Syphilis, Rubella and Enteroparasitosis), as well as guidance from health professionals (doctors, nurses, biomedics, community health agents) regarding

the importance, consequences and adoption of preventive measures against the main congenital infections may lower these rates. This is the main reason behind the need of a training program for such health professionals.

Conclusion

When analyzing the prevalence of the four diseases researched, it could be noticed that when compared to other studies, these diseases did not present a high prevalence, which demonstrates a better preparation of the pregnant women and also to the local health agents regarding knowledge of the infections.

However, as long as there are infected pregnant women, solution must be made in preventing the diseases by the government. In this case, regarding to education and basic sanitation which were presented as variables with significant association in relation to syphilis and enteroparasitosis, diseases that are related to social-economic and behavioral factors, as well as basic sanitation is regarded to the results obtained in enteroparasitosis.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ETHICAL ASPECTS

This project was approved in meeting of the Ethic Committee, in Research involving Human Beings (CEPEH) at Universidade Paranaense (UNIPAR) under protocol 329/985 from 7/September/2013 to Platform Brazil.

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Full Length Research Paper

Shelf life extension study of *ogi* and *fufu* using bacteriocin isolated from *Lactobacillus acidophilus* of fermented dairy products

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Bacteriocins are antimicrobial peptides produced majorly by lactic acid bacteria (LAB), which act against food spoilage and pathogenic bacteria, thereby, extending the shelf life of food products. Bacteriocin produced by *Lactobacillus acidophilus* (isolated from yoghurt and *nono*) was extracted and incorporated in samples of *ogi* and *fufu* to evaluate its bio-preservative potential. Microbiological analysis was done at the beginning of spoilage test lasting for a period of 60 days. Inoculation of bacteriocin resulted in the extension of shelf life of *ogi* to 60 days and *fufu* beyond 60 days under refrigeration conditions as compared to the uninoculated samples of *ogi* and *fufu* which had a shelf life of 45 and 55 days, respectively.

Key words: Shelf life, bacteriocin, *ogi*, *fufu*, bio-preservative.

INTRODUCTION

Food fermentation processes generally involve the conversion of carbohydrates to alcohol and carbon dioxide or organic acids using yeast or bacteria, mostly under anaerobic conditions (William and Dennis, 2011). Various traditional fermented foods are produced in many Africa countries (Chelule et al., 2010). In Nigeria, however, the most common substrates for fermentation are cassava and cereal grains such as maize, sorghum and millet (Adesokan et al., 2010).

Majority of Nigerian fermented foods are products obtained through lactic acid fermentation (Ogunbanwo et al., 2004). The production of many indigenous African foods is often plagued by premature spoilage due to their high moisture content (Adebayo and Famurewa, 2002). Many food preservation techniques including physical, chemical and biological methods have been explored as remedies to food spoilage.

Until now, approaches to seek improved food safety have relied on the search for more efficient chemical preservatives or on the application of more drastic physical treatment (e.g. high temperature). Nevertheless, these types of solutions have many drawbacks such as the proven toxicity of many of the commonest chemical preservatives (e.g. nitrites), the alteration of the

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organoleptic and nutritional properties of foods, as well as the recent consumer trends in purchase and consumption of food that are generally safe, minimally processed and without additives.

To harmonize consumer demands with the necessary safety standards, traditional means of controlling microbial spoilage and safety hazards in food are being replaced by combinations of innovative technologies that include biological antimicrobial system such as LAB and/or their bacteriocins. The use of LAB and/or their bacteriocins may be an efficient way of extending shelf life and food safety through the inhibition of spoilage and pathogenic bacteria without altering the nutritional quality of raw materials and food products (Ross et al., 2002).

Fermented foods are largely consumed in Africa where they constitute a bulk of the diet among the many African traditionally fermented food stuffs like *ogi* and *fufu*. *Ogi* is popular in Nigeria and in most of West Africa. *Ogi* is a fermented semi-solid food product manufactured from cereals. Gelatinized *ogi* is commonly referred to as pap (already prepared *ogi* into porridge in the presence of water and heat). It is commonly used as weaning food for babies and for young children (Opeifa et al., 2015) and as a standard breakfast cereals in many homes. While *fufu* is a product of fermented cassava tuber, fermented cassava roots are mashed and cooked into dough (Oyinlola et al., 2016). However, these food products are plagued with the problem of reduced shelf life due to random inoculation by spoilage organisms and the humid conditions in the tropics (Adeyeye, 2016).

LAB is the main organisms associated with the fermentation of foods. They had been known to produce antimicrobial substances such as organic acids, diacetyl, hydrogen peroxide and bacteriocin (Stellato et al., 2015). They constitute a group of bacteria that have morphological, metabolic and physiological similarities, and they are also closely related phylogenetically. They are Gram-positive, non-sporulating, non-respiring cocci or rods which ferment carbohydrates to produce lactic acid as their major end product (Pei et al., 2017).

LAB made it possible for human to increase the shelf life of food and food products by utilizing their antimicrobial activities without damaging food contents (Tamang et al., 2016). Lactic acid bacteria are usually found in food products, also known as probiotics and produce bacteriocins which are proteinaceous compound.

LAB is also able to produce small organic substances that contribute to aroma and give specific organoleptic attributes to the products (Hattingh et al., 2015). These micro-organisms are found in milk, meat and fermented products; as well as in fermented vegetables and beverages inhibiting the growth of pathogenic and deteriorating micro-organisms, maintaining the nutritive quality and improving the shelf life of foods.

Bacteriocin is the most potent of all the antimicrobial compounds produced by LAB (Aabha and Santosh, 2015). The term "bacteriocin" comprises of a large and diverse group of ribosomally synthesized extracellular antimicrobial low molecular mass proteins or peptides produced by strains of diverse bacterial species. The antimicrobial activity of this group of natural substances against food borne pathogens, as well as spoilage bacteria, has raised considerable interest for their application in food preservation (Gong et al., 2010; Ana, 2012).

In the past years, a lot of work has aimed to detect, purify and characterize bacterocin, as well as their application in food preservation strategies. Application of bacteriocins may help reduce the use of chemical preservatives and/or the intensity of heat and other physical treatments, satisfying the demands of consumers for foods that are fresh tasting, ready to eat, and lightly preserved (Saito and Nitisinprasert, 2015).

The LAB bacteriocins have many attractive characteristics that make them suitable candidates for use as food preservatives such as protein nature, non-toxic to laboratory animals tested and generally non-immunogenic, inactive against eukaryotic cells, and generally thermo resistant (Oduro-Yeboah, 2016).

MATERIALS AND METHODS

Isolation of *Lactobacillus acidophilus*

L. acidophilus was isolated from dairy products (yoghurt and *nono*) in the laboratory of Kaduna State University (KASU), Kaduna State, Nigeria and was maintained on MRS (De Mann Rogosa Sharpe) agar.

Isolation and identification of lactic acid bacteria (*L. acidophilus*)

LAB were isolated using De Mann Rogosa Sharpe agar (oxiode MRS UK) (MRSA-35 gl^{-1}): peptone (10 g); yeast extract (5.0 g); K_2HPO_4 (2.0 g); $NaNO_3$ (4.0 g); $MnSO_4$ (50 mg); $MgSO_4$ (2.0 g); $(NH_4)_2CO_3$ (2.0 g); Tween (0.1 ml); Lab M powder (10.0 g); and distilled water (1 L). The medium was prepared according to the manufacturer's instruction.

1 ml of each diary product (yoghurt and *nono*) was serially diluted by six folds into sterile test tubes containing 9 ml of distilled H_2O. 1 ml of the diluent 10^{-4} was inoculated into MRS agar plates using pour plate technique, incubated anaerobically at 37°C for 24 h. Bacteria colonies were sub-cultured on MRS agar until discrete colonies were obtained. The pure culture was maintained on agar slant for further characterization and identification. The isolates were characterized based on colony morphology, cell morphology, cell arrangements, motility and biochemical test (Fawole and Oso, 1998; Oyeleke and Manga, 2008). The lactic acid bacteria strain was identified by reference to the Bergey's Manual of Systematic Bacteriology and the Genera of Lactic Acid Bacteria.

Bacteriocin production and extraction

To determine bacteriocin production, the *L. acidophilus* was inoculated into 5 ml of MRS broth under anaerobic conditions and incubated at 37°C for 24 h. The culture extract was obtained by

centrifugation at 10,000 rpm for 15 min. The supernatant was decanted and adjusted to pH 7.0 by adding sodium hydroxide (1 M NaOH) to eliminate any effect of acidity; hydrogen peroxide (H_2O_2) was neutralized by the addition of 5 mg/ml catalase. The mixture of supernatant of LAB culture, NaOH and catalase was filtered and sterilized with a 0.2 μm cellulose acetate filter to obtain cell free bacteriocin.

The obtained cell free supernatant was then precipitated with 40% solution of ammonium sulphate. The mixture was stirred for 90 min at 4°C and then centrifuged at 10,000 rpm for 90 min at 4°C. The precipitates were dissolved in phosphate buffered saline (PBS) and then further used in well diffusion assay to check the antimicrobial activity (Savadogo et al., 2006).

Determination of antibacterial activity of bacteriocin using agar well diffusion method

The total volume of 100 μl from the purified Bacteriocin was placed in Mueller Hinton agar wells (wells were bored using 5 mm cork borer) in Petri dishes seeded with the bio-assay strains (*Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Clostridium* spp*. and *Salmonella dysentriae),* separately and incubated overnight at 37°C. The diameters of the zone of inhibition were recorded by measuring the zone of inhibition around the well using a meter rule (Soomro et al., 2002).

Characterization of bacteriocin

The purified bacteriocin sample was characterized with respect to their heat stability, pH stability and stability during storage.

Heat stability of bacteriocin

The effects of temperature on the bacteriocin were tested by heating the bacteriocin in different test tubes at 40, 60, 100 and 121°C for 15 min and incubated for one hour at 37°C. The treated bacteriocin sample was assayed for antimicrobial activity against *S. aureus and E. coli.*

pH stability of bacteriocin

Aliquots of the bacteriocin were placed in test tubes and the pH value of the contents was adjusted to pH 2-12 individually using either NaOH or HCl (1 M NaOH or 1 M HCl solution) and then incubated for 1 h at 37°C. Thereafter, assay for antimicrobial activity against *S. aureus* and *E. coli* was carried out on the treated bacteriocin samples.

Stability of bacteriocin during storage

The purified bacteriocin was incubated at 37°C and refrigerated at 4, 10 and -20°C, respectively, for thirty days. The residual antimicrobial activity against *S. aureus* and *E. coli* was therafter determined.

Preparation of samples (*ogi* and *fufu*)

For *ogi*, guinea corn (*Sorghum bicolor*) was washed and steeped in water and allowed to ferment for 3 days by the natural flora of the grains. After fermentation, the grains were drained, wet-milled into slurry in a grinding mill and sieved through a fine mesh. The chaff was discarded and the resulting starch paste at the bottom of the container was the *ogi*.

For *fufu*, the fresh cassava (*Manihot esculenta*) tubers were peeled and cut into piece. The cassava tissues were then soaked in water to ferment for 4 days at ambient temperature. The soft fermented cassava tissues were meshed manually and later passed through a plastic sieve. The fibres were discarded and the thick paste (mash) that settled under the water was *fufu*.

Shelf life study of *ogi* and *fufu*

A volume of 5 ml of the bacteriocin obtained was added to 10 g of *ogi* and *fufu,* respectively, and the two different combinations (products) were stored under refrigerated conditions (4°C) for 60 days to determine their shelf-lives in comparison with the experimental control (ogi and *fufu* without bacteriocin). The food samples were then observed daily to determine when spoilage would start. The total microbial load was determined at the beginning of spoilage (the growth of microorganisms on the food samples showed sign of spoilage). Initial plate count of samples (ogi and *fufu*) was serially diluted at 10^6 and the plates were incubated at 37°C for 24 h. The colony count was recorded and compared with the control (without bacteriocin) (Narayanapillai et al., 2012).

Statistical analysis

The data generated were subjected to analysis of variance (ANOVA) using the statistical software package SPSS (Statistical package for social science version 20) and standard error of mean (SEM) for all the graphs plotted were represented with error bars.

RESULTS AND DISCUSSION

Isolation and identification of *L. acidophilus*

The fermented milk products (yoghurt and *nono*) analyzed contained lactic acid bacteria (LAB) in varying proportions which included *L. acidophilus.* It was identified based on colony morphology and characterized based on cell morphology and biochemical test (Table 1).

It is clear that the bacteria was Gram positive, rod shaped coccobacilli occurring singly or in chains. The main task of carbohydrate fermentation test was to investigate the ability of bacteria to ferment different types of carbohydrates. Table 1 shows that the isolated bacteria could ferment maltose, lactose, sucrose and glucose, but not sorbitol and arabinose. Thus, the results obtained coincided with *L. acidophilus* strain characteristics.

Antimicrobial properties of bacteriocins

The results of antagonistic effects of the bacteriocin against 5 pathogenic strains are shown in Table 2. The results revealed that the bacteriocin exhibited strong inhibition on the growth of *Pseudomonas aeruginosa, Escherichia coli* and *Clostridium* spp. (inhibition zone of 16, 15 and 10 mm, respectively) and good inhibition against *S. aureus* and *Salmonella dysentriae* with inhibition zones of 8 and 7 mm, respectively.

Table 1. Biochemical, morphological and physiological characteristics of *L. acidophilus.*

Isolate	*Lactobacillus acidophilus*
Cell morphorlogy	Coccobacilli
Colony morphorlogy	Convex, small, rough edges and white
Gram staining	Gram +
Motility	Non motile
Catalase	-
Indole	-
Arabinose	-
Sorbitol	-
Maltose	+
Lactose	+
Sucrose	+
Glucose	+

(-) Negative, (+) positive.

Table 2. Antimicrobial activity (mm) of crude bacteriocin against test organisms.

Test organism	Bacteriocin	Zone of inhibition (in mm) Gentamycin (control)
Staphylococcus aureus	8	14
Escherichia coli	15	12
Salmonella dysenteriae	7	12
Clostridium spp.	10	13
Pseudomonas aeruginosa	16	17

Table 3. Effects of temperature changes on antimicrobial activity of obtained bacteriocin against *E. coli* and *S. aureus.*

Temperature (°C)	Zone of inhibition (mm)	
	Escherichia coli	*Staphylococcus aureus*
37	10.00 ± 0.50^e	10.00 ± 0.30^e
40	9.00 ± 0.60^d	10.00 ± 0.50^e
60	10.00 ± 0.80^e	8.00 ± 0.40^d
80	7.00 ± 0.40^c	6.00 ± 0.20^c
100	4.00 ± 0.20^b	3.00 ± 0.10^b
121	3.00 ± 0.10^a	2.00 ± 0.10^a

Values are Mean ± SEM; values with different superscript within the column are significantly different (P<0.05) by Duncan multiple range test.

The inhibitory activities shown by this bacteriocin against selected microorganisms revealed that bacteriocin was actually produced by the *Lactobacillus* strain. Earlier reports by Ogunshe et al. (2007) revealed the presence of the compound bacteriocins in the *Lactobacillus* strains and bacteriocins have inhibitory effect against several bacteria. Possession of bacteriocin in Lactobacilli strains indicates their probiotic potentials (Ogunshe et al., 2007).

Heat stability of bacteriocin

The bacteriocin produced by the *L. acidophilus* was heat stable after heat treatment at 37, 40, 60, 80 and 100°C for 15 min.

Table 3 shows that at 37 and 60°C, the highest significant (P < 0.05) zone of inhibition in *E. coli* was 10.00 mm, respectively, while at autoclaving temperature 121°C, it had the lowest zone of inhibition of 3.00 mm, respectively. At 37 and 40°C, the highest significant (P < 0.05) zone of inhibition obtained in *S. aureus* was 10.00 mm, respectively while at temperature 121°C, it had the lowest value of inhibition (2.00 mm). Similar results were recorded for a number of bacteriocins produced by *Lactobacillus* strains which was resistant at 100°C for 15 min (Joshi et al., 2006). The phenomenon of heat stability

Table 4. Effects of pH changes on antimicrobial activity against *E. coli* and *S. aureus.*

pH	Zone of inhibition(mm)	
	Escherichia coli	*Staphylococcus aureus*
2	8.00±0.40[cd]	8.00±0.90[d]
4	9.00±0.80[d]	8.00±0.70[d]
6	7.00±0.60[c]	9.00±0.20[d]
8	5.00±0.30[b]	5.00±0.40[b]
10	3.00±0.70[a]	4.00±0.50[b]
12	3.00±0.50[a]	2.00±0.30[a]

Values are Mean ± SEM; values with different superscript within the column are significantly different (P<0.05) by Duncan multiple range test.

of LAB bacteriocins have been reported earlier in literatures (Moigani and Amirinia, 2007; Ogunbanwo et al., 2004). This present research is also in agreement with the above mentioned reports as it was observed that the bacteriocin used in this study still retained its antimicrobial activity after heating at 121°C for 15 min which means it could be placed within the heat stable low molecular weight group of bacteriocins. This quality of the bacteriocin makes it superior in processed foodstuffs where high heat is applied.

Thermostability is a very useful characteristic in case of using bacteriocin as food preservative, because many food processing procedures involve a heating step (Panesar and Bera, 2011). Moigani and Amirinia (2007) also stated that it is a good property of bacteriocin that it remains effective even at 121°C for 15 min. Due to this property, it remains effective during many food safety processes like pasteurization.

pH stability of bacteriocin

The bacteriocin obtained from *L. acidophilus* in this study was observed to be active over a pH range of 2 to 6, but its activity reduced along neutral to alkaline pH range of 7 to 12 (Table 4). This indicates strong probiotic potential because most of the bacteriocins are resistant to acidic pH more than basic pH. It was observed that at pH 4, the highest significant (p < 0.05) zone of inhibition was 9.00 mm against *E. coli*, while at pH 10 and 12, the lowest zone of inhibition of 3.00 mm was obtained respectively. This implies that bacteriocin obtained from *L. acidophilus* will be effective against Gram negative bacteria such as *E. coli* at acidic pH ranges and not at alkaline pH range. Also, against *S. aureus*, the bacteriocin showed maximum activity at pH 6 which had the highest significant (p < 0.05) zone of inhibition of 9.00 mm, while at pH 12, it had the lowest of 2.00 mm, respectively.

Similar results were reported by Adebayo and Famurewa (2002) who opined that the bacteriocin of

Lactobacillus were active over a wide range of pH 2 to 6 and is the optimum pH range for good inhibitory activity of bacteriocin from *Lactobacillus* strains against a wide range of various pathogenic organisms for example *S. aureus.*, while inactivation occurred mostly at pH 12, suggesting an inhibitory effect of acidity on the growth of *S. aureus* and *E. coli*. Most of lactic acid bacteria excrete acid that has been shown to inhibit growth of pathogens. These observations are in agreement with those reported by Tatsadjieu et al. (2009) in their work with LAB bacteriocins with antimicrobial activities against Chicken *Salmonella enteric and E. coli*. The findings of the present study are also in agreement with those reported by Holzapfel et al. (2010) who showed that *L. plantarum* excreted other compounds such as bacteriocins that inhibited the growth of pathogens. Bacteriocins produced by *L. acidophilus* in this investigation proved to have high activity and stability at pH 2, 4 and 6, respectively against the range of pathogenic and spoilage microorganism (Table 4).

Storage stability of bacteriocin

The bacteriocin produced by *L. acidophilus* was stable at 4 and -20°C, slightly stable at 10°C and unstable at room temperature (24±1°C). Table 5 shows that the maximum zone of inhibition was observed at 4°C against *E. coli* (10 mm) and *S. aureus* (9 mm), respectively. The percentage of effectiveness reduced more at 10°C as compared to 4 and -20°C storage temperature. These implies that the bacteriocin can be stored at -20 and 4°C, indicating that cold temperature may be the most appropriate preservation technique for storing bacteriocins. Similar results was reported by Panesar and Bera (2011) that the high stability of bacteriocin during prolong storage makes them superior and can have a positive impact on their use as bio-preservative with a view to improving the hygiene and safety of food products, especially processed foods. Bacteriocin is pH, heat and storage temperature dependent. So, due to these qualities, bacteriocins produced by LAB constitute the best option as bio-preservative for the preservation of food at commercial level.

Bio-preservative efficiency of bacteriocin in *ogi*

The effectiveness of bacteriocin isolated from *L. acidophilus* to act as bio-preservative and its role in increasing shelf life of *fufu* and *ogi* was checked in the presence of bacteriocin as compared to control.

Table 6 presents the result of the shelf life study carried out on inoculated *ogi* and uninoculated *ogi* (as control) which were monitored and compared throughout the 60 days period of study. In the case of the control sample at day zero, the total microbial count in *ogi* was 8.9 × 10^{5}

Table 5. Effects of storage temperature on bacteriocin activity obtained from *L. acidophilus* during storage.

Temperature (°C)	Zone of inhibition (mm)	
	Escherichia coli	*Staphylococcus aureus*
-20	9.00 ± 0.70^b	9.00 ± 0.60^b
4	10.00 ± 0.80^c	9.00 ± 0.30^b
10	6.00 ± 0.50^a	5.00 ± 0.20^a

Values are Mean ± SEM; values with different superscript within the column are significantly different (P<0.05) by Duncan multiple range test.

Table 6. Effects of bacteriocin on microbial count in *ogi* during storage at 4°C.

Day	Uninoculated *ogi* (control) (CFU/ml)	Ogi Inoculated with bacteriocin (CFU/ml)	P-value
0	$8.9\pm0.02\times10^5$	$3.5\pm0.01\times10^5$	0.000
45	$2.20\pm0.20\times10^6$	$2.8\pm0.02\times10^5$	0.000
50	$2.40\pm0.10\times10^6$	$2.2\pm0.02\times10^5$	0.000
55	$2.70\pm0.20\times10^6$	$1.8\pm0.02\times10^5$	0.000
60	$3.00\pm0.20\times10^6$	$1.5\pm0.20\times10^5$	0.000

Values are Mean ± SEM; p-value less than 0.05 is considered significantly different (P<0.05) by independent sample t-test.

Table 7. Effects of bacteriocin on microbial count in *fufu* during storage at 4°C.

Day	Uninoculated *fufu* (control) (CFU/ml)	Fufu inoculated with bacteriocin (CFU/ml)	P-value
0	$2.8\pm0.20\times10^5$	$1.5\pm0.01\times10^5$	0.000
55	$2.20\pm0.02\times10^6$	$1.1\pm0.01\times10^5$	0.000
60	$2.35\pm0.02\times10^6$	$8\pm0.01\times10^4$	0.000

Values are Mean ± SEM; p-value less than 0.05 is considered significantly different (P<0.05) by independent sample t-test.

CFU/ml, while inoculated *ogi* after 24 h was 3.5×10^6 CFU/ml, this could be due to bactericidal effect of bacteriocin on the microorganisms initially present in the sample (Samelis et al., 2005). During storage of the *ogi*, physical examination showed signs of spoilage after 45 days of storage which caused a sharp increase in the microbial count, 2.2×10^6 CFU/ml which could be due to the initiation of spoilage of *ogi* at 4°C, while inoculated *ogi* after 45 days of storage was 2.8×10^5 CFU/ml. The result depicted that the microbial load of bacteriocin treated samples and the control samples after day 45 were not comparable. The result showed that there was a decrease in the microbial count in the inoculated *ogi* throughout the period of study. After 45 days, the microbial analysis was made after every 5 days interval.

The results of this investigation have shown that the uninoculated *ogi* had a shelf life of 45 days before spoilage occurred. With bacteriocin, the shelf life of *ogi* was increased up to 15 days. Ohenhen and Ikenebomeh

(2007) monitored and compared inoculated fermented *ogi* slurry and uninoculated *ogi* slurry throughout a 60 days period of study in which the uninoculated *ogi* slurry had a mouldy flavour by 40 days of study and by the end of 60 days period of study, it was no longer acceptable nor edible in terms of colour and flavour. This observation correlated with the observation of Mensah et al. (2002) that the method of preparation, handling and environmental factors were probably responsible for the early sign of spoilage observed during the study.

Bio-preservative efficiency of bacteriocin in *fufu*

The effect of bacteriocin on the shelf life of *fufu* is presented in Table 7. The total microbial count in *fufu* (control) was 2.8×10^5 CFU/ml as compared to 8.9×10^6 CFU/ml in *ogi* at day zero. There was a significant difference (P<0.05) in the microbial count of uninoculated

fufu and *ogi*. The initial low microbial load in *fufu* as compared to *ogi* might be due to the heat treatment (heating process) involved in the cooking of the fermented mashed cassava root into ready to eat *fufu* (Omafuvbe et al., 2007).

Similarly, previous studies indicate that the treatment of heat during the meat-ball preparation inactivates vegetative cells, which was indicated by zero microbial count in control and treated samples at day zero (Intarapichet and Gosaarak, 2008). The data also shows the bactericidal effect of bacteriocin has drastic decrease in viable cell count in initial sample (day zero), 1.5×10^5 CFU/ml.

Physical examination of the uninoculated *fufu* showed sign of spoilage after 55 days of storage, with microbial load of 2.2×10^6 CFU/ml, while the bacteriocin treated sample showed 1.1×10^5 CFU/ml viable cell counts; there was a decrease in the number of microbial load in the treated sample. The results of this investigation have shown that the shelf life of inoculated *fufu* was over 60 days; this indicates that the product can keep well beyond this period, whereas the uninoculated *fufu* had a shelf life of 55 days before spoilage started.

Previous studies (Intarapichet and Gosaarak, 2008) also reported that crude bacteriocin from *Lactococcus lactis* TISTR 1401 prevented the growth of total aerobic bacteria up to day 6 in treated meatball batter as compared to control.

Conclusion

The *Lactobacilli* strain used in this study, *L. acidophilus* produced an appreciable quantity of bacteriocin that inhibited the pathogenic organisms associated with spoilage of *ogi* and *fufu* under study.

The use of bacteriocin also extended the shelf life of both *ogi* and *fufu* by 15 and 5 days, respectively. From the results obtained, the bacteriocin could be frozen or refrigerated for storage purposes. A maximum reduction of bacterial load was observed when the bacteriocin was introduced into fermented food products.

The production, purification and characterization of bacteriocins are imperative because bacteriocins are important in the improvement of shelf life of foods products. Bacteriocins obtained from LAB are regarded as safe (GRAS).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Interaction between bacterial biocontrol-agents and strains of *Xanthomonas axonopodis* pv. *phaseoli* effects on biocontrol efficacy of common blight in beans

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The bacterial biocontrol agents (BCAs) treatments were used for seed microbiolization; the bacterial biocontrol agents (BCAs) used selected from previous study on the control of *Xanthomonas-axonopodis* pv. *phaseoli* Xap) includes: *Bacillus* (DFs093, DFs348 and DFs769), *Pseudomonas* (DFs513, DFs831 and DFs842), *Rhodococcus* (DFs843 and DFs912), and the combinations C01 (DFs093+DFs769+DFs831), C02 (DFs093+DFs769+DFs842) and C03 (DFs093+DFs769+DFs348). Sixteen (16) *Xap* strains were collected from several Brazilian regions, and were inoculated in cotyledonary leaves. The symptom development was followed for 10 days. The treatments were compared by the area under the disease progress curve for disease incidence, severity, and index. In general, majority of the BCAs reduced, to some degree, the disease caused by different *Xap* strains. The combination C01, and the isolate DFs831 resulted in highest mean disease control. The data from this study suggest a relationship between the region of origin of *Xap* strain and the efficacy of BCA to control the disease caused by it. The use of combinations of these organisms increased the efficacy of the biocontrol of several strains of the same pathogen. A strain-BCA interaction was shown by data collected from this study, which evidence the importance of selecting a BCA or a combination of BCAs with a wider spectrum of action.

Key words: Biological control, co-inoculation, *Bacillus*, *Pseudomonas*, *Rhodococcus,* bean common blight.

INTRODUCTION

The bacterial blight (*Xanthomonas axonopodis* pv. *phaseoli* (Smith) Dye (*Xap*) of common beans (*Phaseolus* *vulgaris* L.) occurs worldwide and is especially serious in regions of high temperatures and relative humidity. In

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Brazil, the disease commonly occurs in the states of Rio Grande do Sul, Rio de Janeiro, São Paulo, Minas Gerais, Paraná, Santa Catarina and in the central-west region. The disease is very damaging in rainy season crop (Almeida et al., 2015; Fourie, 2002) and losses of up to 50% have been reported (Vieira and Souza, 2000).

Being widely distributed in Brazil, difference in the virulence *Xap* strains occurring in different regions are expected, which can affect the control strategies, especially development of resistant cultivars (Vieira and Souza, 2000). The association between the origin of a *Xap* strain and its virulence has been reported. The strains from temperate regions were found to be less virulent than those from tropical regions (Rava and Romeiro, 1990; Mutlu, 2008). Frequent failure of disease control through the use of resistant cultivars and chemicals has been attributed to the differences in the virulence of the pathogen (Mutlu, 2008), which warrants developing alternate methods, including biological control.

The biological control using bacterial biocontrol agents (BCA) is an alternative that has been tested and has shown potential for control of foliar pathogens (Singh and Siddiqui, 2015; Akhtar and Siddiqui, 2010). There are also many researches that show good results for bean diseases such as *Macrophomina phaseolina* (Torres et al., 2016), *X. axonopodis* pv. *phaseoli* (Zanatta et al., 2007; Sallan, 2011), *Pseudomonas savastanoi* pv. *phaseolicola* (Garret and Schwartz, 1998), *Colletotrichum lindemuthianum* (Corrêa et al., 2008; Bardas et al., 2009) and *Pseudocercospora griseola* (Corrêa et al., 2014). However, the studies that evaluate the efficacy of BCA in controlling the diseases caused by different strains of the same pathogen are rare. Corrêa et al. (2014) pointed out that the control of bacterial pathogens by bacterial BCA may be strain dependent, leading to variability in control efficacy. *In vitro* evaluation of BCA to control bacterial blight of beans gave varied results when confronted with different strains of *Xap* (Silva et al., 2008), although *in vitro* studies are not sufficient to evaluate the efficacy of a BCA (Köhl et al., 2011). Thus this study was done to evaluate the potential of several BCAs, alone or in combination, to control the disease development induced by different strains of *Xap* and determine the interaction between them.

MATERIALS AND METHODS

Origin of the isolates of bacterial BCAs and of *Xap*

The isolates of bacterial BCAs *Bacillus cereus* DFs93 and DFs769 (isolated from soil and snap bean respectively), *Bacillus* sp. DFs348 (isolated from onion leaf), *Pseudomonas veronii* DFs513 (isolated from onion tunic), *P. fluorescens* DFs831 and DFs842 (respectively isolated from snap and rhizosphere soil of common bean), DFs843 and DFs912 (*Rhodococcus fascians* isolated from bean leaf) used in this study were selected from a previous work on the control of *Xap* (Zanatta et al., 2007) and are maintained in the collection of the Plant Bacteriology Laboratory of the Federal University of

Pelotas.

The *Xap* strains were collected from several regions of Brazil (Figure 1), and were tested for virulence before use.

Seed treatment with BCA

Bean seeds were treated with either of the BCAs cultivated for 24 h on medium 523. The bacterial suspensions (20 mL) were prepared in saline solution (NaCl 0.85%) and the concentration of the cell suspension was adjusted to $A_{540} = 0.50$. Combinations C01 (DFs093+DFs769+DFs831), C02 (DFs093+DFs769+DFs842) and C03 (DFs769+DFs348+DFs831) were prepared by mixing in equal volumes (20 mL) suspension of each component isolate ($A_{540} = 0.50$). The selection of these combinations was based on an earlier study about control of *Xap* and growth promotion of bean plants (Santos, 2006).

Twenty-five seeds of bean cultivar BRS Valente were immersed in the respective suspensions of BCA (50 mL), for five hours at 10°C under constant orbital agitation. Control seeds were immersed in saline solution only (Zanatta et al., 2007). The treated twenty-five seeds were planted in a commercial substrate Plantmax® in 500 g pots. The pots were randomly distributed on greenhouse benches. Cotyledonary leaves were detached after 12 days for inoculation with the respective strain of the pathogen.

BCAs spectrum to control bacterial blight caused by different strains of Xap

The strains of Xap, belonging to the collections of the Federal Pelotas University and Federal Viçosa University, were cultivated on the medium 523 (Kado and Heskett, 1970) for 48 h at 28°C. The bacterial suspension of each strain was prepared in saline solution and the bacterial cell concentration was adjusted to $A_{540} = 0.2$. The detached leaves from seedlings originating from seeds treated with the either of the BCA (individually or in combination) or from the control were inoculated with the respective *Xap* strain, with the use of the cutting technique. The two cotyledonary leaves were cut at five locations each with a scissor previously dipped into the inoculum suspension, as described previously by Zanatta et al. (2007). The experiment was conducted in three replications of each treatment.

The symptom development was followed for 10 days with evaluation starting 2-days after inoculation and subsequently at 2-days interval. The disease incidence (DI) was determined by counting the number of cuts with symptoms and the disease severity (DS) estimated on the scale of 0 to 6: 0- no symptoms, 1- discontinuous chlorosis at the cuts, 2- continuous chlorosis at the cuts, 3- chlorosis at the cuts and leaf wilting at leaf border, without crossing the lateral veins, 4- chlorosis and wilt that crossed the lateral veins, 5- chlorosis and wilt at the internal levels of the cut and 6- advanced chlorosis and wilt of the cut area (Rava, 1984).The disease index (IDX) was calculated by multiplying the values of incidence and its respective severity at each day (DIx DS). the general mean disease control was also calculated using [(ID + SD + IDX)/3].

The treatments were compared by the area under the disease progress curve (AUDPC) for disease incidence, severity, and index by the by Scott-Knott test with the use of the R Core Team (2015). The results were expressed as percent of disease control relative to the control plants (without BCA) took as 0% control.

The data of incidence, severity and disease index AUDPCs were subjected to analysis of variance and comparison by Dunnet test (p =5%) using statistical software R (2015). The general means disease control were clustered by Toche method (Cruz, 2006) using the software Statistica® using Euclidean distance.

Strain	Origin
Xap02	RS
Xap06	RS
Xap08	RS
Xap12	RS
Xap13	RS
Xap16	RS
Xap19	RS
Xap20	RS
Xap23	SP
Xap24	PR
Xap25	SP
Xap26	MG
Xap28	RS
Xap29	MS
Xap32	MG
Xap36	DF
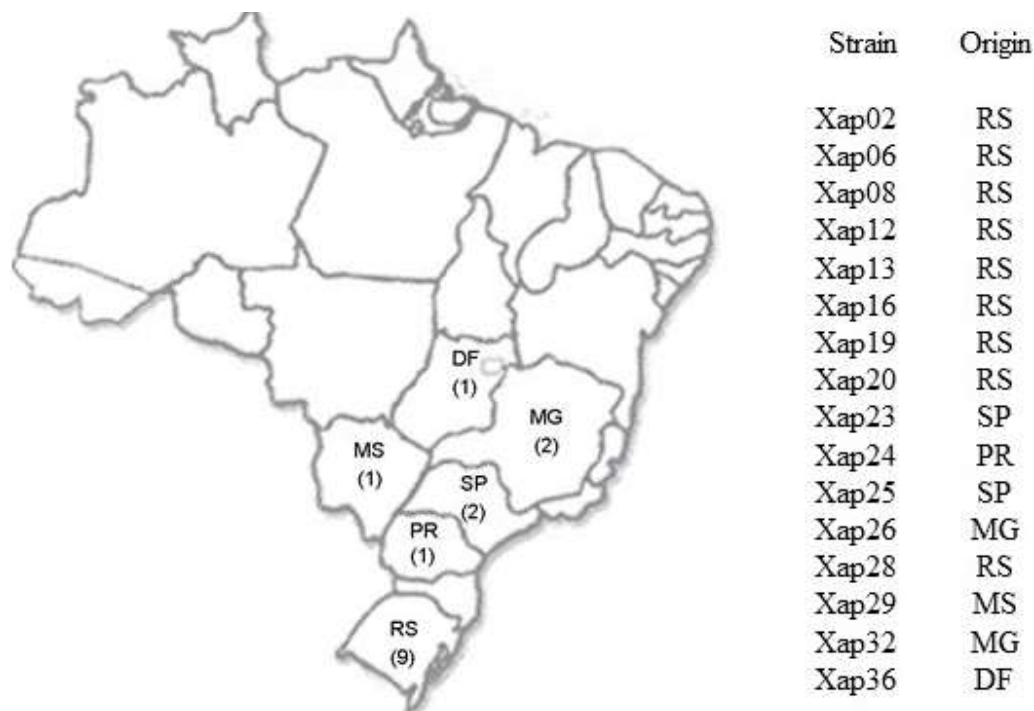
Figure 1. Brazilian states where the pathogenic isolates of *X. axonopodis* pv. *phaseoli (Xap)* came from. DF: Distrito Federal; MG: Minas Gerais State; MS Mato Grosso do Sul State; PR: Paraná State; RS: Rio Grande do Sul State; SP: São Paulo State.

RESULTS

The 16 strains of *Xap* showed different aggressiveness, mainly for severity (ranging10 to 33, average = 25) and disease index (varying from 58 to 260, average = 170) (Figure 2). The most aggressive strains were *Xap*12, *Xap*8*, Xap*13*, Xap*2 and *Xap*28 and the less ones were *Xap*24, *Xap*32*, Xap*6*, Xap*26 and *Xap*25 for all the three diseases variables.

Although the aggressiveness was quite different among the strains, in general, the majority of the BCAs reduced, to some degree, the disease caused by different *Xap* strains (Figures 3 to 5); however, the degree of control as indicated by reduction of DI, DS and the disease index differed significantly among the BCA isolates. Some BCAs did not reduce one (all BCAs), two (six BCAs) or none (nine BCAs) of the variables (DI, DS or IDX) used to quantify the disease induced by a particular *Xap* strain.

The combination C01 and the isolate DFs831 alone, resulted in maximum mean disease control of 36 and 27%, respectively, based on general mean percentage calculated by combining all the three disease indicators, in contrast to only 9% by the least effective isolate DFs093 (Figure 5).

The combination C01 was most efficient of all other combinations and the individual BCA isolates, since it reduced the overall disease induced by any of the *Xap* strain, although no decline in the disease incidence caused by Xap 26 or Xap28 (Figure 3), nor disease severity caused by strains Xap13 or Xap16 or Xap 32 (Figure 4) was observed. For all other *Xap*-strains, despite high variation (0 to 68%) the combination C01 reduced, on an average, DI by 35% (range 0 to 81%), DS by 28% (range 0 to 68%) and disease index by 48% (range 0 to 94%).

The seed treatment with the isolate DFs831 alone (Figures 3 to 5) showed widest spectrum against *Xap* strains compared to the other isolates used singly. The mean DI was reduced by 29% (range 0 to 76%), DS by 28% (range 0 to 82%) and the disease index by 41% (range 0 to 93%). Its performance can be considered only slightly lower than that of the combination C01, not only for general mean disease control, but also because it did not reduce the DS caused by five *Xap* strains only (including same two isolates controlled by C01), and the DI and the disease index caused by only two other strains, which shows that the strain spectrum of DFs831 was narrower than that of the combination C01.

The effects of other BCAs or their combinations were less pronounced. The dendrogram (Figure 6A) shows that seed treatment with the C01 or with DFs831 alone, formed a distinct group, suggesting higher efficacy to control disease induced by strains of *Xap*. The small distance between BCAsDFs769, DFs842 and DFs912 and the combination C02 formed an intermediate group, suggesting a similar spectrum for disease control
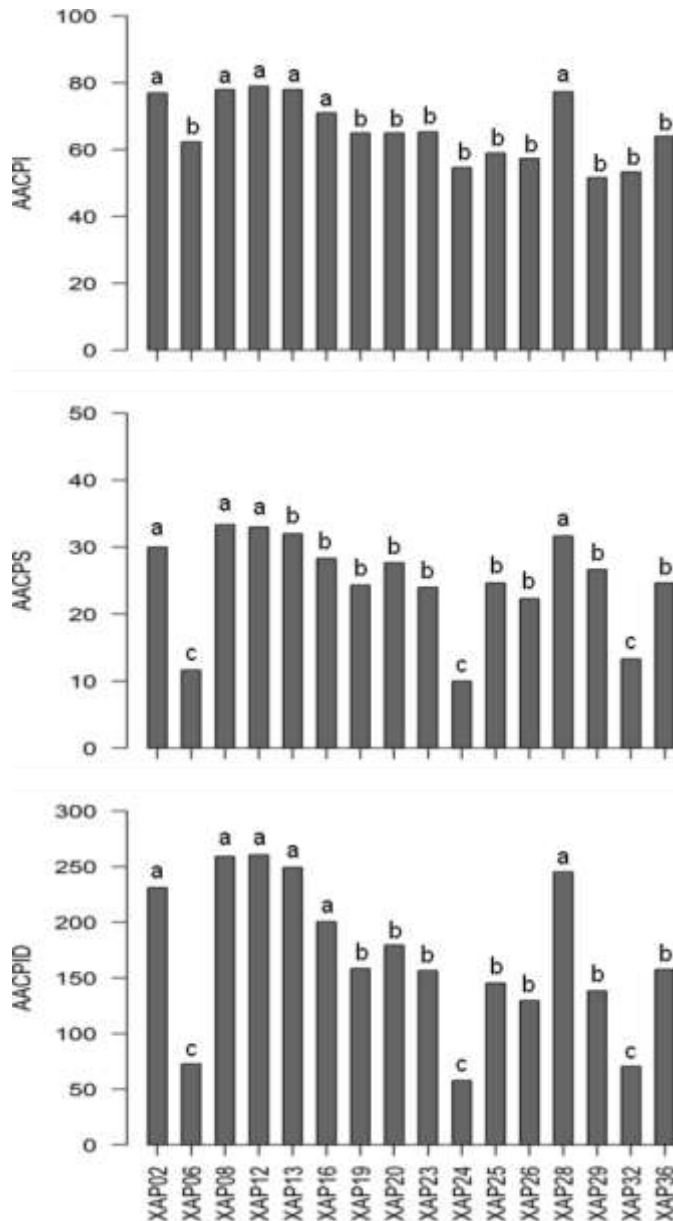
Figure 2. Aggressiveness of different strains of *X. axonopodis* pv. *phaseoli (Xap)* expressed by area under disease progress curve of incidence (AACPI), severity (AACPS) and index (AACPID) on detached cotyledonary leaves of seedlings arising from beans seeds treated with NaCl 0.85% and inoculated by cutting technique. *Means followed by the same letter do not differ by the Scott-Knott test at a significance level of 5%.

(general mean percentage) caused by different strains of *Xap*.

The analysis of the efficacy of all BCAs and their combinations on DI, DS and disease index, showed (Figure 6B) that control of the disease caused by strains *Xap*25 (49.2%) and *Xap*24 (47.8%) formed a separate group, the strain *Xap*12 (38,6%), formed the second group. The BCA shad low disease control efficacy

against *Xap*13 and *Xap*8, each of which formed a single separate group. The control of the disease caused by strains *Xap*6, *Xap*19, *Xap*20, *Xap*23, *Xap*26 and *Xap*29, forming another group was intermediate, showing close similarity among them.

DISCUSSION

The data from this study suggest a relationship between the region of origin of *Xap* strain and the efficacy of BCA to control the disease caused by it. Except for the strain *Xap*12, the strains originating from Rio Grande do Sul were less sensitive to BCAs obtained from the same region, which, at least partially, can be attributed to the co-existence of these organisms (Cook and Baker, 1983). On the other hand, the BCAs which is more efficient in controlling these strains (C01 and DFs831) were obtained from rhizosphere or parts of the bean plant, which confirms the importance of locality or the site from where the BCA was obtained. It is well known that in some pathosystems, the most appropriate site for the finding an effective antagonist is the host itself (Jensen et al., 2016; Mercier, 2006).

The varying effect of BCAs against different strains of the pathogen may also be related to the virulence variability among *Xap* strains (Rava and Romeiro, 1990; Vieira and Souza, 2000; Mutlu, 2008). Generally, in this study, the strains that were more effectively controlled (*Xap*24 and *Xap*25) were less aggressive and in other way, the less controlled (*Xap*8 and *Xap*13) were the more aggressive. However, *Xap*12 was effective controlled (40%) and was one of the most virulent strain (values were same or close to *Xap*8 and slightly bigger than *Xap*13). Additionally, *Xap*6 and *Xap*32 showed low virulence but were in the intermediate group of control (19 and 14% respectively).

The studies showing the disease control ability of BCAs on different strains of the same pathogen are rare, but Naik and Sen (1993) reported considerable variation in the efficacy of a bacterial BCA to control the disease on watermelon caused by nine strains of *Fusarium oxysporum* and *F. solani*, and attributed it to the variability in the virulence of the strains, as also found in this work. Reinforcing results of this study, Corrêa et al. (2014) also observed efficiency variations when the same BCAs of this study to control five isolate of *Curtobacterium flaccumfacien* spv. *flaccumfaciens* in common bean were used.

It is noteworthy that the most effective BCA treatment on different Xap strains was the combination C01, consisting of BCA isolates known for their efficacy when they were used singly (Zanatta et al., 2007; Corrêa et al., 2008; Silva et al., 2008). The use of combinations of these organisms increased the efficacy of the biocontrol of several strains of the same pathogen, and also of the other pathogens, by exercising different modes of action
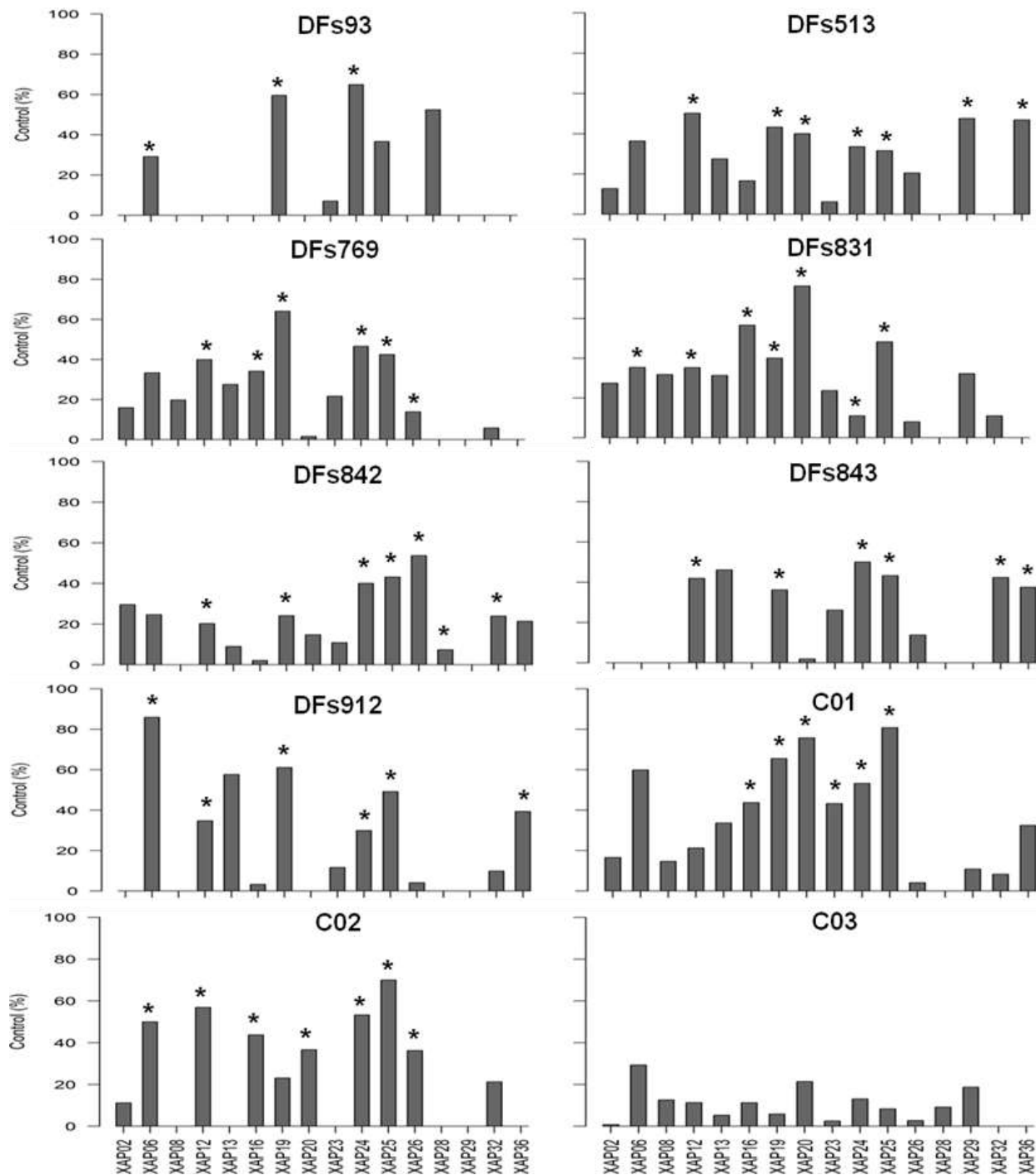
Figure 3. Percent of disease control (incidence) caused by the sixteen strains of *X. axonopodis* pv. *phaseoli* (*Xap*), on detached cotyledonary leaves of seedlings arising from bean seeds treated with biocontrol agents (NaCl control = 0). *Bacillus* (DFs93 and DFs769), *Pseudomonas* (DFs513, DFs831 and DFs842), *Rhodococcus* (DFs843 and DFs912) and the combinations C01 = DFs93+DFs769+DFs842; C02 = DFs93+DFs769+DFs831; C03 = DFs348+DFs769+DFs831. Means followed by * differ by Dunnett test at 5% probability of the control.

(Guetzky et al., 2002; Boer et al., 2003; Wu et al., 2014).

It is also noteworthy that the high spectrum of the isolate DFs831 and its involvement in the combination C01 shows that this isolate has an effective mechanism

to control common bacterial blight, and its efficacy increases when combined with the other isolates, probably by synergism. This synergistic effect is generally due to combination of different modes of action of each
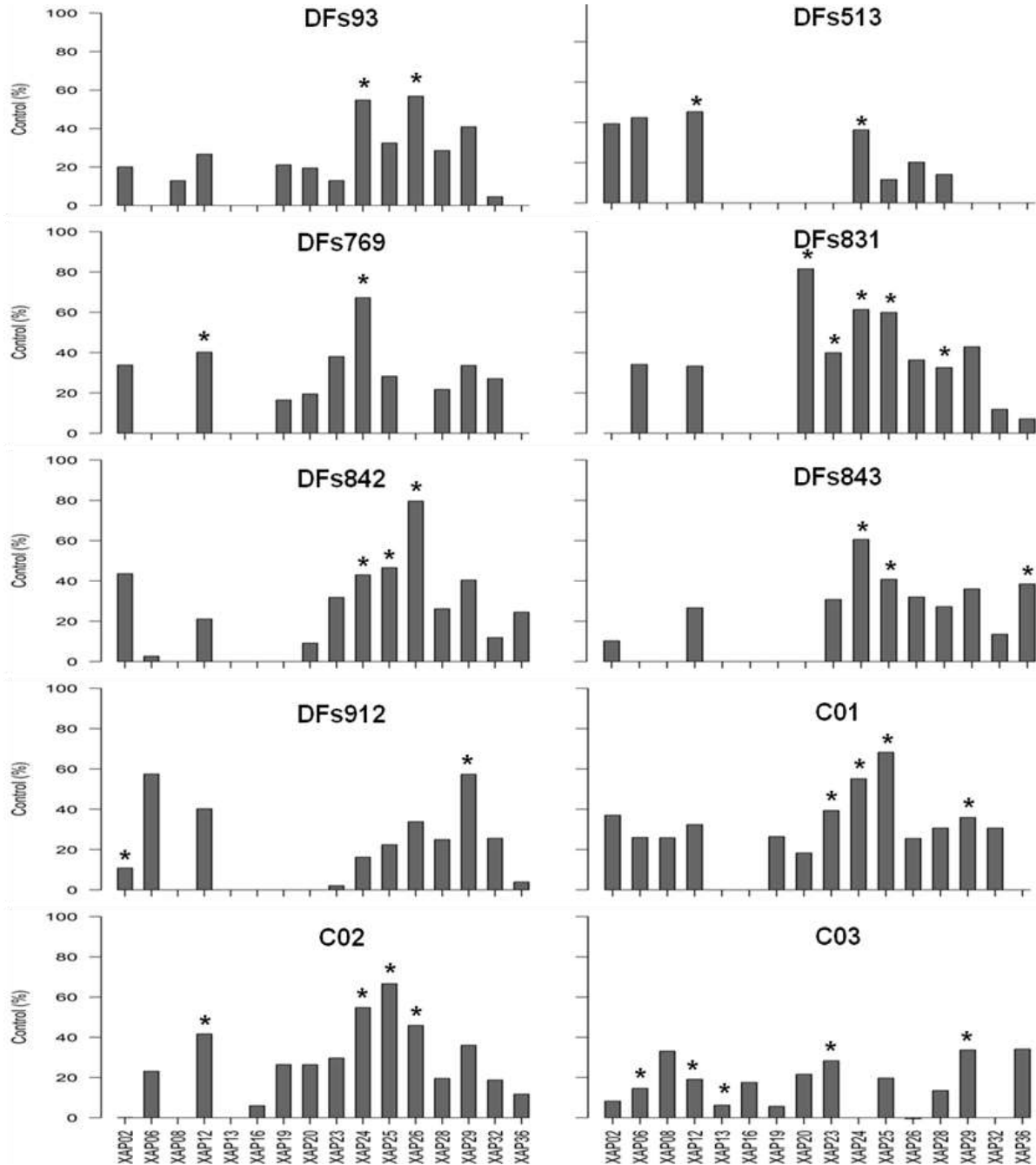
Figure 4. Percent of disease control (severity) caused by the sixteen strains of *X. axonopodis* pv. *phaseoli* (*Xap*), on detached cotyledonary leaves of seedlings arising from bean seeds treated with biocontrol agents (NaCl control = 0). *Bacillus* (DFs93 and DFs769), *Pseudomonas* (DFs513, DFs831 and DFs842), *Rhodococcus* (DFs843 and DFs912) and the combinations C01 = DFs93+DFs769+DFs842; C02 = DFs93+DFs769+DFs831; C03 = DFs348+DFs769+DFs831. Means followed by * differ by Dunnett test at 5% probability of the control.

component BCAs as shown in the combination of *Pichia guilliermondii* and *Bacillus mycoides* to control *Botrytis cinerea* on strawberry leaves, which involves parasitism and production of fungitoxic compounds (Guetzky et al., 2002).

Other mechanisms, however, such as resistance

inducement and competition for iron as shown for isolates of *Pseudomonas putida* (RE8 and WCS358, respectively) should not be underestimated, which have been shown to reduce *Fusarium* wilt in radish (Boer et al., 2003). Involvement of several mechanisms can increase the efficacy of BCAs as shown by Mishra and Arora (2012)
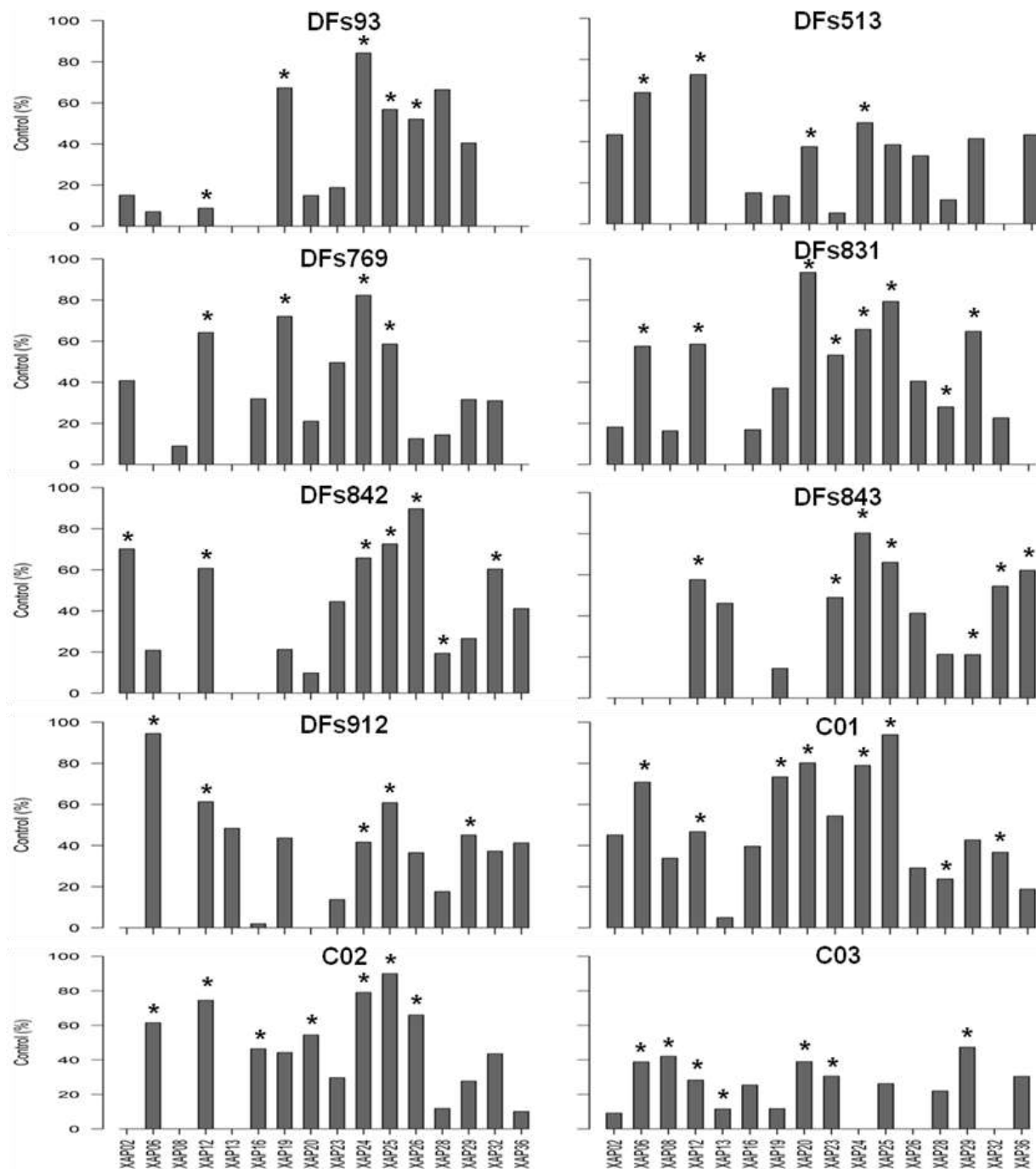
Figure 5. Percent of disease control (index) caused by the sixteen strains of *X. axonopodis* pv. *phaseoli* (*Xap*), on detached cotyledonary leaves of seedlings arising from bean seeds treated with biocontrol agents (NaCl control = 0). *Bacillus* (DFs93 and DFs769), *Pseudomonas* (DFs513, DFs831 and DFs842), *Rhodococcus* (DFs843 and DFs912) and the combinations C01 = DFs93+DFs769+DFs842; C02 = DFs93+DFs769+DFs831; C03 = DFs348+DFs769+DFs831. Means followed by * differ by Dunnett test at 5% probability of the control.

who combined *Pseudomonas* and *Bacillus* to control *Xanthomonas campestris* pv. *campestris,* and found that improved control was due to production of siderphores, autolisines and AHL-lactonases.

The wider spectrum of action and greater control efficacy also can be achieved by combining agents that induce resistance and produce antibiotic as shown for *Bacillus pumilus* (INR7), *Curtobacterium flaccumfaciens* (ME1), *B. subtilis* (GB03) which in different combinations controlled *Pseudomonas syringae* pv. *lachrymans,*
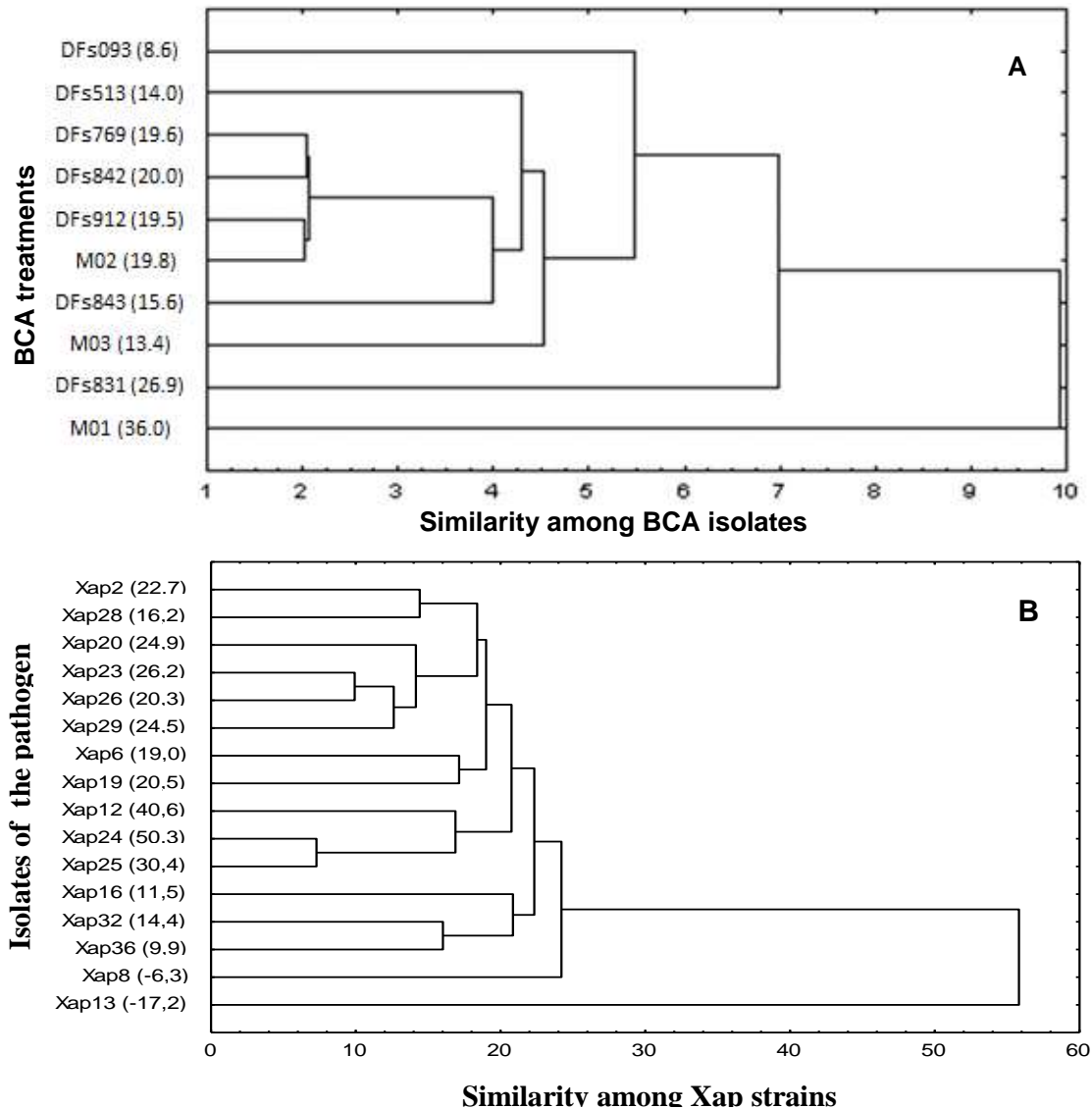
Figure 6. Dendrogram constructed by the software Statistica® relating all the treatments by all biocontrol agents (DFs) considering the general mean percentage of disease control of the sixteen strains of *X. axonopodis* pv. *phaseoli* (A); considering the general mean percentage of control of the sixteen strains of *X. axonopodis* pv. *phaseoli* (*Xap*) (B).

Erwinia tracheiphila and *Colletotrichum orbiculare* (Raupach and Kloepper, 1998) or combining lytic and antibiotic activities with systemic resistance to control bacterial wilt, *Fusarium* wilt, charcoal rot and angular leaf spot of common beans (Corrêa et al., 2014).

The data of this study show that the independent of the virulence of the pathogen´s strain there was strain-BCA interaction, which shows the importance of selecting a BCA or a combination of BCAs with wider spectrum of action, allowing for greater effect under different situations, thus adding to the product stability (Boer et al., 2003; Mercier et al., 2006). It appears that the combination C01 and the isolate DFs831 have the

potential for developing into a practical BCA to control bacterial blight of common beans.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Isolation and characterization of extremely halotolerant *Bacillus* species from Dead Sea black mud and determination of their antimicrobial and hydrolytic activities

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This is the first study that investigated the isolation of extremely halotolerant *Bacillus* species from Dead Sea black mud. Nine isolates obtained from black mud were considered to be extremely halotolerant *Bacillus* based on morphological, physiological, and biochemical properties. Most of their colonies were white to light yellow and circular to irregular. All isolates were Gram-positive rod-shaped endospore-forming bacteria, facultative anaerobes, oxidase negative, catalase positive, mesophilic, extremely halotolerant, reacted positively for tryptophan deaminase and Voges-Proskauer, hydrolyzed gelatin and aesculin, and assimilated potassium gluconate. Most of the isolates were found to hydrolyze o-nitrophenyl-beta-D-galactoside (ONPG) and p-nitrophenyl-β-D-galactopyranoside (PNPG) as well as arginine, and assimilate D-mannose, N-acetylglucosamine, D-maltose, and malic acid. All isolates were considered to be nitrate reducers, six of them were nitrite producers and three were N_2 producers, suggesting that they may play an important role in nitrification-denitrification processes and in the nitrogen cycle in soil. Based on 16S rRNA gene sequence analysis, the isolates were found to share very high identities (97-99%) with their closest phylogenetic relative and they were assigned to eight *Bacillus* species (*B. oceanisediminis*, *B. subtilis*, *B. firmus*, *B. paralicheniformis*, *B. methylotrophicus*, *B. amyloliquefaciens*, *B. sonorensis*, and *B. malikii*). Interestingly, several enzymatic activities were detected from nonhemolytic isolates DSM2 and DSM7 that were identified as *B. paralicheniformis*. It was found that only DSM2 isolate produced promising antimicrobial activities. Its aqueous extract showed the highest significant antifungal activity. Whereas, *n*-butanol and methanol extracts showed significant antibacterial and antifungal activities against human skin pathogens and against other frequent human pathogens.

Key words: Halotolerant, *Bacillus*, nitrification, antimicrobial, hydrolytic.

INTRODUCTION

Halophilic and halotolerant microorganisms that inhabit hypersaline environments can be found in all three domains of life and they exhibit different metabolic pathways. Halophilic microorganisms are classified into mild (require at least 1% NaCl), moderate, and extreme halophiles (require up to 30% NaCl) (Madigan and

Martinko, 2006). Whereas, halotolerant microorganisms possess the ability to grow in the absence of NaCl and also in the presence of high concentrations of NaCl. The halotolerant microorganisms that do not require salt for growth but grow well above 2.5 M salt (that is, above 15% NaCl) are considered extremely halotolerant (Kushner, 1978). Halophilic microorganisms maintain cell structure and function in hypersaline environments by osmoregulation which has been performed by synthesis of compatible solutes strategy, which have been used in industry, and salt-in strategy (Boone and Garrity, 2001; Madigan and Martinko, 2006; Oren, 2006). They play an important role in production of hydrolytic enzymes (Oren, 2006). Halotolerant bacteria are used in numerous industrial processes such as production of salty foods and in maintenance of soil health in saline environment (Vreeland, 1993).

The Dead Sea, in Jordan, is the second largest hypersaline lake in the world after the Great Salt Lake in the western United States. The Dead Sea is the lowest place on earth (Gavrieli et al., 1999). Therefore, it is unique by its high salt concentration especially magnesium, high barometric pressure, high partial oxygen pressure, unique UV radiation that found to be in the range of that reported at high altitudes such as the Alps and the Andes, low humidity, and rarity of rain (Avriel et al., 2011). Furthermore, the Dead Sea is unique in its microbiological environment. The Dead Sea pH is close to neutral (pH 6.1) and from which a large number of novel members of halophilic microorganisms have been provided including extremely halophilic archaea such as *Haloarcula marismortui*, new species of halophilic bacteria such as *Chromohalobacter marismortui*, novel halophilic fungal species such as *Gymnascella marismortui* (Oren, 2010). The Dead Sea was also the natural habitat for the green algae of the genus *Dunaliella* (Oren, 2010).

Dead Sea is rich in highly mineralized and sulfide-rich black mud. Black mudpacks that are abundantly distributed along the shore of the Dead Sea attract patients worldwide, who seek a cure for several skin diseases and rheumatic disorders (Abels et al., 1995; Abels and Kipnis, 1998; Oumeish, 1996; Halevy and Sukenik, 1998; AbdelFattah and Schultz-Makuch, 2004; Portugal-Cohen et al., 2015). Dead Sea black mud minerals have the potential to serve as skincare actives because they affected the expression of various genes that contribute to skin elasticity (Portugal-Cohen et al., 2015). The black mud deposits in three regions where the runoff streams flow into the Dead Sea; including, Jordan Valley, Jordanian Moab Mountains, and Judean Mountains (Rudel, 1993). The mineral mud is also extensively used as an ingredient in cosmetic preparations

(Ma'or et al., 1996). It was reported (Abdel-Fattah, 1997) that the level of trace elements in the Dead Sea mud was less than those in any other sea mud and its major component is carbonate (40%) and less than 1% organic matter.

The purpose of the current study was to isolate and characterize halotolerant *Bacillus* from Dead Sea black mud. The hydrolytic and antimicrobial activities as well as the biochemical properties of the screened isolates were also determined. To our knowledge, this is the first study that investigated the presence of extremely halotolerant *Bacillus* in the black mud of the Dead Sea and examined their enzymatic and antimicrobial activities since they have not been previously reported.

MATERIALS AND METHODS

Collection of samples

Thirty black mud samples were collected from three regions along the shore of the Dead Sea, Jordan (Dead Sea; north, DSN; middle, DSM, and south, DSS). Ten mud samples collected from each region in 500 mL sterile glass containers from 30-40 cm below the surface, 50-100 m away from the sea shoreline and away from human activities.

Isolation of bacteria

An equal volume of sterile distilled water was added to each mud sample and mixed well to get a homogenous mixture. Then, one mililiter from each mixture was serially diluted (10 folds) and 100 μL aliquots were plated by spreading on tryptone soy agar (TSA) plates supplemented with 10% (w/v) NaCl and incubated under aerobic conditions for 72 h at 30°C. The different developing colonies were selected and purified by subculturing on TSA medium, and then, were stored in tryptone soy broth (TSB) containing 20% glycerol at -80°C until usage.

Phenotypic and physiological characterization of the isolates

Colony and cell morphology as well as Gram staining and endospore staining were performed for each isolate according to the standard protocols (Holt et al., 1994). Catalase and oxidase activities were investigated for each isolate. Growth temperature and pH as well as anaerobic growth were also determined. The effect of NaCl on the growth of isolated colonies was also examined.

To determine the growth temperature and pH, the isolates were tested at temperatures in the range 25 to 60°C at 2.5 unit interval and pH in the range 4.0 to 12.0 at 0.5 unit interval in nutrient broth (NB) medium. The growth of isolates was determined after 48 h of incubation by McFarland standards. The effect of NaCl concentration on the growth of isolates was considered by incubating the isolates at 37°C for 48 h in 10 mL NB medium containing 0.0 to 30% (w/v) NaCl at 2.5% interval.

The hemolytic activity of the isolates was tested on blood agar medium containing 5% (w/v) fresh human erythrocytes, by inoculating 50 μL of each prepared extract into each well (5 mm

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i.d.) prepared on the blood agar plates. The type of hemolysis was determined after incubation of plates at 37°C for 48 h (Carillo et al., 1996).

On the other hand, the biochemical properties of the isolates were recognized based on API-20E and API-20NE systems (BioMerieux, USA). In addition, several enzymatic activities of the isolates were determined by API-ZYM system (BioMerieux, USA) and the enzymatic activity of amylase and caseinase were determined for isolates according to Harley and Prescott (2002).

Molecular characterization of the isolates

Genomic DNA extraction

The bacterial isolates as well as the reference strains *B. cereus* ATCC 14579 and *E. coli* ATCC 8739 were inoculated into 20 mL of Luria Bertani (LB) broth and incubated overnight at 37°C with shaking at 150 rpm. Cultures were centrifuged at 14000 rpm for 5 min. Cell pellets were washed three times, then used for DNA isolation using Wizard Genomic DNA purification kit (Promega, USA, part no. A1120) according to the manufacturer's instructions. The extracted genomic DNA was electrophresized in 1% (w/v) agarose gel and photographed by UV Transillumination (Perez-Roth et al., 2001).

PCR amplification of the 16S rRNA gene

The 16S rRNA gene of the isolates and the reference strains was amplified by adding 1 μL of cell culture to a thermocycler microtube containing 5 μL of 5X *Taq* buffer, 0.5 μL of each 10 μM Fd1 and Rd1 primers, 3 μL of 25 mM MgCl$_2$, 0.5 μL of 25 mM dNTPs, 0.25 μL of *Taq* polymerase (5U μL^{-1}), and 38 μL of sterilized distilled water. Universal primers Fd1 and Rd1 (Fd1, 5`-AGAGTTTGATCCTGGCTCAG-3` and Rd1, 5`-AAGGAGGTGATCCAGCC-3`) were used to obtain a PCR product of ~1.5 kb corresponding to base positions 8-1542 based on *E. coli* numbering of the 16S rRNA gene (Winker and Woese, 1991). PCR program used was an initial denaturation for 1 min at 95°C and the samples subjected to 30 cycles for 20 s at 95°C, 30 s at 55°C, and 1 min and 30 s at 72°C. This was followed by a final elongation step for 5 min at 72°C. The PCR products were analyzed on 1% (w/v) agarose gels.

Sequencing and phylogenetic analysis

The sequences of the 16S rRNA gene from PCR products of the isolates and the reference strains were determined with an Applied Biosystems model 373A DNA sequencer by using the ABI PRISM cycle sequencing kit (Macrogen, Korea). The sequences were compared with those contained within GenBank (Benson et al., 1999) by using a basic local alignment tool (BLAST) search (Altschul et al., 1990). The most closely related 16S rRNA gene sequences to the isolates of this study were retrieved from the database. Retrieved sequences were then aligned and the phylogenetic tree was constructed by the use of DNAMAN 5.2.9 sequence analysis software. The obtained sequences were also submitted to GenBank to provide an accession number for each sequence. The reference strains *B. cereus* ATCC 14579 and *E. coli* ATCC 8739 were used as in-group and out-group bacteria, respectively.

Antimicrobial activity

Preparation of extracts

Bacterial cultures were grown in 500 mL NB for two weeks and they were centrifuged at 14,000 rpm for 10 min. The supernatant was extracted with equal volume of different solvent types (*n*-butanol, methanol, ethanol, acetone, and water) for two weeks at room temperature with shaking at 150 rpm. The extracts were then filtered through 0.45 µm membrane syringe filter. The filtrate was evaporated at 40°C in water bath. After evaporation, the remained residues were resuspended in phosphate buffer saline (PBS) to achieve a concentration of 200 mg/mL concentration and used for screening of antimicrobial activity.

Test microorganisms

In order to examine the antibacterial and antifungal activities of the prepared extracts from black mud isolates, 11 reference bacteria; including, *Staphylococcus aureus* ATCC 25923, Methicillin resistant *S. aureus* ATCC 95047 (MRSA), *Streptococcus pyogenes* ATCC 8668*, Salmonella typhimurium* ATCC 14028, *E. coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 27253, *Klebsiella pneumonia* ATCC 7700, *Klebsiella oxytoca* ATCC 13182*, Enterobacter aerogenes* ATCC 35029, *Proteus mirabillis* ATCC 12453, and *Proteus vulgaris* ATCC 33420 and two reference fungi (*Aspergillus brasiliensis* ATCC 16404 and *Candida albicans* ATCC 10231) were used.

Multidrug resistance of test microorganisms to some standard antibiotics

Seven standard antibiotics (Ampicillin 10 µg, Chloramphenicol 30 µg, Erythromycin 15 µg, Nalidixic acid 30 µg, Penicillin G (10 units), Streptomycin 10 µg, and Vancomycin 30 µg) were tested for multidrug-resistance against test bacteria, and two standard antibiotics (Cycloheximide 250 µg, Nystatin 10 µg) were used to investigate the resistance of test fungi. Aliquots of 50 µL from each test bacterium were swabbed uniformly on nutrient agar (NA) medium and thereafter one disk from each standard antibiotic was placed on NA medium surface and incubated at 37°C for 24 h. For test fungi, the same procedure was accomplished but using potato dextrose agar (PDA) medium and incubation at 28°C for 48 h. The antimicrobial activities were determined by measuring the diameter of generated inhibition zones.

Preparation of inoculums

For antimicrobial activity, reference bacteria and fungi were cultured in NB at 37°C for 24 h and Sabouraund dextrose broth (SDB) at 28°C for 48 h, respectively. The cultures were adjusted to achieve 2×10^6 CFU/mL for bacteria and 2×10^5 spore/mL for fungi. The antimicrobial activities were performed by using agar-well diffusion method (Perez et al., 1990). Aliquots of 50 µL from each test microorganism were swabbed uniformly on NA medium for bacteria and on PDA medium for fungi, and allowed to dry for 5 min. Sterile cork borer (6 mm diameter) was used to make wells in the seeded agar. Then, 50 µL aliquot from each prepared extract was added into each well and allowed to stand on the bench for 1 h for proper diffusion and after that incubated at 37°C for 24 h for test bacteria and at 28°C for 48 h for test fungi. The antimicrobial activities were determined by measuring the diameter of formed inhibition zones. Negative controls using 50 µL PBS were also run in the same manner and parallel to the treatments. These studies were performed in triplicates and all data were expressed as the mean ± standard deviation (SD). For statistical evaluation of data for generated inhibition zones, one-way ANOVA (Tukey's studentized range) was applied and significant differences were considered significant at $P < 0.05$.

Table 1. Phenotypic and growth characteristics of extremely halotolerant bacteria screened from black mud of the Dead Sea.

Isolate	DSN1	DSN2	DSN3	DSM2	DSM3	DSM5	DSM7	DSS3	DSS8
Colony shape	Rigid irregular	Rigid irregular	Rigid irregular	Rigid irregular	Circular	Rigid irregular	Circular	Circular	Mucoid irregular
Pigmentation	White	White	Creamy	White	White	White	Translucent	Light yellow	Paige
Cell shape	Single rods	Coccobacilli	Single rods	Single rods	Single rods	Chain rods	Single rods	Chain rods	Chain rods
Sporulation	+	+	+	+	+	+	+	+	+
Gram stain	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+
Anaerobic growth	+	+	+	+	+	+	+	+	+
Growth range									
NaCl (% (w/v)	0-25	0-20	0-20	0-20	0-30	0-25	0-20	0-20	0-20
pH[a] (Optimum)	4.5-9(6.8)	5-9(6.5)	5-9(6.5)	6-9(6.8)	6-9(6.8)	5-11(6.3)	6.5-9(7.0)	4.5-9(6)	5-9(6.3)
Temperature (°C)	25-45(38)	25-45(37)	25-40(36)	25-45(37)	25-45(38)	25-45(38)	25-45(36)	25-45(37)	25-50(37)

[a]Growth of isolates at different pH measured at 37°C.

RESULTS

Phenotypic characterization

Sixty-two aerobic bacterial isolates were harvested from the collected black mud samples with low colony forming unit (CFU) count (18,520-24,733 CFU/g) and most of them (47 isolates) have *Bacillus* characteristics (data are not shown). Based on morphological, physiological, and biochemical properties, only nine isolates which obtained from the Dead Sea black mud met the criteria of extremely halotolerant *Bacillus* (Tables 1 and 2). These isolates were considered extremely halotolerant *Bacillus* and different based on colonial morphology, Gram staining, cell shape, endospore formation, catalase test, and NaCl requirements for growth (Table 1). Most of the developed colonies were white to light yellow circular to irregular on TSA (Table 1). All isolates comprised Gram-positive rod-shaped endospore-forming microorganisms.

Physiological characterization

All isolates grew aerobically and anaerobically, thus the isolates were considered facultative anaerobes (Table 1). All isolates reacted negatively with oxidase and positively with catalase. As shown in Table 1, all isolates were capable to grow at temperature ranging from 25 to 45°C (optimal growth at temperature 36-37°C) except isolate DSN3 grow up to 40°C, at pH ranging from 5 to 9 (optimum pH for growth is between 6.3 to 7), and able to grow in the range of 0-20% salt concentration. Two isolates (DSN1 and DSM5) were found tolerant to 25% (w/v) NaCl and one isolate (DSM3) tolerated up to 30% (w/v) NaCl.

Biochemical characterization

The isolates were tested by different biochemical tests including API-20E and API-20NE (Table 2).

For API-20E test, it was found that all isolates were positive for tryptophan deaminase, Voges-Proskauer, and gelatinase activity. Ortho-Nitrophenyl-β-galactoside (ONPG) hydrolysis and arginine dihydrolase were found to be positive for all isolates except isolate DSM2 and isolate DSN2, respectively. It was observed that only one isolate (DSM2) utilizes citrate, one isolate (DSM7) ferments D-glucose, and one isolate (DSS3) ferments D-mannitol. Whereas, fermentation or oxidation of inositol, D-sorbitol, L-rhamnose, D-sucrose, D-melibiose, amygdalin, and L-arabinose was negative for all isolates. All isolates reacted negatively to lysine decarboxylase, ornithine decarboxylase, H_2S production, urease, and for indol production. Isolates DSN2, DSM2, DSM5, DSM7, DSS3, and DSS8 were found to be nitrite producers. Whereas, the remaining isolates (DSN1, DSN3, and DSM3) were dinitrogen gas producers. For API-20NE (Table 2), it was observed that all isolates were nitrate reducers but they were unable to reduce nitrite, aesculin

Table 2. Biochemical identification of extremely halotolerant bacteria isolated from Dead Sea black mud.

Isolate	DSN1	DSN2	DSN3	DSM2	DSM3	DSM5	DSM7	DSS3	DSS8
API-20E									
ONPG hydrolysis	+	+	+	-	+	+	+	+	+
Arginine dihydrolase	+	-	+	+	+	+	+	+	+
Lysine decarboxylase	-	-	-	-	-	-	-	-	-
Ornithine decarboxylase	-	-	-	-	-	-	-	-	-
Citrate utilization	-	-	-	+	-	-	-	-	-
H_2S production	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-
Tryptophan deaminase	+	+	+	+	+	+	+	+	+
Indol production	-	-	-	-	-	-	-	-	-
Voges-Proskauer	+	+	+	+	+	+	+	+	+
Gelatinase activity	+	+	+	+	+	+	+	+	+
Fermentation/oxidation									
D-glucose	-	-	-	-	-	-	+	-	-
D-mannitol	-	-	-	-	-	-	-	+	-
Inositol	-	-	-	-	-	-	-	-	-
D-sorbitol	-	-	-	-	-	-	-	-	-
L-rhamnose	-	-	-	-	-	-	-	-	-
D-sucrose	-	-	-	-	-	-	-	-	-
D-melibiose	-	-	-	-	-	-	-	-	-
Amygdalin	-	-	-	-	-	-	-	-	-
L-arabinose	-	-	-	-	-	-	-	-	-
NO_2 production	-	+	-	+	-	+	+	+	+
N_2 production	+	-	+	-	+	-	-	-	-
API-20NE									
NO_3 reduction	+	+	+	+	+	+	+	+	+
NO_2 reduction	-	-	-	-	-	-	-	-	-
Hydrolysis of									
Aesculin	+	+	+	+	+	+	+	+	+
PNPG	+	+	+	-	+	+	+	+	+
Assimilation of									
D-mannose	+	+	+	-	+	+	+	+	+
N-acetylglucosamine	+	+	+	-	+	+	+	+	+
D-maltose	+	+	+	-	+	+	+	+	+
Potassium gluconate	+	+	+	+	+	+	+	+	+
Capric acid	-	-	-	-	-	-	-	+	-
Adipic acid	-	-	-	-	-	-	-	-	-
Malic acid	+	+	+	+	+	+	+	-	+
Phenylacetic acid	-	-	-	-	-	-	-	-	-

hydrolyzers, and potassium gluconate assimilators. Except isolate DSM2, all isolates were found to hydrolyze p-nitrophenyl-β-D-galactopyranoside (PNPG), and assimilate D-mannose, N-acetylglucosamine, and D-maltose. All isolates, except isolate DSS3, were positive for malic acid assimilation and only one isolate was positive for capric acid assimilation. None of the isolates assimilates adipic acid and phenylacetic acid.

Molecular characterization

For further identification of the screened isolates from Dead Sea black mud, genomic DNA was extracted from the isolates and amplified by PCR, and then 16S rRNA gene sequences were analyzed. The 16S rDNA of the isolates was amplified with Fd1 and Rd1 primers. The amplified genomic DNA of the isolates and the reference
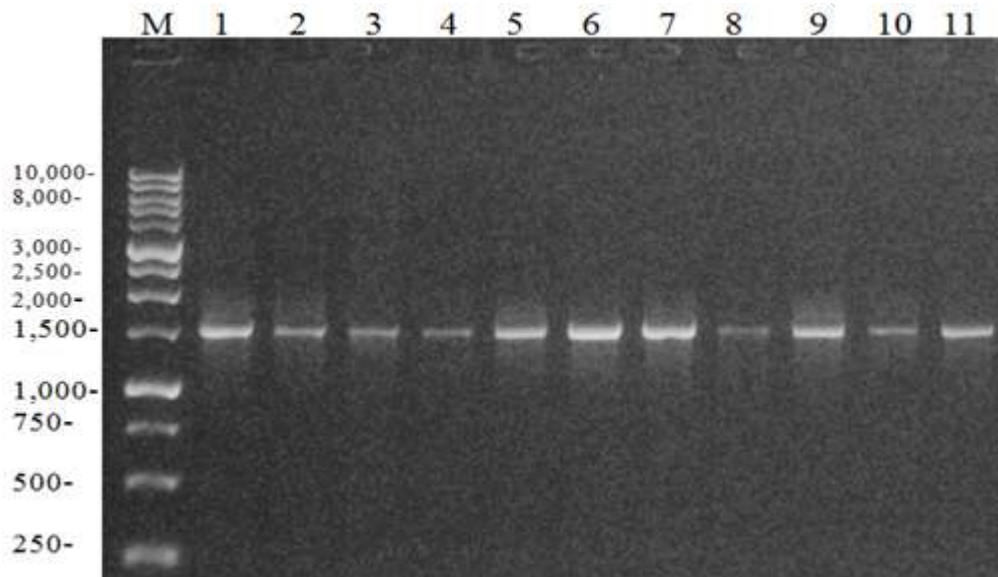
Figure 1. Agarose gel (1%) electrophoresis of PCR amplification of 16S rRNA gene fragments with oligonucleotide forward primer Fd1 and reverse primer Rd1 of nine isolates and two reference strains. Lanes 1-11: Isolates, DSN1, DSN2, DSN3, DSM2, DSM3, DSM5, DSM7, DSS3, DSS8, and the reference strains *B. cereus* ATCC 14579 and *E. coli* ATCC 8739, respectively. Lane M: 1 kb DNA ladder marker (Genedirex, USA).

strains produced PCR band with about 1500 bp in size (Figure 1). The obtained 16S rRNA gene sequences were aligned by BLAST alignment of GenBank sequences. Moreover, the sequences were submitted to the GenBank database and the accession numbers were kindly provided for the submitted sequences (Table 3). Based on BLAST alignment of GenBank sequences to 16S rDNA sequences, all isolates were allocated to the genus *Bacillus* with very high identities ranged from 97 to 99% (Table 3). The reference strain showed 99% similarity to the same species level of its closest phylogenetic relative *B. cereus* ATCC 14579 (Accession no. NR074540). Three isolates (DSN2, DSN3, and DSM5) had 97% sequence identity and the highest GC content. The remaining six isolates showed 99% sequence identity to the closely related phylogenetic *Bacillus* species. In addition, the aligned sequences showed high alignment scores to the closest phylogenetic relative and showed 100% query coverage, 0% gaps, and significant e-values, which equal zero. Based on the sequences alignment, it was clearly observed that the halotolerant *Bacillus* isolates were highly related to eight *Bacillus* species (*oceanisediminis*, *subtilis*, *firmus*, *paralicheniformis*, *methylotrophicus*, *amyloliquefaciens*, *sonorensis* and *malikii*).

Based on the obtained sequences, a phylogenetic tree was constructed (Figure 2). The phylogenetic analysis of the 16S rRNA gene sequences reflected the affiliation of all extremely halotolerant isolates with the genus *Bacillus*, evidencing high bootstrap values at nodes (99-100%), and appeared closely related to the reference strain *B.*

cereus ATCC 14579 with high bootstrap value (97%) (Figure 2). The 16S rRNA gene sequence of the extremely halotolerant isolates were clustered into two subclusters; subcluster-I (99% bootstrap confidence value at the node) that includes six isolates (DSN2, DSM2, DSM3, DSM5, DSM7, and DSS3) and subcluster-II which groups three isolates (DSN1, DSN3, and DSS8) together with 100% bootstrap confidence value at the node.

Hemolytic and hydrolytic activities

The black mud *Bacillus* isolates were tested for their hemolytic activity against human erythrocytes (Table 4). It was found that five *Bacillus* isolates (DSN1, DSN2, DSN3, DSM3 and DSS8) exhibited hemolysis against human erythrocytes but they did not display any of the examined hydrolytic activities including amylase, caseinase, and API-ZYM hydrolysaes (Table 4). However, the remaining non-hemolytic isolates (DSM2, DSM5, DSM7 and DSS3) showed various enzymatic activities. Isolates DSM5 and DSS3 produced alkaline phosphatase only. Isolate DSM7 produced eight enzymatic activities including amylase, caseinase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, and α-chymotrypsin. Whereas, the black mud halotolerant *Bacillus* isolate DSM2 was considered to display several hydrolytic activities which reacted positively for 17 enzymes; including, amylase, caseinase, alkaline

Table 3. The comparison of the 16S rRNA gene sequences of nine extremely halotolerant *Bacillus* isolates harvested from black mud with the 16S rRNA gene sequences in the GenBank.

Isolate[a]	Sequence			Score[e]	% identity[f]
	GenBank accession no.[b]	No. of nucleotides[c] (GC%)	Closest phylogenetic relative[d]		
DSN1	KY848801	1074 (50.6)	*Bacillus oceanisediminis* (HE801980)	1943	99
DSN2	KY848802	1113 (59.8)	*Bacillus subtilis* subsp. *inaquosorum* strain BGSC 3A28 (NR104873)	1890	97
DSN3	KY848803	1086 (50.8)	*Bacillus firmus* strain NBRC 15306 (NR 112635)	1842	97
DSM2	KY848804	1116 (50.5)	*Bacillus paralicheniformis* strain KJ-16 (NR137421)	2050	99
DSM3	KY848805	1306 (49.7)	*Bacillus methylotrophicus* strain CBMB205 (NR116240)	2399	99
DSM5	KY848806	1133 (53.7)	*Bacillus amyloliquefaciens* subsp. *plantarum* strain FZB42 (NR075005)	1921	97
DSM7	KY848807	1115 (49.9)	*B. paralicheniformis* strain KJ-16 (NR137421)	1991	99
DSS3	KY848808	1142 (50.8)	*Bacillus sonorensis* strain NBRC 101234 (NR113993)	2074	99
DSS8	KY848809	1119 (50.6)	*Bacillus malikii* strain NCCP-662 (NR146005)	2043	99
Bacillus cereus ATCC 14579	KY848810	947 (52.2)	*B. cereus* ATCC 14579 (NR074540)	1927	99
Escherichia coli ATCC 8739	KY848811	1291 (51.9)	*E. coli* strain NBRC 102203 (NR114042)	2316	99

[a]*B. cereus* ATCC 14579 was used as reference strain and *E. coli* ATCC 8739 was used as out-group. [b]The accession number for each sequence was provided from GenBank database. [c]The number of 16S rRNA gene nucleotides used for the alignment. [d]GenBank acession number was provided between parentheses. [e]The matching score with the closest phylogenetic relative has 0.0 e-value, 0% gaps, and 100% query coverage. [f]The percentage identity with the 16S rRNA gene sequence of the closest phylogenetic relative of bacteria.

phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-B1-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, and α-glucosidase. However, the hydrolytic activities of β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and β-fucosidase were not detected in all isolates.

Antimicrobial activity

Antibacterial and antifungal activities of the extremely halotolerant *Bacillus* isolated from black mud of the Dead Sea were performed against test bacteria and fungi that exhibited multidrug resistance (Table 5), by preparing bacterial extracts using organic solvents (*n*-butanol, methanol, ethanol, and acetone) and aqueous solvent (water). Unfortunately, it was found that only one isolate (DSM2) exhibited different arrays of antimicrobial activity (Table 5). Aqueous and acetone extracts of DSM2 isolate exhibited no antibacterial activity but produced antifungal activity. Aqueous extract showed the highest significant antifungal activity against test fungi (*A. brasiliensis* ATCC 16404 and *C. albicans* ATCC 10231). Interestingly, both *n*-butanol and methanol extracts of DSM2 isolate were exhibited significant antibacterial and antifungal activities against all test microorganisms investigated in this study. On the other hand, it was observed that ethanol extract of DSM2 exhibited narrower ranges of antibacterial activity against test

bacteria, except *Klebsiella* and *Proteus*, and it did not show inhibitory effects against any test fungus.

DISCUSSION

A little research efforts have focused on isolation of halophilic and halotolerant microorganisms from Dead Sea black mud and no previous study demonstrated the isolation of extremely halotolerant *Bacillus* from black mud. Therefore, this is the first study that investigated the isolation and characterization of extremely halotolerant *Bacillus* from Dead Sea black mud. Furthermore, this is the first study that examined the hydrolytic and the antimicrobial activities of extremely halotolerant *Bacillus* isolated from black mud of

Figure 2. Phylogenetic tree showing the relationships among the 16S rRNA gene sequences of the extremely halotolerant *Bacillus* isolates and the reference strain *B. cereus* ATCC 14579. *E. coli* ATCC 8739 was used as out-group. The accession number for each sequence was provided between parentheses. The phylogenetic tree was built by the neighbor-joining method, using maximum likelihood parameter distance from the partial 16S rRNA gene sequences. The numbers at the nodes are bootstrap confidence values, and are expressed as percentages of 1000 bootstrap replications.

Table 4. Enzymatic and hemolytic activities of the extremely halotolerant *Bacillus* isolated from black mud of the Dead Sea.

Isolate	DSN1	DSN2	DSN3	DSM2	DSM3	DSM5	DSM7	DSS3	DSS8
Hemolysis	+	+	+	-	+	-	-	-	+
Amylase	-	-	-	+	-	-	+	-	-
Caseinase	-	-	-	+	-	-	+	-	-
API ZYM									
Alkaline phosphatase	-	-	-	+	-	+	+	+	-
Esterase (C4)	-	-	-	+	-	-	+	-	-
Esterase lipase (C8)	-	-	-	+	-	-	+	-	-
Lipase (C14)	-	-	-	+	-	-	-	-	-
Leucine arylamidase	-	-	-	+	-	-	+	-	-
Valine arylamidase	-	-	-	+	-	-	+	-	-
Cystine arylamidase	-	-	-	+	-	-	-	-	-
Trypsin	-	-	-	+	-	-	-	-	-
α-Chymotrypsin	-	-	-	+	-	-	+	-	-
Acid phosphatase	-	-	-	+++	-	-	-	-	-
Naphthol-AS-B1-phosphohydrolase	-	-	-	+++	-	-	-	-	-
α-Galactosidase	-	-	-	+	-	-	-	-	-
β-Galactosidase	-	-	-	+	-	-	-	-	-
β-Glucuronidase	-	-	-	+	-	-	-	-	-
α-Glucosidase	-	-	-	+	-	-	-	-	-
β-Glucosidase	-	-	-	-	-	-	-	-	-
N-Acetyl-β-glucosaminidase	-	-	-	-	-	-	-	-	-
α-Mannosidase	-	-	-	-	-	-	-	-	-
β-Fucosidase	-	-	-	-	-	-	-	-	-

Table 5. Antimicrobial activity of extremely halotolerant *Bacillus* isolates DSM2 against multidrug resistant bacteria and fungi.

Test microorganism[a]	Antibiotic resistance[b]	Inhibition Zone[c] (mm)				
		n-Butanol	Methanol	Ethanol	Acetone	Water
Staphylococcus aureus ATCC 25923	A, E, P, V	18.7±1.2[c]	18.3±1.5[c]	12.3±0.6[b]	0[a]	0[a]
MRSA ATCC 95047[a]	A, P	13.3±2.1[c]	15.0±1.7[c]	10.7±1.2[b]	0[a]	0[a]
Streptococcus pyogenes ATCC 8668	A, P, V	19.7±1.6[c]	20.3±3.1[c]	13.0±3.5[b]	0[a]	0[a]
Pseudomonas aeruginosa ATCC 27253	A, C, E, P, S	13.3±0.6[c]	14.0±2.6[c]	10.3±0.6[b]	0[a]	0[a]
Escherichia coli ATCC 8739	A, E, V	19.3±3.0[c]	17.3±0.6[c]	12.7±1.6[b]	0[a]	0[a]
Klebsiella oxytoca ATCC 13182	C, E, N, P, S, V	16.0±1.0[b]	15.7±1.2[b]	0[a]	0[a]	0[a]
Klebsiella pneumonia ATCC 7700	A, P, V	13.7±0.6[b]	17.3±1.5[c]	0[a]	0[a]	0[a]
Enterobacter aerogenes ATCC 35029	A, E, P, V	17.3±3.1[c]	16.7±2.5[c]	11.3±2.5[b]	0[a]	0[a]
Proteus mirabillis ATCC 12453	A, P	13.7±1.6[b]	13.3±3.0[b]	0[a]	0[a]	0[a]
Proteus vulgaris ATCC 33420	A, P, V	16.0±1.7[b]	14.7±1.5[b]	0[a]	0[a]	0[a]
Salmonella typhimurium ATCC 14028	A, N, P, V	20.3±2.5[c]	21.3±1.6[c]	9.7±1.5[b]	0[a]	0[a]
Candida albicans ATCC 10231	NY	15.7±1.2[c]	17.7±1.5[c]	0[a]	11.7±1.2[b]	21.3±1.5[d]
Aspergillus brasiliensis ATCC 16404	CY, NY	19.3±1.5[c]	18.3±2.5[c]	0[a]	9.0±2.0[b]	23.3±0.6[d]

[a]MRSA is methicillin resistant *S. aureus*. [b]A: Ampicillin 10 µg, C: Chloramphenicol 30 µg, E: Erythromycin 15 µg, N: Nalidixic acid 30 µg; P: Penicillin G (10 units), S: Streptomycin10 µg; V: Vancomycin 30 µg; CY: Cycloheximide 250 µg; NY: Nystatin 10 µg. CY and NY activities were not determined for bacteria. The resistance for A, P, and S when inhibition zone (IZ) ≤ 11 mm; for C when IZ ≤ 12 mm, for E, N, and V when IZ ≤ 13 mm; and for CY and NY when IZ ≤ 8 mm. [c]The inhibition ratio was represented as means ± SD. Means ± SD within column followed by the same letter are not significantly different (Tukey's studentized range test: α = 0.05).

the Dead Sea.

In the current study, it was observed that black mud contained low bacterial colony count and dominated by *Bacillus*. This is consistent with Ma'or et al. (2006) findings who reported that most bacteria in black mud of the Dead Sea was endospore-forming bacteria. Based on the phenotypic, physiological, biochemical, and phylogenetic characteristics, nine different bacterial isolates were considered as extremely halotolerant *Bacillus*. All black mud *Bacillus* isolates were Gram-positive, rod-shaped, endospore-forming, facultative anaerobes, and grew at 25 to 45°C, at pH 5 to 9, and at 0 to 20% (w/v) NaCl. Based on those phenotypic and physiological properties, the isolates appeared to be highly closed to extremely halotolerant bacteria of the genus *Bacillus*. Based on biochemical tests, all isolates were found to be positive for tryptophan deaminase, Voges-Proskauer, gelatinase activity, aesculin hydrolysis, and potassium gluconate assimilation. Most of the isolates were found to be positive for arginine dihydrolase, ONPG and PNPG hydrolyses, and for assimilation of D-mannose, N-acetylglucosamine, D-maltose, and malic acid. In terms of phenotypic, physiological, and biochemical characters, the identification of those isolates as *Bacillus* was in agreement with previous studies (Holt et al., 1994; Koneman et al., 1997; Fritze, 2004).

Interestingly, it was found that all isolates were nitrate reducers that are capable to reduce nitrate (NO_3) to nitrite (NO_2) but they were unable to reduce nitrite to dinitrogen gas (N_2). On the other hand, six of the isolates were able to produce nitrite and the remaining three isolates were able to produce N_2 from nitrate. Therefore, the obtained isolates in the current study can perform intermediates of nitrification under aerobic conditions and denitrification under anaerobic conditions or low molecular oxygen levels. This result suggests that extremely halotolerant *Bacillus* isolated from the Dead Sea black mud played an important role in nitrification-denitrification processes and in the nitrogen cycle in soil. It was reported previously (Hall, 1986; Zumft, 1997; Zhang et al., 2012) that facultative anaerobic bacteria including *Bacillus* can perform both nitrification and denitrification. Zhang et al. (2012) reported that *B. methylotrophicus* is an efficient heterotrophic nitrification–aerobic denitrification bacteria, this is in agreement with the results of this study. Denitrification is commonly used in wastewater treatment and to prevent ground water pollution with nitrate due to over use of chemical fertilizers in agriculture (Mulvaney et al., 1997; Foglar et al., 2005). Therefore, extremely halotolerant *Bacillus* isolated from the Dead Sea black mud and possibly the black mud itself can be used in treatment processes of wastewater, in protection of groundwater from nitrate pollution, and in maintenance of soil health.

The phylogenetic analysis of the 16S rRNA gene sequences reflected the affiliation of extremely halotolerant isolates with the genus *Bacillus* and clustered together with the reference strain *B. cereus* ATCC 14579 (Figure 2). All isolates shared very high identities (97-99%) with their closest phylogenetic relative. In addition, the reference strain (*B. cereus* ATCC 14579) as well as the out-group strain (*E. coli* ATCC 8739) showed 99% identities to the same species level.

Furthermore, it was revealed that sequences with identities greater than 85, 95 and 97% are assigned to the same phylum, same genus, and same species, respectively (Schloss and Handelsman, 2005). However, most published libraries are restricted to 97 to 99% identity, thus sequence identity equals to or greater than 97% is assigned to the same species level (Stackebrandt and Goebel, 1994). Therefore, in this study, the standard 97% sequence identity with the closely related *Bacillus* was used to assign *Bacillus* isolates to the same species level. Based on this, extremely halotolerant *Bacillus* isolates screened from Dead Sea black mud were assigned to eight *Bacillus* species (Table 3). The identification of the isolated *Bacillus* species in this study as halotolerant *B. oceanisediminis*, *B. subtilis*, *B. firmus*, *B. paralicheniformis*, *B. methylotrophicus*, *B. sonorensis*, and *B. malikii* was in agreement with previous studies (Garabito et al., 1998; Palmisano et al., 2001; Roongsawang et al., 2002; Berrada et al., 2012; Zhang et al., 2012; Abbas et al., 2015; Dunlap et al., 2015) which demonstrated that those *Bacillus* species can tolerate increased salt concentrations. Whereas, the remained *B. amyloliquefaciens* was not previously defined as a halotolerant bacterium but Zar et al. (2013) demonstrated that this bacterium has the ability to produce halotolerant enzymes. The result of sequence analysis obtained in this study is in agreement with Romanovskaya et al. (2014) who isolated three *Bacillus* strains from black mud of the Dead Sea and found them closely related to *B. licheniformis* and *B. subtilis*.

The results presented in this study indicated that only nonhemolytic bacilli produced enzymatic activities (Table 3). Two *Bacillus* isolates DSM2 and DSM7, which have been defined according to 16SrRNA as *B. paralicheniformis* (accession numbers KY848804 and KY848807, respectively), were found to produce some economically important industrial enzymes such as amylase, lipase and several proteases. Interestingly, 17 enzymatic activities were detected from DSM2; including proteolytic enzymes such as trypsin, saccharolytic/amylolytic enzymes such as amylase, lipolytic enzymes such as lipase, and nucleolytic enzymes such as alkaline phosphatase. The positive results on several enzymes activity are indication of potential applications of such bacterial hydrolyases in biotechnology. Therefore, those two isolates could receive considerable attention due to the production of industrially important enzymes that could be used in food industry, bioremediation, and biosynthesis.

In the purpose of screening antimicrobial activities of the isolates, surprisingly all extremely halotoerant *Bacillus* isolates were found to exhibit neither antibacterial activity nor antifungal activity except DSM2 isolate (assigned to *B. paralicheniformis*). It was clearly observed that aqueous extract of DSM2 showed the highest significant antifungal activity against *A. brasiliensis* and *C. albicans*. Extracts prepared by *n-*butanol and methanol showed significant antibacterial and antifungal activities against multidrug resistant human skin pathogens (*S. aureus*, MRSA, *S. pyogenes, P. aeruginosa*, and *C. albicans*) and against other frequent human pathogens (*S. typhimurium*, *E. coli*, *K. pneumonia*, *K. oxytoca, E. aerogenes*, *P. mirabillis*, *P. vulgaris*, and *A. brasiliensis*) that exhibited resistance for at least two antibiotics. Ethanol extracts exhibited antibacterial activity against some test bacteria but they did not show inhibitory effects against test fungi. Ma'or et al. (2006) screened the antimicrobial activity of the Dead Sea black mud but he did not examine the antimicrobial activities of bacteria naturally occurred in the black mud. It was demonstrated that Dead Sea black mud exhibited slight inhibitory effect against the skin pathogen bacterium *Propionibacterium acnes* and the skin pathogen fungus *C. albicans* but the black mud did not show antibacterial effect against *E. coli* and *S. aureus* (Ma'or et al., 2006). Therefore, this is the first study that evaluated the antibacterial and antifungal activities of bacteria isolated from Dead Sea black mud, in particular extremely halotolerant *Bacillus*. Based on the antimicrobial activity result achieved through this study, the treatment of the Dead Sea black mud by extracting solvents before use in therapy to get better effect against pathogenic microorganisms especially skin pathogens needs to be evaluated by further studies.

Syed and Chinthala (2015) found that three *Bacillus* species (*B. licheniformis*, *B. cereus*, and *B. subtilis*) had significant levels of heavy metal detoxification. On the other hand, Momani et al. (2009) revealed that heavy metals content in the black mud of Dead Sea of Jordan was less than their contents in other types of mud. This might be due to detoxification of heavy metals by halotolernat *Bacillus* that dominate black mud as described in this study. Moreover, Abbas et al. (2015) reported that *B. malikii* is heavy metal tolerant, suggesting that extremely halotolerant *Bacillus* in black mud may play a role in lowering heavy metal content in black mud by detoxification processes. This needs further experimental studies.

The results of this study demonstrated that extremely halotolerant *Bacillus* isolated from Dead Sea black mud could be used in several industrial applications such as wastewater treatment, groundwater protection, food industry, and enzyme industry. In addition, the byproducts of DSM2 isolate can be used for pharmaceutical and medicinal purposes for instance treatment of bacterial infections, in particular multidrug resistant bacteria such as methicillin-resistant staphylococcus aureus (MRSA), skin and soft tissues therapies, and other potential medical applications.

CONFLICT OF INTERESTS

The authors has not declared any conflict of interests.

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