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Table of Contents: Volume 10   Number 31   21 August, 2016

ARTICLES

Full Length Research Paper

Relationship between the probiotic *Lactobacillus rhamnosus* and *Enterococcus faecalis* during the biofilm formation

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One of the factors that make the treatment of *Enterococcus faecalis* infections difficult is their ability to form biofilm, as well as their natural and acquired resistance to antibiotics which does not have specific drugs for their inhibition. This fact makes essential the search for alternative treatments, as the use of probiotics strains of *Lactobacillus rhamnosus* has been effective in the treatment of some diseases. In this investigation, the relationship between the probiotic strain of *L. rhamnosus* and *E. faecalis* during the biofilm formation was analyzed. Standardized suspensions used in biofilm development and treatment in different stages of the biofilm formation were prepared. The *L. rhamnosus* suspension was placed in contact for 90 min with *E. faecalis* freshly created biofilms (initial adherence) in the 24 h biofilms. The same was made with *E. faecalis* suspension on *L. rhamnosus* biofilms. *L. rhamnosus* showed no inhibitory effects on *E. faecalis* biofilms formation, with an increase in the counting of colony forming units in the treated groups (p=0.0047, p=0.0060). About the *L. rhamnosus* biofilms, there was no significant difference for both treatment stages. The probiotic strain interfered *in vitro* with the *E. faecalis* biofilm formation, thereby intensifying the growth of *E. faecalis* biofilm.

Key words: *Enterococcus faecalis*, *Lactobacillus rhamnosus*, biofilm, virulence factor, probiotic.

INTRODUCTION

Enterococcus faecalis is the main cause agents of nosocomial infections and even being present on human intestinal microbiota, has been related to many cases of infections in imunossupressed individuals and or/those treated by broad-spectrum antibiotics. This bacterium, is known by its natural resistance to some antibiotics; large

Table 1. *E. faecalis* and *L. rhamnosus* groups divided according to the time of biofilm formation (90 min or 24 h) and interaction solution (*L. rhamnosus* or *E. faecalis* or control – saline solution).

First Suspension	Time of biofilm formation		Interaction solution
	90 min.	24 h	90 min.
Enterococcus faecalis	G1	-	Control - Saline solution
	G2	-	*L. rhamnosus* suspension
	-	G3	Control - Saline solution
	-	G4	*L. rhamnosus* suspension
Lactobacillus rhamnosus	G5	-	Control - Saline solution
	G6	-	*E. faecalis* suspension
	-	G7	Control - Saline solution
	-	G8	*E. faecalis* suspension

capacity of genetic shares between the microbial cells, this potentiates its resistance to some antimicrobial agents such as vancomycin (Arias and Murray, 2012; Heintz et al., 2010; Sartelli, 2010).

The biogenesis and biofilm formation ability also contribute to the treatment of infections caused by *E. faecalis*. A matrix of exopolysaccharides surrounding offers protection against the action of antibiotics and cells of immune system (Aparna and Yadava, 2008). It stimulates the persistence of bacterial infections and supports the cells of this community (Jefferson, 2004; Mohamed and Huang, 2007; Paganelli et al., 2012; Rabin et al., 2015).

With the dissemination of resistant bacterial strains, the development of new drugs and also the search for alternative treatments, such as phytotherapy (Bhardwaj et al., 2013; Castilho et al., 2013; Sponchiado et al., 2014) and phagoterapy (Khalifa et al., 2015). There is also the use of probiotics strains with the intention of colonization and/or growth inhibition (Chapman et al., 2014).

The term probiotic was defined by the World Health Organization (WHO), in 2002, as "the use of live microorganisms administrated in adjusted amounts to promote positive physiological effects in the host". The use is more frequent as biotherapeutics agents, especially in the preventive medicine. The most used bacteria as probiotic are those belonging to the lactic acid bacteria group, where the genus *Lactobacillus* is enclosed (Bubnov et al., 2015). This can intervene with the colonization and proliferation of pathogenic microorganisms, by the production of antimicrobials substances (Fukuda et al., 2011, Oelschlaeger, 2010; Todorov et al., 2011), or by means of immunomodulatory effects (Remus et al., 2011; Suzuki et al., 2008).

Currently, the specie of *Lactobacillus* most studied is *Lactobacillus rhamnosus* because it has good characteristics of growth and adhesion in gut epithelium and this helps in competing with pathogenic micro-organisms on the gastrointestinal tract and intervening in immune system, intensifying the IgA production, stimulating the local release of interferons facilitating the antigenic transport to the lymphoid cells, thus, serving to increase the presentation of these to the Plate of Peyer (Vandenplas et al., 2015; Segers and Leeber, 2014, Gupta and Garg, 2009). In this investigation, the relation between probiotic strain of *L. rhamnosus* and *E. faecalis* during biofilm formation was analyzed.

MATERIALS AND METHODS

E. faecalis (ATCC 29212) and *L. rhamnosus* (ATCC 1465) were cultivated, respectively, on Brain Heart Infusion broth (BHI, Kasvi, Roseto degli Abruzzi, Italy) and MRS *Lactobacillus* (Hymedia, Mumbai, India), and later incubated at 37°C for 24 h, with tension of (5%) of CO_2.

Each 24 h culture was centrifuged (Centribio TDL80-2B) at 843 g for 10 min, and the supernatant was discarded. The pellet was resuspended in sterilized saline solution (NaCl 0.9%) and centrifuged again, with the supernatant discarded at another time. This procedure was repeated three times to remove the culture way residues. From the last deposit was prepared standardized suspensions for spectrophotometry (Femto 432C, São Paulo, Brazil) in wave length of 530 nm, adjusted in 10^7 cells/mL for *E. faecalis* and 10^8 cells/mL for *L. rhamnosus* (absorbance at 0.020 and 0.600 respectively).

These suspensions were used in different biofilm assays, divided in groups according to Table 1.

To the wells of 96-well microtitration plates had been added 200 µL of *E. faecalis* and *L. rhamnosus* suspensions. The plates were incubated at 37°C under agitation (75 rpm, multi-functional agitator Biomixer TS-2000) per 90 min. After this time, the wells were washed three times with sterilized saline solution to remove the cells not adhered. The groups of 90 min experiment (G1, G2, G5 and G6) immediately received the interaction solution. The groups pertaining to the 24-hours experiment (G3, G4, G7 and G8) received 200 µL of BHI broth, was incubated for more than 24 h in 37°C, and was washed three times and then, received the interaction solution.

The interaction solution was 200 µL of *L. rhamnosus* suspension (G2 and G4) or 200 µL of *E. faecalis* suspension (G6 and G8), and the control groups received 200 µL of sterilized saline solution (G1, G3, G5 and G7). The plates were placed under agitation on 75 rpm at 37°C per 90 min. A new laundering was done with sterilized saline solution, for three times, to remove the cells not-adhered. After that, 200 µl of BHI broth was added to each well. The groups G1, G2, G5 and G6 (90 min) were incubated at 37°C for more 48 h (with broth renovation after 24 h), and the groups G3, G4, G7 and
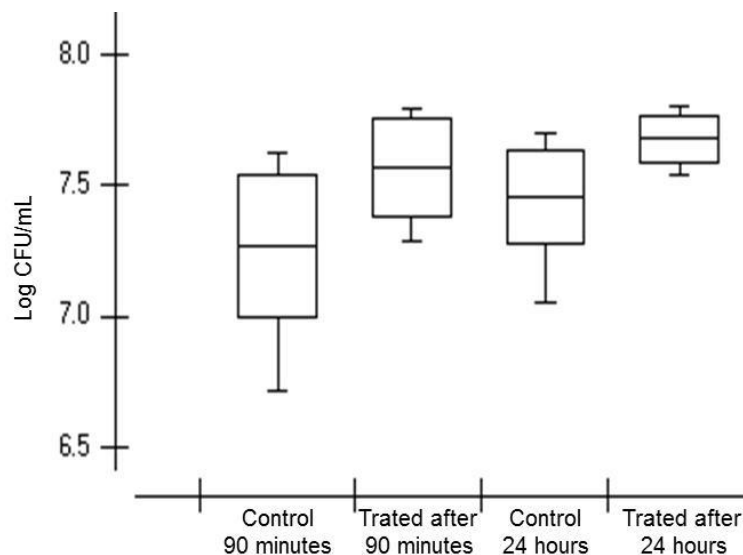
Figure 1. Average and shunting line standard of colonies units' formation for milliliters (in logarithms in base 10) after 90 min of *E. faecalis* biofilm contact (initial stage of biofilm formation and after 24 h of formation) with saline solution (control) or *L. rhamnosus*.

G8 (24h) were incubated for only 24 h. The wells were later washed by sterilized saline solution, three times, to remove the cells not-adhered. Then, 200 µL of sterilized saline was added. The loosed bacterial biofilms were carried by means of friction and homogenization for each well with sterilized tips.

Dilution series (10^{-2} up to 10^{-8}) was prepared for the loosen cells suspension and plating for the drop method (Herigstad et al., 2001), on triplicate, in *Enterococci* agar (Vetec, Rio De Janeiro, Brazil) for groups 1, 2, 3 and 4 and agar *Lactobacillus* MRS (Hymédia, Mumbai, India) for the groups 5, 6, 7 and 8. These were incubated for 24 h in 37°C with CO_2 tension of (5%), for *L. rhamnosus* groups.

The reading was carried out by counting and calculating the number of colony forming units per milliliter (CFU/mL). The number of CFU/mL was transformed into logarithms to base 10 and after analysis of normality, the data was analyzed by Wilcoxon test (program Bioestat 5.3) considering the level of significance of 5%.

RESULTS AND DISCUSSION

After 90 min of exposition, there was a significant positive interference of *L. rhamnosus* probiotic strain, with increase in the CFU/mL counts of *E. faecalis* in biofilms of 90 min (p=0.0047) and of 24 h (p=0.0060) of formation (Figure 1). The average increase was 85% in biofilms of 90 min and 58% within the 24 h biofilm, when compared with the counting in the control groups. There was no significant interference of *E. faecalis* on biofilm formation by *L. rhamnosus* (90 min, p=0.5751 and 24 h, p=0.2300) (Figure 2).

L. rhamnosus has been of the most studied probiotic strain, and its use is considered safe (Vandenplas et al., 2015), however, the interaction with different micro-organism, pathogenic or not, is still unclear. Thus, the present study is considered to evaluate if the *L. rhamnosus* probiotic strain would be capable to interfere with the growth of *E. faecalis* biofilms hindering its formation or reducing the number of cells, as well as if *E. faecalis* could interfere with the biofilm formation by *L. rhamnosus*.

The suspension contained 10^8 CFU/mL of *L. rhamnosus* opted for the use by reason of, the majority of the lyophilized *Lactobacillus*, commercialized in pharmacies, contains enters 10^8 and 10^{11} CFU/g in each dose and in microbial ecology, it is considered that a microorganism influences in the ecosystem where it only meets when its population is equal or superior to 10^7 CFU/g or mL (Stefe et al., 2008).

The choice of the species was because, in case of probiotic consumption, *E. faecalis* and *L. rhamnosus* would interact in the gastrointestinal mucosa, forming biofilms. *E. faecalis* biofilm formation ability is a key-factor in the persistence of bacterial infections and difficulty of treatments (Hoiby et al., 2011; Zoletti et al., 2011). The extracellular polymeric matrix prevents the host cells defenses or restraint to the penetrations of antimicrobials agents (Donlan and Costerton, 2002). *L. rhamnosus* and *E. faecalis* occupy inverse extremities in the current microbiological scene, *E. faecalis* is responsible for innumerable cases of infection in imunossupressed individuals, with strains resistant to antibiotics of broad spectrum, while *L. rhamnosus* is commonly used in probiotic therapy (Vandenplas et al., 2015; Rabin et al., 2015).

Thus, as it has been stated that *L. rhamnosus* presents an ample antimicrobial potential (Dubourg et al., 2015), it
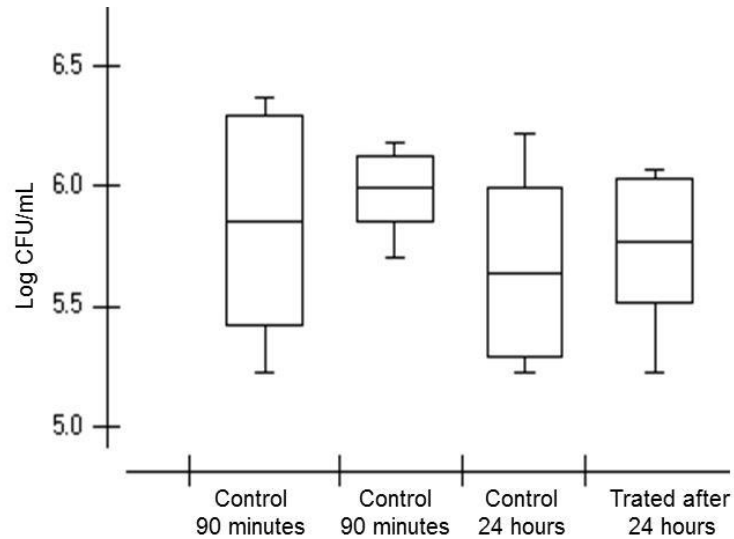
Figure 2. Average and shunting line standard of colonies units' formation for milliliters (in logarithms in base 10) after 90 min of *L. rhamnosus* biofilm contact (initial stage of biofilm formation and after 24 h of formation) with saline solution (control) or *E. faecalis*.

was expected that, when interacting with *E. faecalis*, *L. rhamnosus* would interfere with its growth and harmed the biofilm formation. However, with the methodology used in this research, *L. rhamnosus* not only did not inhibit the growth of *E. faecalis* in biofilm, but also enhanced its growth.

The metabolic and structural features of *E. faecalis* allow its adaptation (modification) in accordance to the ambient and nutritional environmental conditions (Stuart et al., 2006). It is known that in conditions with low glucose availability, the biogenesis of *E. faecalis* biofilm decay, and mechanisms, as increase of the hydro-phobicity of the cell surface, increase for the maintenance of its viability (Ran et al., 2015).

Thus, the results of the *in vitro* interaction of these microorganisms must be considered as the existence of an intraspecific competition between *E. faecalis* and *L. rhamnosus*, and the availability of nutritional resources as determinative for the increase in the counting of microorganisms in the experiment.

Factors like pH and temperature act directly in cell generation time and metabolic taxes of *E. faecalis*. When leaving a favorable environment, pH 6.5 and 37°C, the time of generation cellular is extended, however, this fact is compensated by the increase of the metabolic activity (Morandi et al., 2005). The use of BHI media, which pH is around 7.2 ± 0.2, created an initial favor to the growth of *E. faecalis*, and even the possible posterior production of metabolites, as ascetic and latic acids, by L. rhamnosus was not enough to inhibit its overgrowth.

In vivo tests with administration of *L. rhamnosus* probiotic strain in children colonized by *E. faecalis* strain resistant to vancomycin (VRE), is a significant elimination of the carrier state and increase in gastrointestinal counting of colonies of *Lactobacillus* spp. was observed (Szachta et al., 2011). *In vivo,* conditions are totally different from our experimental condition, where other microorganisms strains are present besides host epithelial and immune cells.

Therefore, although the probiotic strain of *L. rhamnosus* did not present inhibitory effects on *E. faecalis* biofilm *in vitro*, it must be considered its immunomodulatory effect in the host, and does not discard it a prophylactic measure.

Thus, from the methodology used in the present research, it can be concluded that the probiotic strain of *L. rhamnosus* intervened on *E. faecalis* biofilm and intensified its growth.

Conflict of interest

The authors declare that they have no competing interests.

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Full Length Research Paper

Relative efficiency and response of promiscuous soybean to rhizobia inoculant in Savanna region of Nigeria

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Inoculation of promiscuous soybean with rhizobia has been proven to improve yield. Screenhouse pot experiments were conducted to assess the response of promiscuous soybean, TGx1448-2E to rhizobia inoculation and to determine the relative efficiency of indigenous rhizobia from soils collected from the Northern and Southern Guinea and Sudan Savanna agro-ecological zones of Nigeria. Soils were collected at 0 to 20 cm depth from 45 sites spread in 15 local government areas of Kaduna and Kano states. The treatments used were uninoculated control, mineral nitrogen and Legumefix, a commercial rhizobia inoculant which were arranged in randomized complete block design and replicated three times. Nodulation and biomass were assessed at eight weeks after planting. Response to inoculation and relative efficiency were also determined. The results showed that response to inoculation was higher in the Sudan Savanna than in the Northern Guinea Savanna. These were observed in 12 out of 21 and 9 out of 19 sites in the Sudan Savanna and Northern Guinea Savanna, respectively. Of these, inoculation response reached 62 and 90% in two sites in the Sudan Savanna while it barely reached 20% in the remaining sites. The results also showed a relative efficiency which is greater than 100% in some locations. This implies that the indigenous rhizobia populations were superior to mineral nitrogen and Legumefix inoculant. Notable among the locations were ALB 1, GRK 1, SOB 4 and KOL, which spread across all the agro-ecological zones. Strains from such locations have high potential for use as inoculants.

Key words: Legumefix, mineral nitrogen, inoculation, indigenous strains, agro-ecological zones.

INTRODUCTION

Soybean can contribute to the N economy of nutrient-poor soils through its capacity to form symbiotic relationship with different rhizobia species. It is estimated that rhizobia can fix about 50 to 300 kg N ha^{-1} (Bokhtiar and Sakurai, 2005). Thus, their contribution to the N economy of the soil can be quite substantial. Soybean

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Table 1. Sampling sites and their codes.

Location (LGAs)	Code	Site
Garko	GRK	6
Bunkure	BKR	2
Albasu	ALB	3
Minjibir	MJB	1
Bichi	BCH	3
Wudil	WDL	2
Dawakin Kudu	DKD	2
Warawa	WRA	2
Giwa	GWA	5
Soba	SOB	6
Igabi	IGB	4
Tudun Wada	TWD	4
Kachia	KCH	3
Kolosok	KOL	1
Zonkwa	ZKW	1
Total	-	45

has the capability to improve soil fertility in cereal based cropping systems in the Guinea Savanna (Yusuf et al., 2006). It can fix as much as 100 kilograms of nitrogen per hectare (Sanginga et al., 2003).

In the early 1980s, it was assumed that most tropical countries did not have the facilities and personnel required for inoculum production, its storage, and distribution. They depended on the importation of the crop's final product (Pulver et al., 1982). Soybean breeders at the International Institute of Tropical Agriculture (IITA), Nigeria developed promiscuous soybean genotypes in order to take advantage of high yielding American and promiscuous Asian soybean varieties that were introduced to the country. These genotypes, known as Tropical Glycine cross (TGx) nodulate effectively with Bradyrhizobium spp. populations that are indigenous to African soils (Abaidoo et al., 2007). Okereke et al. (2000) reported that promiscuous soybean may also need to be inoculated with exotic Bradyrhizobia depending on the effectiveness and population of the indigenous bradyrhizobia in the locality.

Furthermore, in order to ensure optimum productivity by these promiscuous genotypes, it is essential to assess the relative effectiveness of the indigenous rhizobia in the soil. The number of indigenous Bradyrhizobia as well as the soil available nitrogen has been cited as some of the main factors that have a significant effect on symbiotic relationships (Keyser and Li, 1992; Maingi et al, 2006). Sanginga et al. (1999) found that the need for inoculation of promiscuous soybean depended on the effectiveness of indigenous Bradyrhizobia population. An increase in nodulation and N_2 fixation of promiscuous soybean cultivars using commercial Bradyrhizobium inoculant was reported by Okereke and Eaglesham (1993) and Yusuf et

al. (2012), while Wasike et al. (2009) reported nodulation of promiscuous soybean varieties with indigenous rhizobia in Western and Eastern Kenya. Most of the studies conducted in Nigeria are limited in terms of number and location. They are few studies mostly conducted on-station.

This study was conducted to assess the response of promiscuous soybean to inoculation in soils of the moist Savanna of Nigeria and also to examine the relative effect of the indigenous rhizobia population on soybean biomass. The present investigation attempts to expand on the existing studies on the response of promiscuous soybean to inoculation in these agro-ecologies.

MATERIALS AND METHODS

Study location

The Northern Guinea Savanna of Nigeria covers an area of about 34 million hectares in West and Central Africa (Kolawole et al, 2007). Its temperatures range from 27.3-34.0°C (maximum) and 18.6-21.6°C (minimum). The mean annual rainfall of the area is between 1200 and 1700 mm, with a growing period between May and October (Wall, 1979). The Southern Guinea Savanna is characterized by a mean annual rainfall of 1284mm and a maximum temperature of about 33.5°C (Ojanuga, 2006). The Sudan Savanna zone covers about 22.8 million hectares (Manyong et al., 1995), and it has high annual average temperature of 28 to 32°C (Sowunmi and Akintola, 2010) and annual average rainfall of 884 to 1200 mm (Shehu et al., 2015).

The experiment was set up in a screenhouse in the Department of Soil Science, Ahmadu Bello University, Zaria (110 9' N, 70 36' E). Soil samples were collected at 0 to 20 cm depth from 45 sites in 14 local government areas (LGAs) spread across three agro-ecological zones of Nigeria (Table 1). Figure 1 shows a map of the sampling distribution. The samples were bulked, air dried, crushed and sieved through a 4 mm mesh (for planting) and 2 mm and 0.5 mm mesh (for physicochemical analysis). Plastic pots of about 16.5 cm in diameter and 16.5 cm height with a capacity of 3.5 litres and drainage holes were filled with 4 kg of soil samples.

The trial was made up of 3 treatments. The first treatment had mineral nitrogen otherwise known as the reference; the second treatment had a known commercial inoculant (Legumefix) which contained *Bradyrhizobium japonicum* USDA 532c while the third treatment was an uninoculated control with no N applied. All treatments were replicated three times and arranged in Randomized Complete Block Design (RCBD) due to shading effect of trees around the screenhouse.

The following fertilizer treatments were applied to all pots to ensure adequate nutrient supply:

$ZnSO_4$ (0.079 mg/pot), $MgSO_4$ (0.085 mg/pot), TSP (0.507 mg/pot), $NaMoO_4.2H_2O$ (0.004 mg/pot), KCl (0.117mg/pot) and Urea (0.263 mg in each of the relevant pots).

The urea was applied in split form: the first dose at planting and the second dose 14 days after planting. The test crop used was a promiscuous soybean cultivar (TGx1448-2E). The seeds were surface sterilized to rid them of contamination especially from rhizobia as described by Somasegaran and Hoben (1985). Five seeds were planted per pot and then later thinned to two plants per pot at two weeks after planting. The plants were irrigated once or twice daily depending on the moisture content of the soil.
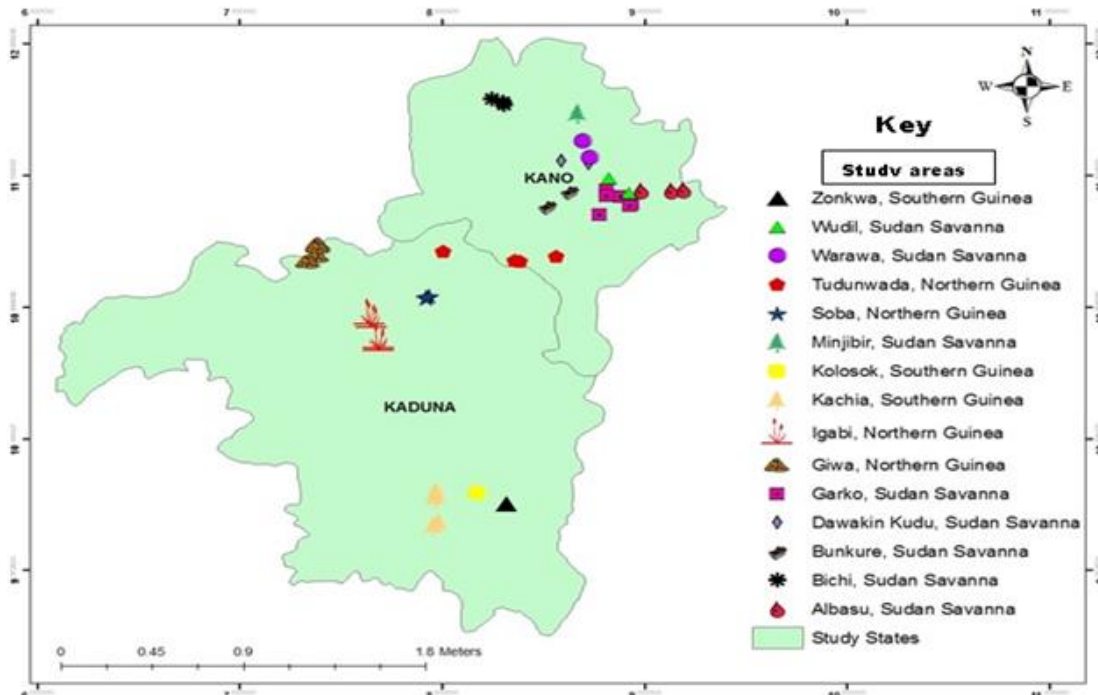
Figure 1. Map showing distribution of sampling sites.

Soil analysis

Particle size distribution was determined by the hydrometer method, as described by Gee and Bauder (1986). The soil texture was determined using the USDA soil textural triangle. Soil pH was measured in both water and 0.01 M CaCl$_2$ solution using a 1:2.5 soil to solution ratio (Hendershot et al., 1993).

Organic carbon was determined by the Walkey-Black (chromic acid) method as described by Nelson and Sommers (1982). Total nitrogen was determined by micro-Kjeldahl digestion method (Bremner and Mulvaney, 1982). Available phosphorus was determined by the Bray 1 method (Olson and Sommers, 1982). Exchangeable bases were extracted with 1N ammonium acetate at pH 7.0 buffer (Chapman, 1965). Exchangeable K and Na was determined using flame photometer while exchangeable Ca and Mg was determined using Atomic Adsorption Spectrophotometer (AAS) (Jackson, 1958). The soil CEC was determined by 1N ammonium acetate saturation method (Anderson and Ingram, 1993) (Table 1 and Figure 1).

At eight weeks after planting, the plants were harvested and the following parameters were assessed: nodule number, nodule fresh weight, shoot dry weight and root dry weight. The data used for the soil analysis were subjected to descriptive statistics. The total dry matter yield (TDMY) was used to calculate the relative effectiveness index which gives an indication of the relative efficiency (RE) and inoculant response (IR) for each of the agro-ecological zones.

Relative Efficiency (RE) = 100a/b
Inoculant Response (IR) = 100 (c-a)/a

Where:

a = dry matter weight in control treatment
b = dry matter weight in plus N treatment
c = dry matter weight of inoculated treatment

RESULTS AND DISCUSSION

Physicochemical properties of soil samples

The soils of the experimental areas are generally slightly acidic to moderately acidic (Table 2). This is a common feature of savanna soils (Jones and Wild, 1975). The pH value falls within the normal range of 5.5-7.0 reported to be optimum for the release of plant nutrients (Sharu et al., 2013). The organic carbon total nitrogen contents were generally low for all the agro-ecologies. Jones and Wild (1975) have earlier reported low values of available P in addition to organic carbon and total nitrogen in Nigerian Savanna soils. These ratings are according to FMANR (1990).

Relative efficiency (RE) and inoculant response (IR) in the three agro-ecological zones

In the Sudan Savanna (Figure 2), the IR ranged from -26.15 to 90.5%, while the RE ranged from 55.06 to 112.5%. The figure shows that WDL 1 has the highest response to inoculation (90.5%), followed by ALB 3 (65.11%); WRA1 has the lowest negative response to inoculation by -26.15%. ALB 1 has the highest relative efficiency of 112.5% followed by GRK 1 which has RE of 111.11% while BKR 1 has the lowest RE of 55.06%.

In the Northern Guinea Savanna (Figure 3), the IR ranged from -16.67 to 22.2% while the RE ranged from
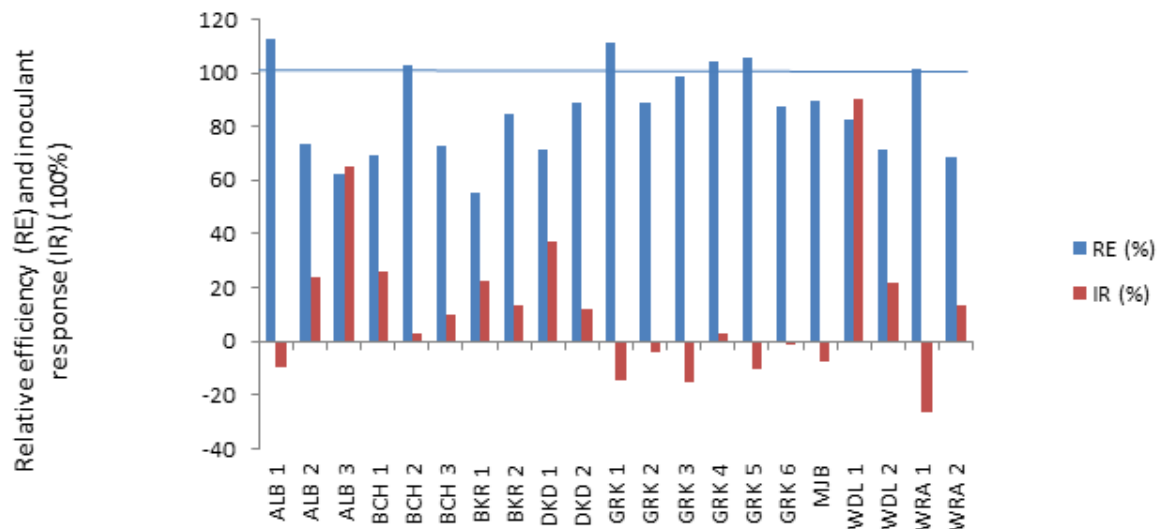
Figure 2. Relative Efficiency (RE) and inoculant response (IR) in the Sudan savanna.
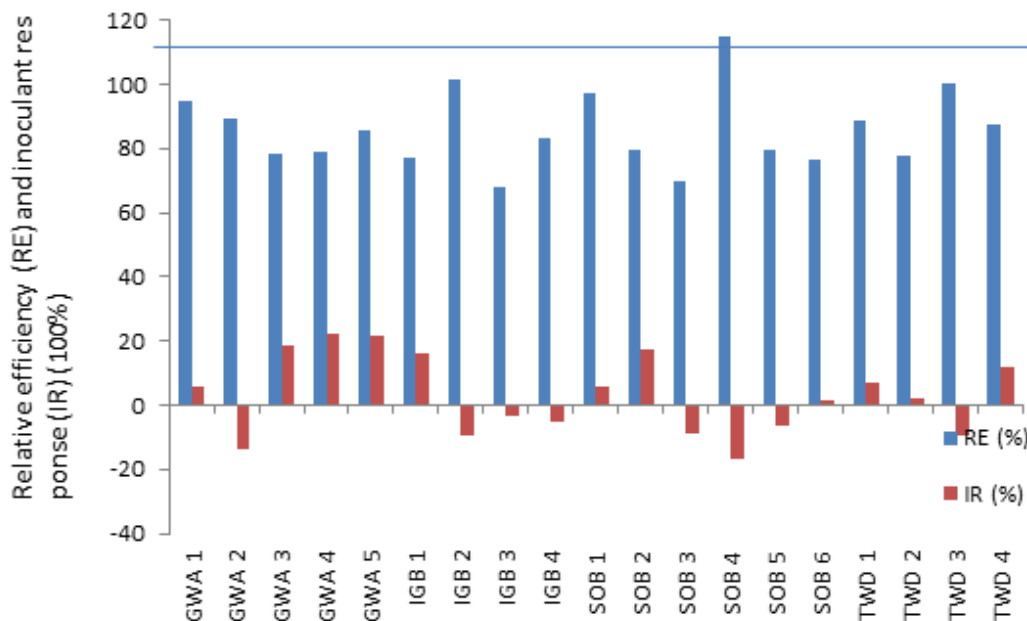
Figure 3. Relative efficiency (RE) and inoculant response (IR) in the Northern Guinea savanna.

67.74 to 114.7%. The highest relative efficiency was observed for SOB 4 with RE of 114.7% followed by IGB 2 with RE of 101.4%; the lowest was IGB 3 (67.74%). The highest response to inoculation was in GWA 4 (22.2%), followed by GWA 5 (21.8%); while the lowest was SOB 4 (-16.67).

In the Southern Guinea Savanna (Figure 4), the IR ranged from -25 to 30.3% while RE ranged from 82.5 to 128.6%. The highest IR was in KCH 4 (30.3%) followed by KOL (22.22%); while the lowest was in KCH 3 (-25%).

The highest RE was observed for KOL (128.6%) followed by KCH 1 (100%); while the lowest RE was in KCH 4 (82.5%). However, samples were collected from relatively few sites, which is not sufficient to provide information that will confidently allow for suitable recommendation. The high relative efficiency observed in some locations could be as a result of the interactions of the population of the native rhizobia in those locations. It is suggested that successful nitrogen fixation depends on the interaction between legume genotype, rhizobium strain
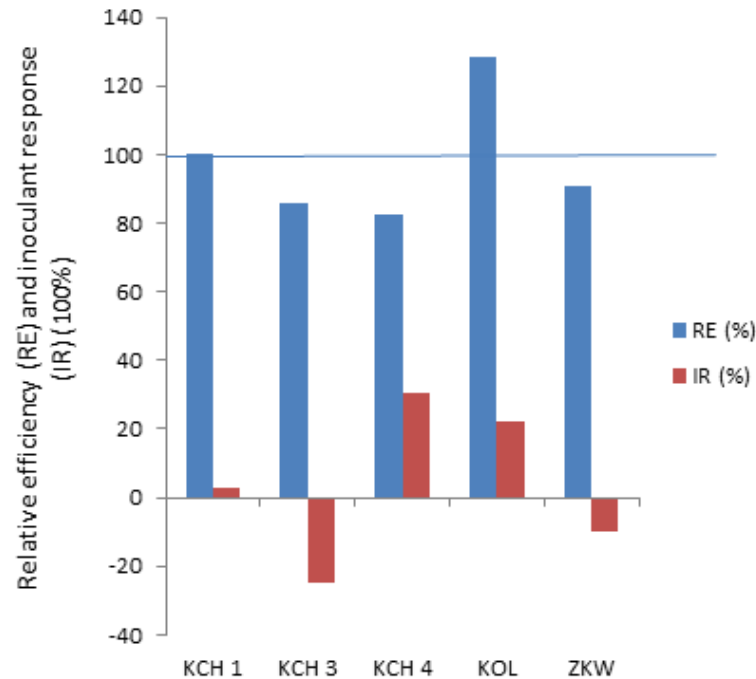
Figure 4. Relative efficiency (RE) and inoculant response (IR) in the southern Guinea savanna

and environment (Giller, 2001).

Relative efficiency greater than 100% implies that the indigenous population of rhizobia was superior to the application of mineral N. The strains isolated from these location(s) have very high potential for use as inoculant. Hence, it is not surprising that very few such cases occurred. Notable among such locations are BCH 2 (102.9%), GRK1 (R111.1%), GRK 4 (104.35%), GRK 5 (105.3%), WRA 1 (101.6%) in the Sudan Savanna. In the Northern Guinea Savanna, there is IGB 2 (101.4%), SOB 4 (114.7%).

In the Southern Guinea Savanna, there is KOL (128.6%). Locations with IR greater than 100% show that inoculation with effective rhizobia will improve soybean biomass. The higher inoculant response observed in the Sudan Savanna could be as a result of high competitiveness of the introduced strains. The presence of large population density of compatible rhizobia does not preclude the possibility that responses to inoculation can be obtained if competitive and highly effective strains are introduced (Giller, 2001).

Similarly, the population of indigenous rhizobia could have been very low in the Sudan Savanna. In a study conducted by Aliyu et al. (2013) in Shanono which lies in the Sudan Savanna, it was reported that the native rhizobia population was 1.10×10^2 which was quite low, suggesting that the soils have little or no N fixing ability for soybean. Hence, introduction of a commercial inoculant would have resulted in high response. Kumaga

and Etu-Bonde (2000) demonstrated that nodulation and N_2 fixation of promiscuous soybean may be increased by inoculation with effective Bradyrhizobia.

The negative response observed in certain locations could be as a result of the competitiveness of the indigenous rhizobia that were more efficient than the introduced strain. It was reported by Meade et al. (1985) that successful competition for nodule sites by native rhizobia is one reason for the failure to achieve a response to inoculation with elite rhizobial strains.

In the Northern Guinea Savanna, SOB 4 interestingly had the highest RE and the lowest IR. This suggests that the population of the indigenous rhizobia may have been highly competitive for nodule occupancy and highly effective in their nitrogen fixing ability. Therefore, there will be no need for inoculation or mineral N application. It is generally argued that indigenous populations are highly adapted to their local soil environments and may form more effective symbioses than commercial inoculants isolated from a distant and un-related soil environment (Gandee et al., 1999).

Similarly, this AEZ has a long history of soybean cultivation and inoculant use (Sanginga et al., 1995, 2003; Okogun et al., 2004; Yusuf et al., 2012). The previously introduced strains might have replaced the indigenous population, thus response to inoculation may be very minimal. This situation can be circumvented if superior strains are present in inoculants to be used in the zone.

Table 2. Means and ranges of some of the physico-chemical properties of experimental sites

Agro-ecological zone	Clay (%)	Silt (%)	Sand (%)	pH water (1:2.5)	pH CaCl$_2$ (1:2.5)	OC (g kg^{-1})	TN (g kg^{-1})	Avail. P (mg kg^{-1})	Exchangeable bases (cmol kg^{-1})				CEC (cmol kg^{-1})
									Ca	Mg	K	Na	
Northern Guinea savanna													
Minimum	8	8	56	5.7	5.0	0.18	0.11	1.75	4.2	0.38	0.11	0.26	6.2
Maximum	18	28	84	7.5	6.4	1.12	0.28	12.25	11.6	4.83	0.24	0.74	17.2
*Mean	13.16	21.05	65.79	6.46	5.3	0.44	0.19	6.31	7.26	2.63	0.18	0.39	11.34
Southern Guinea savanna													
Minimum	14	4	66	5.6	4.6	0.4	0.14	1.75	4.6	2.4	0.09	0.23	8.6
Maximum	22	16	82	6.6	4.7	1.12	1.89	26.3	12.2	5.8	0.26	0.54	18.7
**Mean	18	10.29	71.71	5.8	4.7	0.71	0.57	15.76	8.03	3.7	0.19	0.31	13.18
Sudan savanna													
Minimum	8	2	70	5.4	4.4	0.04	0.11	1.75	4.4	0.84	0.06	0.18	7.3
Maximum	18	16	90	7.3	5.8	0.39	0.74	31.5	10.6	4.64	0.38	2.78	16.8
***Mean	9.9	8.09	82	6.24	5.18	0.24	0.36	19.13	7.8	2.88	0.19	0.45	12.18

*Mean of 19 sites; **Mean of 5 sites; ***Mean of 21 sites.

Conclusion

The results show variation in the response to inoculation of rhizobia with respect to plant biomass in the various agro-ecological zones. The results showed better response to inoculation in the Sudan Savanna compared to the Northern and Southern Guinea savannas. This is not surprising as response to inoculation is usually lower in areas where soybean has been extensively grown. Of all the three agro-ecological zones, Sudan Savanna will benefit more from inoculation. Also, locations with high relative efficiency would produce promising strains if explored further. While the results show response to inoculation and high relative efficiency of indigenous strains, further field studies need to be carried out in order to improve their use as inoculants.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Diagnosis of *Helicobacter pylori* infection in children and their mothers using some non-invasive techniques

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Helicobacter pylori (*H. pylori*) is an important global public health concern. It infects approximately one half of the world's population. Considering the broad spectrum of available *H. pylori* diagnostic methods and since a reliable diagnosis is mandatory both before and after eradication therapy; thus it is crucial to study the different methods to be able to select the highly accurate laboratory tests that could be used to efficiently diagnose *H. pylori* infection in clinical practice. In this descriptive-cross sectional study we aimed to evaluate some non-invasive techniques for the diagnosis of *H. pylori* infection in children and their mothers. Forty children and their 21 mothers who had gastrointestinal symptoms were included. Stool, serum and saliva samples were collected from each enrolled child and mother to detect *H. pylori* antigens (Ags) in stool using chromatographic immunoassay and IgG antibodies (Abs) in serum and saliva by enzyme linked immune sorbent assay (ELISA). Collected data were analyzed using IBM SPSS software package version. Thirty (75%) children were positive for stool Ags, 10 (25%) were positive for serum Abs and only 5.4% were positive for saliva Abs. *H. pylori* stool Ag test was the most reliable test in all studied individuals and testing *H. pylori* IgG Abs in serum was more useful in adults than in children.

Key words: *Helicobacter pylori*, *H. pylori* stool antigen, *H. pylori* antibodies, chromatographic immunoassay, ELISA.

INTRODUCTION

Helicobacter pylori (*H. pylori*), one of the most common bacterial pathogens in the world, is an important global public health concern (Cheng et al., 2009). This organism infects approximately one half of the world's population and represents a key factor in the etiology of various gastrointestinal diseases, ranging from chronic active gastritis without clinical symptoms to peptic ulceration, gastric adeno-carcinoma, and gastric mucosa-associated lymphoid tissue lymphoma (Estakhri et al., 2008). The prevalence varies greatly between countries and between

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population groups within the same country and is inversely associated with socioeconomic status or household hygiene and sanitation (Hanafi and Mohamed, 2013). In developing countries, more than 80% of the population is *H. pylori* positive, even at young ages, while in developed countries the prevalence generally remains under 40% and is considerably lower in children and adolescents than in adults and elderly people (Yan et al., 2013).

H. pylori infection is commonly acquired in childhood (Luzza et al., 1997).The prevalence of infection among children ranges from 2.4 to 13.6% in developed countries and from 22.6 to 80% in developing countries. This difference may be attributed to poor socioeconomic status and overcrowded conditions during childhood (Ravelomanana et al., 2013). In Egypt, infection is alarmingly high (Mohammad et al., 2008). A prevalence rate of 33% has been reported among children less than 6 years and 72.38% among school children (Frenck et al., 2006).

The diagnosis of *H. pylori* can be done using invasive and non-invasive methods. Invasive methods need endoscopy and biopsy [histological examination, rapid urease test (RUT), culture, polymerase chain reaction (PCR), and fluorescent in situ hybridization (FISH)]. Non-invasive methods include ^{13}C, ^{14}C urea breath test (UBT), detection of *H. pylori* antigen (Ag) in stool, and detection of antibodies (Abs) against *H. pylori* in serum, urine, and saliva (Koletzko et al., 2011; Leal et al., 2011).

Non-invasive tests for *H. pylori* infection have been helpful in epidemiological studies of prevalence, mode of transmission and spontaneous clearance of the infection. Considering the broad spectrum of available *H. pylori* diagnostic methods and since a reliable diagnosis is mandatory both before and after eradication therapy; thus it is crucial to study the different methods to be able to select the highly accurate laboratory tests that could be used to efficiently diagnose *H. pylori* infection in clinical practice (Leal et al., 2008). In this study we aimed to evaluate some non-invasive techniques (Detection of *H. pylori* stool Ags using rapid chromatographic Immunoassay and *H. pylori* IgG Abs in serum and saliva by ELISA technique) for the diagnosis of *H. pylori* infection in children and their mothers.

MATERIALS AND METHODS

This descriptive-cross sectional study was conducted at El-Shatby University Hospital and the Microbiology Department of the High Institute of Public Health (HIPH), in Alexandria, Egypt during a five-month period from May to December 2014.

Forty children and their twenty one mothers who had gastrointestinal symptoms suggestive of *H. pylori* infection (Heart burn, epigastric pain, nausea, vomiting, anorexia, vague abdominal pain, diarrhea, indigestion, bloating, weight loss, bleeding) were included in the present study. Any child or mother who had received antimicrobial agents (including metronidazole) and /or proton pump

inhibitors (PPI) within 2 weeks prior to samples collection was excluded from the study.

Sample size

Using a power of 80% to detect the sensitivity of salivary IgG at predicting the *H. pylori* in children = 87% and specificity = 73% (Roxo-Rosa et al., 2013), assuming the prevalence of *H. pylori* in children =50% with precision = 5 and at alpha = 0.05 (Mohammad et al., 2008); a minimum sample size required was calculated using STATA to be 30 children and their mothers for whom all methods were applied.

Data collection method

A questionnaire sheet was completed for every individual enrolled in the study with all the relevant information including gastrointestinal symptoms and risk factors related to *H. pylori* infection. This study was approved by the HIPH Ethics Committee, and written informed consents were obtained from all the mothers of examined children.

Samples collection and processing

Stool, blood and saliva* samples were collected from each of the 40 children and their 21 mothers enrolled in the present study. *Saliva samples were collected from 37 children as it was not feasible to collect saliva from three babies.

At the laboratory

Stool samples were tested for *H. pylori* Ag using rapid chromatographic Immunoassay (Abon one step test code no: 1155976703) (ABON Biopharm Co 2012).

Small portions of stool samples collected from three different parts (to collect approximately 50 mg) were transferred to the sample collection tube containing extraction buffer. It was then vigorously agitated and after two minutes of resting the tube, two full drops were dropped into the round window of the test cassette. Reading was made after 10 minutes of incubation at room temperature.

Interpretation of the results

Based on the appearance of colored lines across the central window of the cassette, two lines, C (control) and T (test), indicated positive test. Only one line in C indicated negative result. A pale colored line in T was also considered positive. Invalid test was considered if no line appeared in C line region.

The obtained sera were stored at -20°C until used for detection of serum *H. pylori* IgG using the Immunospec Helicobacter pylori IgG ELISA kit (Catalog No.E30-145) (Immunospec Co 2006).

Qualitative results

The cut-off control corresponds to calibrator one. If the absorbance of the sample was higher than that of the cut-off, the sample was positive for the presence of specific IgG. The ratio between optical density (OD) value of the sample and that of the cut-off was calculated.

Table 1. Kappa coefficiency test analysis.

Kappa	Interpretation
< 0	Poor agreement
0.0 – 0.20	Slight agreement
0.21 – 0.40	Fair agreement
0.41 – 0.60	Moderate agreement
0.61 – 0.80	Substantial agreement
0.81 – 1.00	Almost perfect agreement

The sample was considered:

- Positive: if the ratio was > 1.1.
- Doubtful: if+/- 10% of the cut-off.
- Negative: if the ratio was < 0.9.

Quantitative results

Units (U): Positive results were expressed in U, the OD values of the 5 calibrators were interplotted and the value of the sample was compared to this curve. Containers with collected saliva samples were stored at -80^0 C till ELISA test for salivary *H. pylori* IgG was performed using the WKEA Ab H. pylori IgG Ab ELISA Kit (WH-354) (Wkea Med Supplies Co 2009).

Preparatory work

Preparation of saliva samples: A 0.5 ml of saliva was placed in a sterile centrifugal tube, and then one ml of phosphate buffer solution (PBS) with pH value of 7.4 was added by an automatic pipette. The tube was then centrifuged for 20 min (2000-3000 rpm) and the supernatant was collected carefully in a clean Eppendorf tube.

Interpretation of results

Critical cut off was calculated:

Critical cut off = The average of negative control well + 0.15.

- If sample OD < calculated critical cut off, it is negative.
- If sample OD ≥ calculated critical cut off, it is positive.

Statistical analysis of the data

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0 (Kirkpatrick and Feeney, 2013). Qualitative data were described using number and percent. Comparison between different groups regarding categorical variables was tested using Chi-square test. When more than 20% of the cells have expected count less than 5, correction for chi-square was conducted using Fisher's exact test or Monte Carlo correction. Significance of the obtained results was judged at the 5% level. Calculation of diagnostic sensitivity and specificity were also chosen at the optimal cut off which has a highest positive likelihood ratio ($^+$LR)

$$^+LR = \frac{Sensitivity}{1-specificity} = \frac{TPrate}{FPrate}$$

Where $^+$LR =

N.B. $^+$LR > 1 denotes good performance of a test.

The agreement between different tests was analyzed using Kappa coefficiency test as shown in Table 1:

RESULTS

A total of forty children and their 21 mothers who attended the outpatient clinic at El-Shatby University Hospital and had symptoms suggestive of *H. pylori* infection were included in this study. The 40 studied children included 22 (52.5%) males and 18 (47.5%) females. Their ages ranged from 7 months to 15 years, with a mean age of 6.3. The mean age of enrolled mothers was 32.6 and their ages ranged from 23 to 52 years.

Eleven (52.38%) of the 21 examined mothers and their children (20 children [50%]) were classified as of high socioeconomic class, 9 (42.85%) and their children (19 children [47.5%]) were of average socioeconomic class and only one (4.76%) and her child (one child [2.5%]) belonged to low socioeconomic class according to modified score for social leveling of families (Fahmy and El Sherbini, 1983).

Of the 21 mothers, 8 (38%) and their children (13 [32.5%]) were from urban areas, while 13 (61.9%) and their children (27 [67.5%]) were from rural areas. In addition, seven of these 21 mothers (33.33%) and 14/40 children (35%) had previous *H. pylori* infection.

Of the 40 children, 30 (75.0%) were positive for stool Ags, 10 (25.0%) were positive for serum Abs, while two (5.4%) out of 37 children were saliva Ab positive, as it was not feasible to collect saliva from 3 babies. These results were found to be statistically significant. ($p1<0.001$, $p2<0.001$, $p3= 0.026$) (Table 2)

As regards the 21 studied mothers, 19 (90.4%) were positive for each of stool Ags and serum Abs and only 5 (23.8%) were positive for saliva Abs. These results were statistically significant ($p_2<0.001$, $p_3<0.001$) (Table 2).

Twenty seven (27) children together with their mothers (18) were concordantly positive for *H. pylori* stool Ags, while 9 and their mothers (7) were positive for serum Abs and 2 children and their mothers were positive for saliva Abs. These results were statistically significant ($p_1<0.001^*$, $p_2<0.001^*$, $p_3= 0.032^*$) (Table 3).

As regards children, the sensitivity of the serum Ab test in relation to stool Ag test was 30 % with a specificity of 90%; positive predictive value (PPV) equals 90% and negative predictive value (NPV) 30%. The agreement between the two tests in the diagnosis of *H. pylori* infection was 45% (slight agreement) (Table 4). While for mothers, the sensitivity of the *H. pylori* serum Ab test in relation to stool Ag test was 94% with a specificity of 50%. PPV was 94.74% and NPV was 50%. The agreement between the two tests in the diagnosis of *H. pylori* infection was 90.48% (moderate agreement)

Table 2. Results of *H. pylori* stool Ag, serum and saliva IgG Abs among studied children and their mothers.

Results of *H. pylori tests* Studied individuals	Stool Ag				Serum Ab				Saliva Ab			
	Positive		Negative		Positive		Negative		Positive		Negative	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Children (40)	30	75.0	10	25.0	10	25.0	30	75.0	2*	5.4	35*	94.6
Significance between groups	$p_1<0.001^*$, $p_2<0.001^*$, $p_3= 0.026^*$											
Mothers (21)	19	90.4	2	9.6	19	90.4	2	9.6	5	23.8	16	76.2
Significance between groups	$p_1= 1.000$, $p_2<0.001^*$, $p_3<0.001^*$											

*A total of 37 saliva samples were collected from children as we could not obtain saliva from 3 of them. (Their ages were 7months, 2 years and 5 years). p_1: p value for Chi square test or Fisher Exact test for comparing between stool and serum results. p_2: p value for Chi square test for comparing between stool and saliva results. p_3: p value for Chi square test for comparing between serum and saliva results. *: Statistically significant at $p \le 0.05$.

Table 3. Relation between results of *H. pylori* stool Ag, serum and saliva IgG Abs among studied children and their mothers.

Studied tests for *H. pylori* Results of studied individuals	Stool Ag	Serum Ab	Saliva Ab
Positive children and their mothers	27children /18 mothers	9 children /7 mothers	2 children /2 mothers
Significance between groups		$p_1<0.001^*$, $p_2<0.001^*$, $p_3= 0.032^*$	
Negative children and their mothers	0 children /0 mothers	2 children /1 mother	25 children /16 mothers
Significance between groups		$^{FE}p_1= 0.494$, $p_2<0.001^*$, $p_3<0.001^*$	
Negative children and positive mothers	10 children /9 mothers	28 children /17 mothers	10 children /4 mothers
Significance between groups		$p_1<0.001^*$, $p_2= 0.204$, $p_3<0.001^*$	
Positive children and negative mothers	3 children /2 mothers	1 child /1 mother	0 children /0 mothers
Significance between groups		$^{FE}p_1= 0.615$, $^{FE}p_2= 0.241$, $p_3= 1.000$	

p_1: p value for Chi square test or Fisher Exact test for comparing between stool and serum results. p_2: p value for Chi square test or Fisher Exact test for comparing between stool and saliva results. p_3: p value for Chi square test for comparing between serum and saliva results. *: Statistically significant at $p \le 0.05$.

Table 4. Relation between results of *H. pylori* serum IgG Abs and stool Ag in 40 studied children.

Stool Ag results Serum Abs results		Stool Ag		Sensitivity	Specificity	PPV	NPV	Agreement
		Positive	Negative					
Serum Abs	**Positive**	9	1	30.0	90.0	90.0	30.0	45.0
	Negative	21	9					
	Total (40)	30	10					
	Kappa (p)							0.120(0.206) Slight agreement

(Table 6).

Regarding saliva test, the sensitivity of the saliva Ab test in children in relation to stool Ag test was 6.67 %, with specificity and PPV of 100% each, and NPV of 26.32%. The agreement between the two tests in the diagnosis of *H. pylori* infection was 30% (slight agreement) (Table 5). While for mothers, the sensitivity of the saliva Ab test in relation to stool Ag test was 15.79%, with specificity and PPV of 100% each, while NPV was 11.11%. The agreement between the two tests in the

Table 5. Relation between results of *H. pylori* saliva IgG Ab and stool Ag among 40 studied children.

Stool Ag results Saliva Ab results		Stool Ag		Sensitivity	Specificity	PPV	NPV	Agreement
		Positive	Negative					
Saliva Ab	Positive	2	0	6.6	100.0	100.0	26.3	30.0
	Negative	28	7					
	Total (37)	30	7					
	Kappa (p)							0.026(0.482) Slight agreement

*Total collected saliva samples were 37.

Table 6. Relation between results of *H. pylori* serum IgG Ab and stool Ag among 21 studied mothers.

Stool Ag results Serum Ab results		Stool Ag		Sensitivity	Specificity	PPV	NPV	Agreement
		Positive	Negative					
Serum Ab	Positive	18	1	94.0	50.0	94.7	50.0	90.4
	Negative	1	1					
	Total (21)	19	2					
	Kappa (p)							0.447[*](0.040*) Moderate agreement

Table 7. Relation between results of *H. pylori* saliva IgG Ab and stool Ag among 21 studied mothers.

Stool Ag results Saliva Ab results		Stool Ag		Sensitivity	Specificity	PPV	NPV	Agreement
		Positive	Negative					
Saliva Ab	Positive	5	0	15.7	100.0	100.0	11.1	23.8
	Negative	14	2					
	Total (21)	19	2					
	Kappa (p)							0.064(0.406) Slight agreement

* Total collected saliva samples were 37.

diagnosis of *H. pylori* infection was 23.81% (slight agreement) (Table 7).

Of the 30 children with positive stool Ags, 10 (34%) were living in urban areas and 20 (66%) were from rural areas. Of the 10 children with positive serum Abs, 6(60%) were from urban areas and 4 (40%) were from rural areas. Regarding the 2 children with positive saliva Abs, one (50%) was from an urban area and the other (50%) was from a rural area. As regards mothers, of the 19 mothers with positive stool Ags and serum Abs, 7 (37%) were from urban areas and 12 (63%) were from rural areas. Of the 5 mothers with positive saliva Abs, 3 (60%) were from urban areas and 2 (40%) were living in rural areas. There was no statistical significant difference between these results.

As regards family size, 27/30 children (90%) with positive stool Ags, had families of 4-6 members and 3(10 %) belonged to families with more than 6 members. For the mothers, 18 /19 (95%) who were positive for each of stool Ags and serum Abs had a 4-6 family size.

Regarding Unhealthy eating habits, of the 30 children who were positive for stool Ags, 25 (83%) shared spoons, glasses and plates with their family members and 8 (62%) of them, their mothers chewed food before giving it to them. While concerning the 19 mothers who were positive for each of stool Ags and serum Abs, 15 (78%) shared spoons, glasses and plates with their family members and 7 (36%) chewed food before giving it to their children.

As regards serum Abs, of 13 mothers who had symptoms suggestive of *H. pylori* infection, 12 (92.3%) mothers were positive for serum Abs and had 6 (25%) of their children positive for serum Abs. These results were found to be statistically significant (p<0.001*).

DISCUSSION

H. pylori, one of the most common bacterial pathogens of humans, is the main cause of chronic gastritis all around

the world. It colonizes the gastric mucosa and appears to persist throughout the host's life unless the patient is treated. It is the only bacterium to be classified as a carcinogen by the world health organization (WHO) because of its role in gastric cancer development. Gastric cancer is the third leading cause of cancer deaths worldwide, and 89% of gastric cancer cases are attributable to *H. pylori* infection. Thus an accurate and reliable method for diagnosis is crucial (Ahmed and Shammari, 2015; Plummer et al., 2015; Moon et al., 2013). The invasive techniques for diagnosis of *H. pylori* are difficult, very expensive and not accepted by the patients (Ni et al., 2000). So in clinical settings, a rapid and cost-effective detection method for diagnosis of *H. pylori* infection is desirable especially in children. Non-invasive testing for *H. pylori* has been strongly recommended as it is less expensive, more patient friendly than invasive methods and does not need a very complicated laboratory facility (Osman et al., 2014).

Stool Ag test is one of the non-invasive methods that is widely used in the diagnosis of *H. pylori* infection and had been known with its accuracy and comparability to invasive methods (Ni et al., 2000). In the present study *H. pylori* stool Ag test was considered the gold standard method for diagnosis of *H. pylori* infection. This is due to its high sensitivity and specificity in children and adult patients and excellent positive and negative predictive values regardless of *H. pylori* prevalence as reported by Bakri (2012). In addition, Pourakbari et al. (2013) has recorded that the performance of stool Ag test in adult patients was excellent, with a sensitivity, specificity, and accuracy of 91, 79 and 85%, respectively. Furthermore in 2014, a review done by Garza-González et al. documented that *H. pylori* stool Ag test seemed to perform well in children with sensitivity of 97%, specificity of 97%, PPV of 88%, and NPV of 99% (Garza-González et al., 2014).

Also in comparison with UBT, stool Ag proved to be equivalent in its sensitivity specificity as reported by Frenck et al. at Cairo University, who concluded that UBT and stool Ag test had the highest sensitivity (98, 94%, respectively) and specificity (89%, 81%, respectively) and that the use of the stool Ag test has been evaluated as equivalent to the UBT (Frenck et al., 2006).

In the present study, stool Ag test was positive in 30/40 examined children (75%) (Table 2). A higher percentage was recorded by Douraghi et al. (2013), where 93.1% of the children were positive for *H. pylori* stool Ags (Douraghi et al., 2013). Jafar et al. (2013) found that stool samples were positive for *H. pylori* Ags in 294 (64.2%) children (Jafar et al., 2013). A lower percentage was reported by Issa et al. (2014) where stool Ags were positive in only 45.7% of tested children. Even a much lower percentage (30%) was recorded in an early study that was conducted by Kato et al. (2003).

As regards the studied mothers in this work, 19 (90%)

were positive for stool Ags (Table 2). This was in line with Karim et al. (2012) who found that *H. pylori* stool Ag was positive in 92% of the patients. Lower percentages were reported by Naji et al. (2014) and Segamwenge et al. (2014) where stool Ags were positive in 49 and 33.5%, respectively (Naji et al., 2014; Segamwenge et al., 2014). Korkmaz et al. (2015) reported a much lower percentage where *H. pylori* stool Ag test was positive in only 19.7% of patients. In this study, immuno-chromatographic assay was used for stool Ag detection as it has the advantage over other immune assay methods that the test results are available within minutes and it does not require the use of expensive laboratory equipment. In a study done in 2013 by Jekarl et al., *H. pylori* stool Ag had high sensitivity and specificity (84.5 and 96.2%, respectively) (Jekarl et al., 2013). In the current work, all collected stool samples were well formed. This fact could be the reason for the enhanced stool Ag test results, as the accuracy of the stool Ag test is lowered when stool samples are unform or watery, because *H. pylori* specific Ags in these stool samples are diluted. Therefore, watery stools should not be used, particularly in the determination of the results of eradication therapy. The sensitivity of this test is also lowered in patients with upper gastrointestinal bleeding (Shimoyama, 2013). All our studied individuals didn't have gastrointestinal bleeding.

In addition to stool Ag test, serological testings are also useful non-invasive methods for the diagnosis of *H. pylori* infection. It is easy for patients to accept the test because of its non-invasiveness; the results can be quickly obtained, less likely to be affected by colloidal bismuth, PPIs, or antibiotics (Shah et al., 2014).

In the current study, serum and the saliva IgG Ab tests were done and they were compared to the stool Ag test that was considered as the gold standard method for diagnosing *H. pylori* infection. It was found that serum IgG Ab was positive in 19 of the 21 enrolled mothers (90%) with sensitivity, specificity and agreement of 94, 50 and 90%, respectively) (Table 6). These results were in concordance with the findings of Pandya et al. (2014) who reported that the IgG Abs evaluated in their study, had a sensitivity of 100% in adults, which permits the safe use of the test in epidemiologic surveys. Also, they recorded a very low specificity, indicating that the ELISA test must be validated for different populations (Pandya et al., 2014). In addition, Shah et al. (2014) reported that serum IgG sensitivity ranged between 90 and 97%, but the specificity ranged between 50 and 96%. In contrast to the results of the current study, Iqbal et al. (2013) reported a higher specificity rate of 80%. As regards children in the present study, 10 out of 40 children (25%) were positive for IgG Abs in serum with sensitivity, specificity and agreement of the test were 30, 90 and 45%, respectively (Table 4). Sensitivity was found to be lower than that of the mothers and this could be attributed

to the immature immune response or tolerance to *H. pylori* that exists in childhood and thus serodiagnosis of *H. pylori* infection is less useful in children. Similar findings were reported by Okuda et al. (2002) who compared *H. pylori* IgG and IgA Abs with *H. pylori* stool Ags (Okuda et al., 2002). Our results were in concordance with that of Pourakbari et al. (2013) who found that ELISA-IgG serum tests in children showed high specificity, but low sensitivity. These findings have significant clinical implications, since a negative test would not be reliable for ensuring the absence of *H. pylori* infection. Low sensitivity may be attributed to the weak or immature immune response observed in young children (Pourakbari et al., 2013). IgG was also much more specific in children than adults, corroborating the fact that adults are more likely to have been frequently exposed to *H. pylori* in the past so if it is present in a child, it is so specific to the infection (She et al., 2009). This may explain the difference in specificity.

In this study, it was found that 18 out of the 19 positive mothers for stool Ags (94%) were positive for both stool Ags and serum Abs (Table 6) while in children only 9/30 positive children for stool Ags (30%), were positive for both stool Ags and serum Abs and 21 children were positive for stool Ags but negative for serum Abs. This may be explained by the fact that negative results do not preclude the absence of Abs to *H. pylori*. Colonization may be present, however it may be in its very early stages or the Ab titer may be too low for the assay to detect. Similar findings were reported by Couturier (2013).

Saliva samples have advantages over serum samples, since their collection is easy, non-invasive, less hazardous and there is a greatly reduced risk of blood-borne infections (Krishnaswamy et al., 2012). In this study we have collected saliva samples from 37 children, as we couldn't collect saliva from 3 children (their ages were 7 months, 2 years and 5 years) due to their physical resistance and continuous crying.

In the present study, the results of the salivary Abs were inferior to that of stool Ags and serum Abs. Only 5 mothers out of 21(23%) were positive for salivary Abs (Table 7) and only 2 out of 37 children (5%) were positive for salivary Abs. (Table 2) This may be due to the fact that IgG which appears in saliva by trans-capillary leakage is present in low concentration as compared to that present in serum. Factors such as low concentration and variable trans-capillary leakage may be the reasons why salivary IgG assay is less reliable than the serum IgG assay as recorded by Kabir (2003), who in his research concluded that salivary IgG assay has provided inconsistent results with less than optimum sensitivity and specificity.

The calculated sensitivity, specificity and agreement for mothers' salivary Ab tests in the current study were 15.79, 100 and 23.81%, respectively (Table 7) and the corresponding figures for children were 6.67, 100 and 30%, respectively (Table 5). A higher sensitivity and lower specificity percentages were recorded by El-Fakhfakh et al. (2014) who evaluated the frequency of anti-*H. pylori* serum and salivary Abs positivity among Egyptian patients with gastric disorders and the validity of salivary and serum serological tests for diagnosis of *H. pylori*, comparing them with tests performed on endoscopy biopsy; where salivary IgG succeeded to diagnose 19 cases from the 31 positive *H. pylori* patients with a sensitivity of 63.33% and specificity of 92.86% (El-Fakhfakh et al., 2014).

The prevalence of *H. pylori* infection seems to depend mostly not only on the rate of acquisition, but also on the rate of loss of infection and the length of the persistence period between acquisition and loss. Based on these factors, *H. pylori* prevalence differs from one country to another and may differ between different ethnic, social, or age groups within the same country. Among Egyptian children, *H. pylori* prevalence was highest in children attending school in socially-deprived areas. Inhabitants of Cairo residing in an overcrowded home had the highest prevalence among the locations studied (Muhammad et al., 2012).

In our study, *H. pylori* infection was recorded in 37% of mothers who lived in urban areas and 63% of those who lived in rural areas. It represented 34% in children who lived in urban areas and 66% among those who were from rural areas. This was comparable to what was documented by Laszewicz et al. (2014) where they demonstrated statistically significant higher prevalence of *H. pylori* infection both in adults and children in rural areas. This could be attributed to inadequate sanitary conditions. Absence or poor personal hygiene may also play a role in increasing *H. pylori* infection. This may be observed in developing countries, which may reflect the combined effects of poor living conditions, poor hygiene and overcrowded cities (Laszewicz et al., 2014). This work showed higher levels of positivity in children (83%) and mothers (78%) who shared spoons, plates and glasses. This was in accordance with the study of Kaya et al. (2014) who reported that some practices that are characterized by lack of hygiene such as sucking the teat before giving it to the baby and sharing spoons, forks and cups were associated with higher prevalence of infection.

Infection by *H. pylori* remains the most frequent and persistent bacterial infection worldwide; therefore, accurate diagnosis of infection is imperative. Determining the diagnostic method and therapies to use for each patient depends on several factors, such as the patient's clinical condition and the prevalence of infection (Garza-González et al., 2014). The ideal test for diagnosis of *H. pylori* infection should be noninvasive, highly accurate, widely available and inexpensive. Furthermore, it should be able to discriminate the colonization from *H. pylori* associated disease. That is why non-invasive tests are

useful for primary diagnosis, when a treatment indication already exists, or to monitor treatment success or failure (Jafar et al., 2013).

Limitations of the study

One of the limitations of the present study was that we could not compare our 3 tested non invasive techniques to one of the invasive techniques that need endoscopy/biopsy, as this wasn't feasible during the study period. Another limitation was the relatively small sample size, though we have abided by the statistically calculated sample size based on the prevalence rate of *H. pylori* in children. Increasing the number of patients would have yielded more profound data.

Conclusions

1) *H. pylori* stool Ag test was the most reliable, rapid, simple and easy to perform non invasive test in all studied individuals.
2) Testing *H. pylori* IgG Ab in serum was more useful in adults than in children in the diagnosis of *H. pylori* infection.
3) Although saliva IgG Ab test was easy especially among adults, yet it wasn't reliable as its results were inferior to those of stool Ag and serum IgG for the detection of *H. pylori* infection in both children and adults.

Conflict of Interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Bioprospecting and plant growth-promoting bacteria tolerant to salinity associated with *Atriplex nummularia* L. in saline soils

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This study aimed to bioprospect and select halotolerantes bacteria and promoting plant growth associated with the plant *Atriplex nummularia* L. in saline soils. For bioprospecting of bacteria, samples were collected in five niches and two field experiments located in Serra Talhada and Ibimirim, Pernambuco, Brazil. After collecting the material it was performed the isolation and selection of bacteria based on plant growth promotion mechanisms. 107 bacterial salt tolerant isolates were obtained in which the population density of bacteria was higher in the rhizosphere (10^7 CFU g^{-1} soil), the cultivated soil (10^6 CFU g^{-1} soil) and uncultivated soil (10^5 CFU g^{-1} soil). For the solubilization rate of inorganic phosphate was obtained 65 and 25% positives isolated in 0 to 5% NaCl concentration, respectively. For the characteristics of biological fixation nitrogen, indole acetic acid production, exopolysaccharides and quorum sensing molecule, reached up to 87 percent; 100; 83.33 and 96.66% of the bacteria, respectively. Therefore, the bacterial isolates UAGAt 89 and UAGAt 101 expressed greater tolerance to salinity when analyzed in relation to the characteristics that promote plant growth, making it promising for future studies in order to contribute to the development of *Atriplex* plants and rehabilitation of soil affected by salts.

Key words: Halophytes, quorum sensing, exopolysaccharide, phytoremediation

INTRODUCTION

Salinity is an abiotic factor that negatively affect crop yields worldwide, especially in arid and semiarid regions (Silini-Chérif et al., 2012). Salinization occur due to inadequate irrigation management and excessive

fertilization, contributing to the increase in areas with high concentrations of salts in the soil. The improvement of these soils is important to adopt recovery techniques and, among those, there is the possibility of using halophytes

with phytoremediation function which *Atriplex nummularia* L. is highlighted by its adaptability to salinity and water deficits (Souza et al., 2011; Santos et al., 2013).

On the other hand, excess salts in the soil may or may not interfere effectively in microbial communities depending on tolerance to salts thereof. Thus, select isolates that support this type of stress, as well as bacterial isolates bioprospect with growth-promoting characteristics associated with salty environments provides a possible alternative strategy to improve plant growth and would also benefit the biological and chemical soil characteristics (Upadhyay et al., 2012; Damodaran et al., 2013).

In addition, sustainable agriculture is important to find technologies that increase efficiency and reduce the use of chemical fertilizers, therefore, a viable alternative is the halotolerantes bacteria with plant growth promotion. Thus, microorganisms can act expressing different mechanisms, such as inorganic phosphate solubilization, nitrogen fixation, synthesis of phytohormones, exopolysaccharide, as well as the expression of quorum sensing molecules. These processes are performed by different species of bacteria with the ability to solubilize inorganic phosphate in the soil, leaving it available for the plant, and also include the conversion of atmospheric nitrogen into ammonia, production of auxins able to exert function in the regulation of plant growth, protection against plant stress such as salinity, drought and high temperatures (Pereira et al., 2012; Dawwam et al., 2013). These actions of microorganisms decrease the use of chemical fertilizers, representing an economic benefit and minimizing the impacts of fertilizers on the environment (Salamone et al., 2012).

Despite being the largest soil microbial biodiversity of the planet, studies on the biotechnological potential of salt tolerant bacteria associated to plants in saline soils are still scarce (Flores-Fernández et al., 2010). Mapelli et al. (2013) point out that the influence of these bacteria on plant growth has been recognized in conventional and extreme habitats, where the ability of bacteria to facilitate the adaptation of plants and promote growth and productivity has been proven. The extremes to which these microorganisms survive trigger an intense curiosity of the scientific community in the understanding of the physiology of these organisms.

However, the major driving force behind these studies is the biotechnological potential of these mechanisms and expressions from bacteria (Ramadoss et al., 2013). Thus, the interactions of soil, plant and microorganisms play a vital role in the mobilization of nutrients and substances, positively influencing the yield of crops can contribute in growing *Atriplex* and aid in recovery from two saline soils. In this context, this study was conducted in order to bioprospect and to select salt tolerant bacteria and promoter of plant growth associated with the *A. nummularia* L. in saline soils.

MATERIAL AND METHODS

Study material

For bioprospecting of bacteria, samples were collected in five niches: 1) soil area without *Atriplex* cultivation (control) (0-20 cm depth); 2) soil cultivated with *Atriplex* collected at a distance of 1 meter from the plant; 3) soil under cultivation of *Atriplex*, collected in the rhizosphere of plants; 4) Roots of *Atriplex* plants; 5) Leaves of *Atriplex* plants. The samples were collected in two field experiments with *A. nummularia* L. grown in plots in the irrigated areas of Serra Talhada and Ibimirim in the Pernambuco state, Brazil (Table 1). Soon after the collected, the samples were placed in box containing ice and transported to the Laboratory of Genetics and Microbial Biotechnology (LGBM) of the Academic Unit of Garanhuns - Rural Federal University of Pernambuco, for the isolation of bacteria and further analysis.

Isolation of bacteria

The isolation of endophytic and rhizosphere bacteria was performed according to the methodology proposed by Araujo (2010). The number of colony forming units (CFU) per gram of soil and fresh plant tissue was estimated by counting grown colonies, resulting in the population density of the bacteria on solid medium (TSA - Tripcase Soy Agar), plus 5% NaCl. Then purification was made by bacterial strains depletion technique of striae, which were subsequently stored at -20°C, and then performed in vitro plant growth promotion analysis: inorganic phosphate solubilization, biological nitrogen fixation, synthesis indole acetic acid, exopolysaccharide production, and expression of the quorum sensing molecule.

Solubilization inorganic phosphate

In order to evaluate inorganic phosphate solubilization were used 107 bacterial isolates: 65 isolates in the Serra Talhada area. The bacteria were inoculated onto solid medium containing insoluble calcium phosphate (Verma et al., 2001) supplemented with 0 to 5% NaCl with three replications. The plates were incubated at 28°C and readings were taken at 3, 6 and 10 days after inoculation.

The presence of clear area around the bacterial colonies indicated the solubilization of phosphate. Thus, it was calculated the solubilization index (SI), expressed by the average diameter ratio of solubilization halo by the average diameter of the colony halo (Berraquero et al., 1976).

Biological fixation nitrogen

From the phosphate solubilization test in the absence of NaCl (0%) 71 bacteria were selected for N2 fixation test *in vitro*. The bacteria

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Table 1. Chemical characterization of the soil used in the isolation of bacteria.

Attribute		Serra Talhada			Ibimirim		
		SNC[1]	SWC[2]	RS[3]	SNC[1]	SWC[2]	RS[3]
pH water (1: 2.5)		8.15	9.04	9.4	7.3	7.6	7.6
Ca^{2+}	($cmol_c$ kg^{-1})	6.45	4.99	5.31	26.32	36.62	17.1
Mg^{2+}	($cmol_c$ kg^{-1})	0.15	0.12	0.13	0.64	0.57	0.37
Na^+	($cmol_c$ kg^{-1})	10.72	9.93	5.98	7.77	8.32	3.19
K^+	($cmol_c$ kg^{-1})	0.53	0.49	0.67	0.72	0.82	0.51
P	(mg kg^{-1})	6.05	8.04	6.81	11.75	9.85	16.87
CEes	(dS m^{-1})	41.59	38.73	13.69	59.89	54.44	44.29
Ca^{2+}	($mmol_c$ L^{-1})	270.32	217.6	160.8	438.5	229.8	148.1
Mg^{2+}	($mmol_c$ L^{-1})	44.66	19.66	3.83	991.66	904.16	383.33
Na^+	($mmol_c$ L^{-1})	160.08	88.6	13.39	263.45	238.62	127.45
K^+	($mmol_c$ L^{-1})	0.41	0.44	0.15	0.76	0.85	0.67
COT	(dag kg^{-1})	0.86	0.82	0.78	1.83	1.36	1.28

[1]SNC- Soil no cultivation; [2]SWC- Solo with cultivation; [3]RS- Rhizosphere soil of *Atriplex* plants.

were inoculated into test tubes containing 10 mL of culture medium NFb (5 g L^{-1} of malic acid; 0.5 g L^{-1} of K_2HPO_4; 0.2 g L^{-1} of $MgSO_4.7H_2O$; 0.1 g L^{-1} of NaCl; 0.01 g L^{-1} of $CaCl_2.2H_2O$; 4 mL L^{-1} of Fe-EDTA (solution 1.64%); 2 mL L^{-1} of bromothymol blue (0.5%); 2 mL L^{-1} micronutrients solution, (0.2 g L^{-1} of $Na_2MoO_4.2H_2O$; 0.235 g L^{-1} of $MnSO_4.H_2O$; 0.28 g L^{-1} of H_3BO_3; 0.008 g L^{-1} of $CuSO_4.5H_2O$); 1.8 g L^{-1} of agar; and pH 6.8) semisolid without nitrogen, with two replicates incubated at 28 °C and evaluated after seven days of growth. The positive result was characterized qualitatively by film formation or bacterial growth halo of clear color, within the culture medium, indicating fixation capacity of nitrogen (Dobereiner et al., 1995).

Synthesis of indole acetic acid

In the selection and quantification of indole acetic acid production (IAA), positive bacteria were used with respect to inorganic phosphate solubilization. Bacterial isolates were evaluated in vitro by means of calorimetric and specific method which characterizes the production of phytohormone (Crozier et al., 1988). The experiment was conducted with three replications and the positive result was characterized by the formation of pink color. The samples were evaluated in a spectrophotometer, which measures the absorbance at 530 nm. To convert readings was used a standard curve (Barbosa, 2010) from an IAA solution of different concentrations.

Production exopolysaccharide

For the selection and production of exopolysaccharides (EPS), we used the methodology proposed by Kavamura (2012). Bacterial isolates were inoculated in a modified solid culture (2% yeast extract, 1.5% K_2HPO_4, 0.02% $MgSO_4$, $MnSO_4$ 0.0015%; 0.0015% $FeSO_4$ 0.003% $CaCl_2$; 0.0015% NaCl, 1.5% agar), plus 10% sucrose and grown for 24 hours at pH 7.5 at 28°C.

The experiment was performed in triplicate using 30 bacterial isolates originating from both environment described above, characterized positive for inorganic phosphate solubilization (1% NaCl), biological nitrogen fixation and indole acetic acid synthesis. The production of EPS was visually characterized by the presence

or absence of halo production thereof, characterized as positive or negative, and then rated according to the size of the halo EPS produced was made a classification (+ for halo halo with little production- <10 mm in diameter; ++ for halo with average production - halo> 10 <14 mm; +++ for halo halo with great production-> 14 mm).

Molecule expression quorum sensing

The identification of bacteria producing the quorum sensing molecule (N-acyl homoserine lactone - AHL) was performed by *Agrobacterium tumefaciens* biosensor AHLs in bioassays with the test bacteria. *A. tumefaciens* NT1 was inoculated vertically on Petri dishes containing LB (Luria Bertani) + X-gal (10 µg mL^{-1}) (5-bromo-4-chloro-3-indolyl-beta-D galacto-pyranoside). Bacterial isolates were inoculated transversely to A. tumefaciens, containing the promoter TraR (TRAG: LacZ fusion gene), forming a complex which regulates the expression of the LacZ operon. In the presence of AHLs, these TraR bind to the promoter activating the expression of the lacZ gene, encoding the enzyme β-galactosidase, which breaks the molecule X-gal, making the blue cell (Gold et al., 2013). Thus, after inoculation at 28°C for 48 h, the observation of A. tumefaciens colonies with blue pigment indicated by the production of AHLs bacterial isolates In this test, we evaluated 30 positive bacterial isolates as the inorganic phosphate solubilization (1% NaCl), biological nitrogen fixation and indole acetic acid synthesis. The experiment was performed in duplicate.

Statistical analysis

The results for the density of bacterial isolates were subjected to analysis of variance F-test, for up to 5% of significance and means were compared by Tukey test. For the average index of inorganic phosphate solubilization, production of acid indole acetic and exopolysaccharides was applied Scott-Knott test up to 5% probability, using the statistical program, Sisvar 5.3. Frequency data were subjected to relative chi-square (x^2) to confirm the influence of the factors area and bacterial colonization niche on the distribution of phosphate solubilizing isolates, biological nitrogen fixers and producers of IAA.
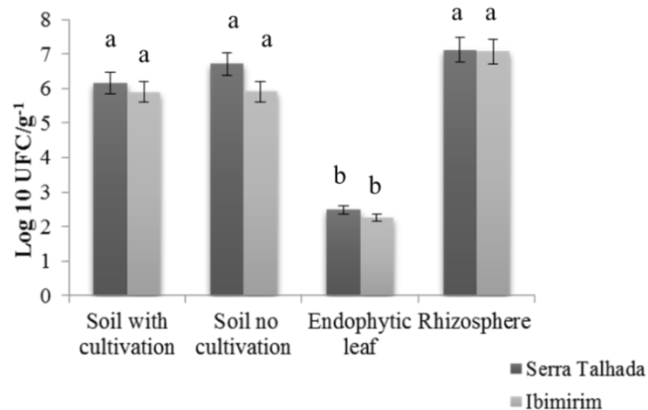
Figure 1. Population density of the bacterial community (Log10 CFU g^{-1} soil or g^{-1} fresh plant tissue) in two areas cultivated with *Atriplex nummularia* L. Columns with same letters within each area, does not differ statistically between the niches (Tukey's test, p < 0.05).

RESULTS

Isolation of bacteria

107 bacterial isolates were obtained from the two areas of study. Thus, it was observed that the bacterial density in both areas and niches present variation 10^2 to 10^7 CFU g^{-1} soil or g^{-1} fresh plant tissue (CFU- colony forming units). There was a significant difference (p<0.05) between niches of the areas studied, observing that the population density of bacteria in the rhizosphere soil, soil with and without cultivation of *Atriplex* were statistically similar, differing from endophytic leaves niche (smaller population of bacteria isolated) (Figure 1). The rhizosphere provided average value of 10^7 CFU g^{-1} soil for the two areas studied; followed with 10^6 and 10^5 CFU g^{-1} soil, soil with and without cultivation in the areas of Serra Talhada and Ibimirim. It is worth noting that the ground without the cultivation of *Atriplex*, with cultivation and rhizosphere soil associated with plants, they are salinized (Table 1) and therefore, bacteria in these environments formed groups tolerance to this stress factor in each environment studied.

Solubilization of inorganic phosphate

Among 107 bacterial strains evaluated, 65% were able to solubilize inorganic phosphate in the absence of NaCl and 25% at concentration of 5% NaCl, indicating tolerance of these microorganisms to salinity. Consequently, as higher salt concentrations, lower the mechanism of inorganic phosphate solubilization (SIF), as well was noted at 5% NaCl. However, does not inhibit this activity, confirming the existence of solubizer bacteria salt-tolerant of phosphate associated with *Atriplex*.

The inorganic phosphate solubilization index (SI) differs among the bacterial strains tested (Table 2), as the days of cultivation and NaCl concentrations. Similarly to inorganic phosphate solubilization index (SI) by the bacterial isolates in relation to absence and presence of NaCl (5%), there was only significant difference for isolated UAGAt89 (without soil cultivation) and UAGAt101 (soil with cultivation), resulting with higher values in the concentration of 5% NaCl with 3.91 and 4.10 for SI, respectively.

For the three days of cultivation it is clear that in the absence of NaCl, after 3 days of culture, isolated UAGAt19 (without soil cultivation-Serra Talhada) had the highest SI (9.53) (Table 2). After 6 days of culture in the absence of NaCl, it was observed a greater number of isolated solubilizers, when compared after 3 days of cultivation, especially isolated UAGAt34, root endophytic area Serra Talhada, with a maximum value of 9.73 (SI). After 10 days of culture in both areas, there was the largest SI (5.57) for isolated UAGAt01 soil without soil cultivation of Serra Talhada (Table 2).

With the assessment of the bacteria at 5% NaCl in 3 days of culture, it was observed that there was no solubilization of inorganic phosphate by the bacterial isolates for any of the areas and niches, while at 6 days of culture, had solubilization, for 15% of the isolates, specially UAGAt101 (soil cultivation without-Ibimirim) and UAGAt02 (soil cultivation without-Serra Talhada) with SI of 4.10 and 3.48, respectively. After 10 days of cultivation, there was an increase in the solubilization rate of the cultured bacteria at this concentration of NaCl to afford 21.5% of positive isolates under these conditions. However, bacteria that significantly expressed the highest value of SI was UAGAt89 (6.0) associated with soil cultivation without of Ibimirim area (Table 2). Phosphate solubilization ratio in the cultivation time in the absence and presence of NaCl (5%) it is perceived that the 6 days of cultivation, no significant difference from the isolated, while the 10 days of cultivation the SI isolates were higher in the absence of NaCl comparing with the concentration of 5% NaCl.

Biological nitrogen fixation

For biological nitrogen fixation (BNF), among 71 bacterial isolates evaluated, 87% were able to grow in culture medium without nitrogen source, indicating potential for fixation nitrogen biological in vitro, with emphasis on high frequency, bacteria niche root endophytic (Serra Talhada) and leaf (Ibimirim).

By analyzing the BNF of bacteria bioprospected area Serra Talhada, exceeding 27.9% was observed relative frequency for endophytic bacteria in the root niche, however, endophytic leaves had lower frequency (13.94%) in for positive isolates. Thus, there was a variation of this growth promotion mechanism between

Table 2. Index inorganic phosphate solubilization of isolated bacterial salt-tolerant associated with *Atriplex nummularia* L. due to the absence and presence of NaCl at different time evaluation.

Isolated	Niche	0% of NaCl			5% of NaCl		
		3 days	6 days	10 days	3 days	6 days	10 days
UAGAt 01	SNC	-	1.38Ac^{a}	5.57Aa^{a}	-	-	3.62Ab^{b}
UAGAt 02	SNC	2.41Ac	2.31Ac^{a}	2.26Ac^{a}	-	3.48Aa^{a}	-
UAGAt 06	SNC	-	1.84Ac^{a}	2.31Ac^{a}	-	-	-
UAGAt 08	SNC	1.88Ac	2.51Ac^{a}	2.63Ac^{a}	-	-	-
UAGAt 09	SNC	1.83Ac	2.51Ac^{a}	2.63Ac^{a}	-	-	-
UAGAt 13	SNC	1.87Ac	2.80Ac^{a}	-	-	-	-
UAGAt 14	SNC	1.59Ac	1.57Ac^{a}	2.01Ac^{a}	-	1.42Ac^{a}	
UAGAt 15	SNC	1.90Ac	2.14Ac^{a}	1.96Ac^{a}	-	-	-
UAGAt 19	SNC	9.53^{a}	-	-	-	-	-
UAGAt 21	SNC	1.16Ac	1.94Ac^{a}		-	-	1.60Ac^{b}
UAGAt 22	SNC	-	2.44Ac^{a}	-	-	1.14Ac^{a}	-
UAGAt 23	SWC	1.52c	-	-	-	-	-
UAGAt 25	SWC	1.89Ac	2.91Ac^{a}		-	1.50Ac^{a}	1.93Ac^{b}
UAGAt 33	ER	2.67Ac	1.92Ac^{a}	-	-	-	1.45Ac^{b}
UAGAt 34	ER	6.82Ab	9.73Aa^{a}	-	-	-	-
UAGAt 35	ER	2.17Ac	-	-	-	-	1.59Ac^{b}
UAGAt 36	ER	2.04Ac	-	-	-	-	1.48Ac^{b}
UAGAt 37	ER	2.15Ac	1.68Ac^{a}	-	-	1.13Ac^{a}	
UAGAt 38	ER	-	1.99Ac^{a}	-	-	-	1.64Ac^{b}
UAGAt 39	ER	2.72c	3.82b^{a}	2.92b^{a}	-	-	-
UAGAt 40	ER	3.80c	4.83b^{a}	4.19b^{a}	-	-	-
UAGAt 41	ER	3.75c	4.59b^{a}	4.82a^{a}	-	-	-
UAGAt 42	ER	2.06c	1.96c^{a}	-	-	-	-
UAGAt 43	ER	3.47c	4.36b^{a}	3.75b^{a}	-	-	-
UAGAt 45	ER	2.43c	2.76c^{a}	2.88b^{a}	-	-	-
UAGAt 51	EL	-	2.19Ac^{a}	1.73Ac^{a}	-	-	1.65Ac^{b}
UAGAt 53	EL	-	2.02c^{a}	-	-	-	1.686c^{b}
UAGAt 54	EL	2.04Ac	1.92Ac^{a}	-	-	1.10Ac^{a}	-
UAGAt 61	EL	-	2.35c^{a}	2.90b^{a}	-	-	-
UAGAt 63	EL	-	-	-	-	-	1.67c^{b}
UAGAt 69	RS	1.74Ac	-	-	-	2.50Ab^{a}	1.68Ac^{b}
UAGAt 71	RS	1.95c	1.94c^{a}	-	-	-	-
UAGAt 72	RS	-	1.45c^{a}	-	-	-	-
UAGAt 73	RS	2.12c	2.52c^{a}	-	-	-	-
UAGAt 75	RS	1.84c	2.09c^{a}	-	-	-	-
UAGAt 76	RS	-	2.68c^{a}	-	-	-	-
UAGAt 77	RS	-	3.03Ac^{a}	-	-	2.79Ab^{a}	1.98Ac^{b}
UAGAt 89	SNC	-	2.33Bc^{a}	3.916Bb^{a}	-	1.81Ac^{a}	6.02Aa^{b}
UAGAt 92	SNC	-	1.88Ac^{a}	2.59Ac^{a}	-	-	2.26Ac^{b}
UAGAt 93	SNC	-	1.93Ac^{a}	1.90Ac^{a}	-	-	-
UAGAt 94	SNC	-	1.79Ac^{a}	1.77Ac^{a}	-	-	-
UAGAt 95	SNC	1.47c	1.75c^{a}	-	-	-	-
UAGAt 98	SWC	1.85c	2.09c^{a}	-	-	-	-
UAGAt 99	SWC	-	2.20c^{a}	2.36c^{a}	-	-	-
UAGAt 101	SWC	-	1.89Bc^{a}	3.15Bb^{a}	-	4.10Aa^{a}	4.2Ab^{b}
UAGAt 104	SWC	-	-	1.08c^{a}	-	-	-
UAGAt 107	SWC	-	1.836c^{a}	-	-	-	-
UAGAt 108	SWC	1.37Ac	1.43Ac^{a}	-	-	1.68Ac^{a}	
UAGAt 113	EL	1.67Ac	1.94Ac^{a}	-	-	-	-

Table 2. contd.

UAGAt 114	EL	1.59Ac	2.49Ac[a]	-	-	2.41Aa[a]	-
UAGAt 115	EL	-	1.18c[a]	-	-	-	-
UAGAt 119	EL	1.64Ac	1.87Ac[a]	-	-	-	1.72Ac[b]
UAGAt 120	EL	-	1.87Ac[a]	-	-	-	1.53Ac[b]
UAGAt 122	EL	1.83Ac	1.69Ac[a]	-	-	-	1.42Ac[b]
UAGAt 123	EL	-	1.83Ac[a]	2.17Ac[a]	-	-	1.47Ac[b]
UAGAt 124	EL	1.62Ac	1.91Ac[a]	-	-	-	1.28Ac[b]
UAGAt 125	EL	-	1.46Ac[a]	1.62Ac[a]	-	-	1.33Ac[b]
UAGAt 126	EL	-	1.28c[a]	-	-	-	-
UAGAt 127	EL	1.17Ac	-	-	-	-	1.61Ac[b]
UAGAt 128	EL	-	-	1.97c[a]	-	-	-
UAGAt 133	RS	-	1.54Ac[a]	-	-	1.61Ac[a]	-
UAGAt 135	RS	-	1.37Ac[a]	-	-	1.49Ac[a]	-
UAGAt 140	RS	2.54Ac	1.69Ac[a]	-	-	1.92Ac[a]	-
UAGAt 141	RS	-	1.88Ac[a]	-	-	2.59Ab[a]	2.04Ac[b]
C.V		54.48					

Means with same uppercase letter in the line comparing the rate of solubilization by bacterial isolates compared to NaCl concentrations (0 and 5%); Lowercase letters in the column comparing the rate of solubilization by bacterial isolates in the three culture times - 3, 6 and 10 days; Letters in exponential line compare the solubilization index by bacterial isolates in relation to time and the concentrations of NaCl. Same letter do not differ at 5% probability by Scott-Knott's test. (SNC- soil no cultivation; SWC- soil with cultivation; ER- endophytic root; EL: endophytic leaf; RS: rhizosphere soil); (UAGAt 01-UAGAt 77: Isolates of Serra Talhada; UAGAt 89-UAGAt 141: Isolates of Ibimirim)

the different niches associated with the plant *Atriplex*. For Ibimirim area, different responses were observed, evidencing significant value in endophytic niche leaves (42.85%), where in the rhizosphere percentage of 6.28% was observed in the relative frequency of FNB. It is observed that Serra Talhada excelled in relation to Ibimirim, with values of 60.55 and 39.43%, respectively.

Synthesis of indole acetic acid

Regarding of synthesis of indole acetic acid (IAA), it was observed that 100% of the tested isolates were able to synthesize this phytohormone. The values of the IAA production varied from 61.10 to 1.0 µgmL^{-1} corresponding to the UAGAt21 bacteria (soil with crop - Serra Talhada) and UAGAt119 (endophytic leaf-Ibimirim) respectively and a significant difference between bacteria evaluated (Table 3). Production of IAA in Serra Talhada show 31.57% positive isolates for endophytic root niche, especially in relation to the studied environments. For samples arising Ibimirim, isolated endophytic leaf shown positive relative frequency of 63.15%. In generally, 100% of the isolates producing IAA, (66.66%) are Serra Talhada and (33.33%) to Ibimirim. Thus, it is possible to point the most potential of endophytes isolated for mechanism of plant growth promotion in Serra Talhada.

Production of exopolysaccharides

Among the 30 bacterial isolates studied, 83.33% were positive to the test and was possible to confirm the production of EPS by bacteria tolerant to salt associated with *Atriplex* (Figure 2). Given the classification described in Table 4, it is observed that five isolates (16.66%) were negative for the production of EPS, while 13.33% have provided little production, 10% medium production and 60% optimum production of EPS. Difference also was observed between isolates according to their EPS production (Table 5). The isolated UAGAt89, from the soil without cultivation of area Ibimirim provided the highest average value, with the halo of producing 60.33 mm, differing from the other, except UAGAt90 and UAGAt128.

In addition, the isolates showed greater diameter halo originating from the soil without cultivation for endophytic leaf of Ibimirim area, which confirms the classification result shown in Table 4.

Molecule expression quorum sensing

It was found 29 positive bacterial isolates tested for production of the quorum sensing (AHL) molecule because bacterium *A. tumefaciens* colonies showed blue pigmentation (Figure 3) while only isolate belonging to UAGAt77 rhizosphere from Serra Talhada, did not express the production of this molecule. For that, it can be highlighted the high rate (96.66%) of isolates producing the AHL molecule, a fact which shows the adaptation of these isolates to the saline environment under *Atriplex* cultivation.

Table 3. Production of indole acetic acid (IAA) in vitro by salt-tolerant bacterial isolates associated with *Atriplex mummularia* L.

Isolated bacterial	Niche	IAA µg mL^{-1}
UAGAt 01	SNC	3.82^e
UAGAt 02	SNC	15.38^d
UAGAt 06	SNC	3.13^e
UAGAt 08	SNC	1.36^e
UAGAt 09	SNC	3.04^e
UAGAt 12	SNC	4.42^e
UAGAt 13	SNC	3.73^e
UAGAt 14	SNC	18.35^d
UAGAt 15	SNC	11.67^d
UAGAt 19	SWC	7.57^e
UAGAt 21	SWC	61.10^a
UAGAt 22	SWC	6.15^e
UAGAt 23	SWC	21.72^d
UAGAt 25	SWC	53.25^a
UAGAt 27	SWC	4.42^e
UAGAt 34	SWC	41.64^b
UAGAt 33	ER	2.14^e
UAGAt 35	ER	42.59^b
UAGAt 36	ER	2.05^e
UAGAt 37	ER	3.22^e
UAGAt 38	ER	24.62^c
UAGAt 39	ER	11.88^d
UAGAt 40	ER	21.80^d
UAGAt 41	ER	16.67^d
UAGAt 42	ER	1.23^e
UAGAt 43	ER	14.77^d
UAGAt 45	ER	39.19^b
UAGAt 51	EL	1.83^e
UAGAt 53	EL	31.77^c
UAGAt 54	EL	2.40^e
UAGAt 61	EL	27.41^c
UAGAt 63	EL	14.95^d
UAGAt 66	RS	1.79^e
UAGAt 74	RS	4.38^e
UAGAt 77	RS	7.14^e
UAGAt 93	RS	9.94^d
UAGAt 71	RS	3.34^e
UAGAt 73	RS	11.45^d
UAGAt 75	RS	10.72^d
UAGAt 89	SNC	2.91^e
UAGAt 90	SNC	6.79^e
UAGAt 92	SNC	21.76^d
UAGAt 95	SNC	2.40^e
UAGAt 99	SWC	1.23^e
UAGAt 101	SWC	1.49^e
UAGAt 107	SWC	2.39^e
UAGAt 113	EL	6.19^e
UAGAt 114	EL	2.52^e
UAGAt 115	EL	1.10^e
UAGAt 119	EL	1.01^e

Table 3,contd.

UAGAt 120	EL	1.10^{e}
UAGAt 122	EL	1.58^{e}
UAGAt 123	EL	1.96^{e}
UAGAt 124	EL	1.58^{e}
UAGAt 125	EL	16.33^{d}
UAGAt 126	EL	15.25^{d}
UAGAt 127	EL	5.63^{e}
UAGAt 128	EL	42.68^{b}

(Coefficient of Variation: 23.84%) Same letters in the columns do not differ by the Scott-Knott's test at 5% probability. (SNC- (Soil without cultivation; SWC- Solo with cultivation; ER- Endophytic root; RS- Rhizosphere soil; EL: Endophytic leave of *Atriplex* plants).
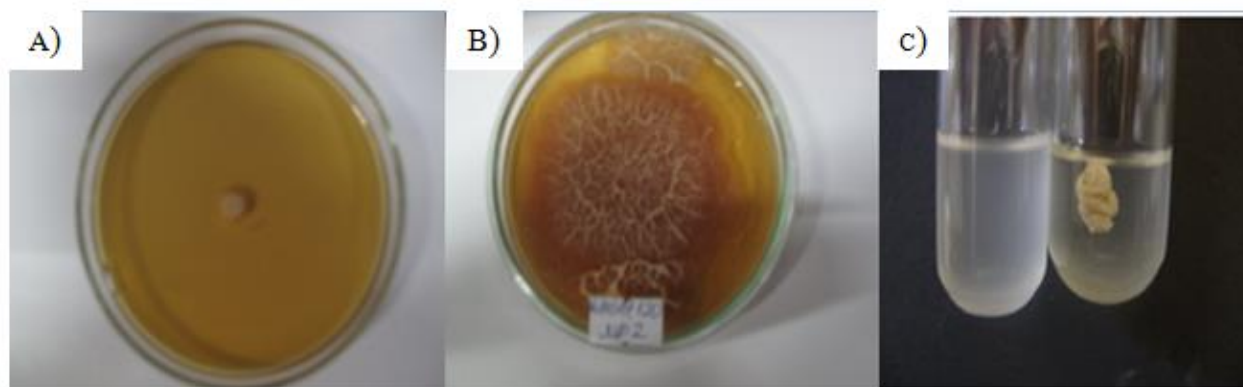
Figure 2. Negative bacterial isolate (A); Isolated positive bacterial (B); Confirmation of EPS production (negative: cloudy / pipe on the left; positive: precipitate / all right) (C).

Table 4. Classification of exopolysaccharide (EPS) by bacterial isolates possibly plant growth promoters associated with *Atriplex*.

Production of exopolysaccharides							
Isolated bacterial	**Little**	**Mean**	**Optimum**	**Isolated bacterial**	**Little**	**Mean**	**Optimum**
UAGAt 08	-	-	-	UAGAt 71			+++
UAGAt 09			+++	UAGAt 75			+++
UAGAt 14	+			UAGAt 77		++	
UAGAt 21		++		UAGAt 89			+++
UAGAt 22	-	-	-	UAGAt 90			+++
UAGAt 25		++		UAGAt 92			+++
UAGAt 33			+++	UAGAt 93	+		
UAGAt 35			+++	UAGAt 95	-	-	-
UAGAt 37			+++	UAGAt 99			+++
UAGAt 51			+++	UAGAt 101			+++
UAGAt 53	+			UAGAt 107			+++
UAGAt 54	+			UAGAt 114			+++
UAGAt 63			+++	UAGAt 122			+++
UAGAt 66	-	-	-	UAGAt 125			+++
UAGAt 69	-	-	-	UAGAt 128			+++

Table 5. Halos of exopolysaccharide production by bacterial isolates possibly plant growth promoters associated with *Atriplex*.

Isolated bacterial	Niche	Halo de EPS (mm)
UAGAt 09	SNC	16.78[g]
UAGAt 14	SNC	7.46[g]
UAGAt 21	SNC	10.65[g]
UAGAt 25	SWC	10.86[g]
UAGAt 33	ER	23.26[e]
UAGAt 35	ER	27.76[d]
UAGAt 37	ER	20.30[e]
UAGAt 51	EL	18.05[f]
UAGAt 53	EL	9.83[g]
UAGAt 54	EL	7.72[g]
UAGAt 63	EL	39.75[c]
UAGAt 71	RS	29.83[d]
UAGAt 75	RS	31.63[d]
UAGAt 77	RS	10.67[g]
UAGAt 89	SNC	60.33[a]
UAGAt 90	SNC	57.08[a]
UAGAt 92	SNC	18.42[f]
UAGAt 93	SNC	8.00[g]
UAGAt 99	SWC	41.00[c]
UAGAt 101	SWC	25.66[e]
UAGAt 107	SWC	50.38[b]
UAGAt 114	EL	14.12[f]
UAGAt 122	EL	17.67[e]
UAGAt 125	EL	53.96[b]
UAGAt 128	EL	60.28[a]
C.V (%)	14.54	

Similar letters in the same columns do not differ according to Scott-Knott's test at 5% probability. (Soil no cultivation; SWC- Solo with cultivation; ER- Endophytic root; RS- Rhizosphere soil; EL: Endophytic leave of *Atriplex* plants).

DISCUSSION

Isolation of bacteria

The microbial community may be vary depending on the plant species and soil type, since these organic compounds influence the quantity and quality of exudates, which, in turn, will select or favor specific nutritional groups and organisms in the rhizosphere soil (Ahemad and Kibret, 2014).Similar results were found by Santos (2010), studying the bioprospecting of bacteria associated with *Atriplex nummularia* L. in saline-sodic soil in Pesqueira, Wasteland of Pernambuco (Brazil), revealing significant amount of population density in the rhizoplane niche (UFC 10^9 g^{-1} soil). Mapelli et al. (2013) studied bacteria associated with rhizosphere of Salicornia plant in hypersaline soils, detected values from 10^4 a 10^{10} CFU g^{-1} soil for population density, bigger those found

in this study. These authors also report that the interaction with the plant and the presence of root exudates may account for the higher abundance of halotolerantes bacteria detected in the rhizosphere.

Regarding to salt tolerant bacteria associated with *A. nummularia* L. in this research, some factors must have influenced the population density of these bacteria, such as climate change, plant species, and the type of soil in which the plant is being grown, giving conditions to varying results.

Solubilization of inorganic phosphate

Several factors can influences this mechanism. Thus, by the attributes shown in Table 1, the soil cultivation without Ibimirim area has higher electrical conductivity (59.89 dS m^{-1}), being more saline than the soil cultivation without area Serra Talhada (41.59 dS m^{-1}). These data may explain the predominance of larger SI area Serra Talhada when tested in the absence of NaCl, while a concentration of 5%, the bacteria isolated from Ibimirim area stood out this characteristic. This can be justified by the possible formation of more bacteria groups adapted to each environment, promoting change in SIF. Many microorganisms are able to solubilizing inorganic phosphate, but their transformative capacity can associate to the ecological conditions, including soil characteristics and vegetation. Regarding the expression of SIF in salinity conditions associated with *Atriplex* it can be explained by the fact that adversely affect salt growth and proliferation of microbial cells, resulting in a loss of efficiency of growth promotion mechanism, which may be variable among bacterial species. Thus, bacteria appears to adapt constantly their physiology to changes in physic and chemical factors of the environment, including the accumulation of osmoprotectors expressing adaptive behaviors to achieve osmotic adjustment and to ensure the stability of certain active protein (Chérif-Silini et al., 2013).

In another research, Nakbanpote et al. (2013) addressing tolerance of bacteria to salinity and plant growth-promoting, SIF bacteria observed in 8% NaCl conditions, confirming tolerance to salinity of these microorganisms. Regarding to more expressive values for the SI (9.73) were found in this study with bacteria tolerant to salinity associated with plants of *Atriplex*, indicating the biotechnological potential of salt-tolerant isolated. It can be noticed that the inorganic phosphate solubilization rates found with bacteria associated with Atriplex this study provide potential for promoting plant growth, which indicates the efficiency of these micro-organisms coming from saline soils, contributing to plant growth.

However, for the enhancement of inorganic phosphate solubilization process, there is still need for further knowledge of these micro-organisms associated with halophytes, as the Atriplex in saline soils, for this
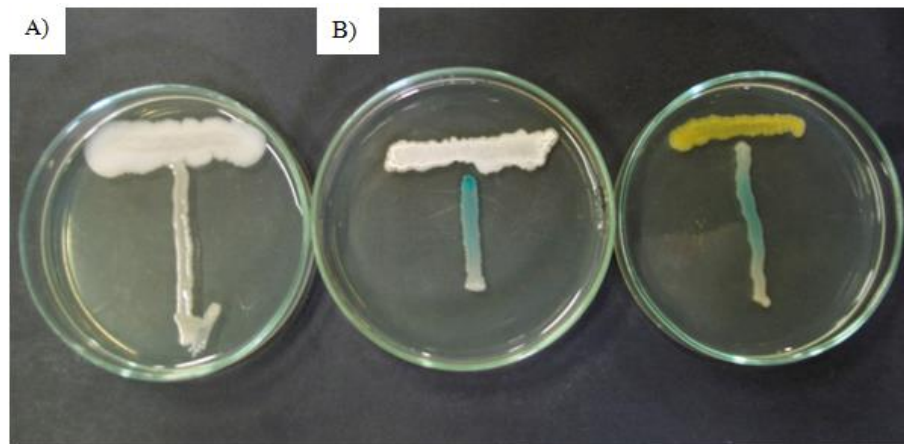
Figure 3. Absence of blue staining (negative) (A); Presence of indicator blue staining of quorum sensing molecule expression (AHL) (B). *A. tumefaciens* is peaked in the vertical and horizontal test bacteria.

process to be safely indicated in phytoremediation of soils and in sustainable agriculture.

Biological nitrogen fixation

The existence of nitrogen-fixing bacteria associated with different niches and areas is very variable. Santi et al. (2013), report that endophytic bacteria can have advantages over the rhizospheric bacteria, because they colonize the interior of the plant tissues and consequently can be established in niches providing more appropriate conditions for effective nitrogen fixation, suffering less competition the bacteria present in the soil, possibly, excreting part of the nitrogen, occurring the transfer of fixed nitrogen to the plant. This may have occurred with the endophytic root tested in this study, especially in the FNB mechanism.

In research carried out by Santos (2010) is mentioned the existence of salt-tolerant nitrogen-fixing bacteria associated with plants of *Atriplex*, where there was a greater number of bacteria with this feature in endophytic root niche, a result similar to that found in soil Serra Talhada. Pereira et al. (2012), studying endophytic bacteria of sugarcane in relation to salinity, they observed the NFB in the tested strains, detaching the importance of exploring bacteria tolerant to salinity so that they can be used as inoculants, in order to minimize the use of chemical fertilizers and thus the salinization increasing.

Synthesis of indole acetic acid

Regarding to stronger association of *Atriplex* roots with bacterial community may be related to the compounds exuded by the roots, rich system sugars, polysaccharides, phenolic compounds and aliphatic attracting these

microorganisms, with consequently greater interaction bacteria and plant. Thus, there are better conditions for IAA phytohormone synthesis (Compant et al., 2010; Ahemad and Kibret, 2014). Kuklinsky-Sobral et al. (2004) report that the habitat associated with the plant is a dynamic environment, which may occur interference of several factors on composition of the bacterial community, which colonizes the niches associated to the plants. Microorganisms can select a different pathway depending on the environment and can reveal interesting results.

According to other results obtained by Jha et al. (2011) about salt-tolerant bacteria and plant growth-promoting associated with *Salicornia brachiata*, was observed that all isolates tested produced IAA in amounts ranging from 30 to 100 µg mL^{-1}. In another study, Sgroy et al. (2009) evaluated endophytic bacteria associated to the halophyte *Prosopis strombulifera* and found the highest values for IAA production of 2.2 µg mL^{-1}. In this context, the findings of this study are significant for the production of IAA by bacteria salt-tolerant with dependent pathway of tryptophan.

Production of exopolysaccharides

The observation of great production of exopoly-saccharides is more visible in bioprospected bacteria of Ibimirim area. Therefore, it is because bacteria are associated with more stressful environments in relation to soil salinity and these microorganisms can to express more sharply, in order to protect the plant against raised stress (Qurashi et al., 2012). Thus, it is believed that bacteria have been induced to produce EPS in large quantities, since the accumulation or formation of this substance tends to increase from the moment in which the salt stress or other stress is expressed most

pronouncedly the environment. Thus, the bacteria tend to produce this substance in order to protect the plant against stress causes, such as saline strees, drought and heat stress, benefiting crop growth and development, especially in saline soils, where *Atriplex* is grown.

On the production of EPS Qurashi et al. (2012) studied this mechanism under influence of salt stress and reported increase of EPS production at higher salinity levels promote formation of biofilm and protects the plant by maintaining a water layer around the cells, contributes significantly to the improvement of soil fertility and plant growth. Ashraf et al. (2005), evaluating producing bacteria EPS, the observed an increase in soil aggregation around the roots of inoculated wheat plants, grown in saline soil, positively affecting their physico-chemical characteristics. The authors also realized that to offset the stress imposed by salinity, the production of exopolysaccharides are significant strategies to help in the metabolism of tolerance to salts by bacteria adapted to this environment.

Molecule expression quorum sensing

Associating with the results found in this work to ready the literature, it was noticed a higher percentage of bacteria producing the quorum sensing (AHL) molecule. Leite et al. (2014) studied bacteria salt-tolerant associated to sugar cane and analyzing the production of quorum sensing among 102 isolates, 49% positive isolate were found.

Bhattacharyya and Choudhury (2008) report that environmental conditions often change rapidly and the bacteria need to respond very quickly to this change in order to survive. These responses can be different, including adaptation, availability of nutrients and defense against other microorganisms. However, quorum sensing communication helps bacteria to coordinate their behavior face to the adverse environmental conditions.

Thus, the importance of detecting quorum sensing, especially the AHL molecule, the adaptation of microorganisms in general and particularly in salty environments, is indispensable. However, the role of this molecule in microbial biosphere is still relatively unknown, requiring further studies for biotechnology in agriculture purposes.

Conclusions

In general, bacteria tolerant to salinity associated with plant *A. nummularia* L., provided plant growth promoting confirmed in the areas studied (Serra Talhada and Ibimirim). The study indicates high levels of SIF at a concentration of 5% NaCl by isolated UAGAt 89 (without soil cultivation-Ibimirim) and UAGAt 101 (soil with cultivation-Ibimirim). The endophytic bacterial isolates

stood out with NFB and production of IAA. It is worth noting that tolerant to salt bacteria have potential for EPS production and expression of N-acyl homoserine lactone molecule by the communication system quorum sensing.

Thus, the bacterial isolates UAGAt89 and UAGAt101 expressed greater tolerance to salinity, making it promising for future studies and may contribute to the development of *Atriplex* plants and soil reclamation affected by salinity.

Conflict of Interests

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Morphogenesis of edible gall in *Zizania latifolia* (Griseb.) Turcz. ex Stapf due to *Ustilago esculenta* Henn. infection in India

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The *Zizania latifolia* Griseb. Turcz. ex Stapf., a perennial wetland wild rice species get infected by a basidiomycetous fungus *Ustilago esculenta* Henn that produces gall. Here we report the morphogenesis behaviour of this plant during gall transformation. The identity of *Z. latifolia* and *U. esculenta* were confirmed by comparing microsatellite and Internal Transcribed Spacer region of nuclear ribosomal DNA sequence with the public database. *U. esculenta* infection transforms the *Z. latifolia* into two distinct morphotypes such as gall forming and non-gall forming. The non-gall bearing morphotype exhibited pinkish microporous lamellar discs (15-20 numbers) filled with mycelia in the internodal spaces. Whereas the gall bearing morphotype fuses 3-4 nodes into compact spindle shaped hypertrophic tissue measuring 2.5 cm diameter x 8-10 cm length tapering at the tip end where dark brown coloured teliospores are deposited in linear sori. In vitro culture of lamellar discs and teliospores in Potato Sucrose Agar medium initially develops white colony 2.6- 4.0 cm diameter later turned into yellowish brown after 7 -10 days and subsequent sporulation within 8-12 days. Spherical sporangium of 28 μm diameter developed on long sporangiophore 7 μm diameter filled with large number of spores 1-1.5 μm in diameters. The unsuccessful sporulation of *Ustilago* even after infection failed to develop gall in the host plants behave normal life cycle and are not consumed. The spore along with hypertrophic soft tissue is consumed as vegetable in the oriental countries.

Key words: Manchurian wild rice, teliospore, microsatellite.

INTRODUCTION

Manchurian wild rice or water bamboo (*Zizania latifolia* Griseb, Turcz. ex Stapf.) grows luxuriantly in wetland. Its

perennial stems are comprised of rhizomes with scale leaves, stolons, and culms with relatively broad leaves. The grains contains high protein, carbohydrate and low anti-nutritional components such as cyanogenic glycosides, phytate, oxalate, saponin and tannin thus makes it more nutritious than any other rice and safe as human food (Umar et al., 2013), grains are used as food and rhizomes as diuretics and as medicines for anaemia, heart disease and liver disease (Stapf, 1909) and preventing obesity and liver lipotoxicity (Han et al., 2012) and the gall due to presence of tricin derivatives for allergy and inflammation treatment (Lee et al., 2015). *Z. latifolia* is one of the four wild rice species indigenous in northeastern India, Burma, China, Japan, in parts of Siberia and Russia. The biomass yield recorded as 109.9 tonnes dry weight ha^{-1} where 80% are in below ground (Champion and Hofstra, 2006).

Ustilago esculenta Henn, a basidiomycetous fungus has very restricted host range and till date *Z. latifolia* is the only known host. The fungus stimulates enlargement of the host culms into an edible gall that contains ascorbic acid, protein, water soluble pectin and free radical-scavenging properties besides angiotensin-converting enzyme (Qian et al., 2012). *U. esculenta* infection interfere flower initiation thereby affect seed production in *Z. latifolia* (Chan and Thrower, 1980; Yang and Leu, 1978). Two unique strains of *U. esculenta* such as sporidial (T) and mycelial (M-T) strains are reported in *Z. latifolia* (Yang et al., 2014). Cultivars of *Z. latifolia* are divided into green, red, and white according to the colour of the outer skin of the gall.

Identification of fungi through sequence similarity search of nuclear DNA internal transcribed spacer (ITS) region with the reported sequence in the public database is one of the most commonly used method. The ITS region being highly conserved at species level and variable in higher taxa (Bruns et al., 1991) has many advantages in molecular identification of fungi due to their high copy numbers that can easily be amplified and sequenced with universal primers (Bruns et al., 1991; Henson and French, 1993).

Z. latifolia population in India is grows only in and around Loktak Lake of Manipur, a wetland of Ramsar site. This lake has a unique ecosystem called 'phoomdi' (a Manipuri word meaning floating mats of soil and heterogeneous vegetation includes *Z. latifolia*) that forms the Keibul Lamjao National Park (40.5 km^2) home to the Sangai the brow-antlered deer (*Cervus eldi eldi*). The nutrient analysis of *Zizania* gall collected from Loktak Lake and its uses by ethnic communities are well described (Jain et al., 2012).

This paper investigates the authenticity of *Z. latifolia* and its pathogen *U. esculenta* found in Loktak Lake of Manipur, through microsatellite sequence alignment for *Z. latifolia* and ITS1-4 sequence alignment for *U. esculenta* and illustrates the features pertaining to gall bearing and non-gall bearing.

MATERIALS AND METHODS

Sample collection

Zizania plants were collected for both gall bearing and the non-gall bearing from Loktak freshwater wetlands of Manipur, India during August-November 2013. The whole plants were transported to Laboratory stationed at Guwahati, India about 550 km away from the source of collection for further studies and established there.

Gall bearing and non-gall bearing *Z. latifolia*

Both qualitative and quantitative parameters were recorded for leaf, stem, rhizome, stolons using measuring tape and calipers. The non-gall bearing stem were dissected longitudinally and recorded lamellae numbers in internodal space. The lamellae were observed under microscope (Axioscope A1.0, Carl Zeiss). The gall was cleaned by removing the leaves attached to it and dissected transversely into slices and longitudinally thereafter teliospores were collected by gentle tapping. The teliospore morphology was observed under microscope.

Microbial studies

After surface sterilization with 0.2% sodium hypolchlorite (Fisher Scientific) for 10 min, followed by four times washing with doubled distilled water thin slices of lamellae from no gall bearing internodal space were inoculated into the PSA (Potato Sucrose Agar) media and incubated at 28°C for 6–10 days in incubator (Max 8000, Thermofisher). On the second day, watery white coloured spot on the lamellae was visible and on 3rd day white mycelium developed that was aseptically transferred to PSA plates and incubated for sporulation. Further, the teliospores from gall tissue were also inoculated into same media and incubated as stated. These fungal cultures were subcultured once every month. PSA media was used for the regular maintenance of the culture and a temperature of 28°C was maintained for all the experiments. The pH of the medium was adjusted to 6.5 and was sterilized at 121°C for 20 min. The solid medium contained 1.7% agar agar (Himedia). After several passages of sub-culturing, the pure culture were obtained and allowed for sporulation in the culture plate.

The spores were germinated in PSA (both in semi-solid and liquid) medium at 28°C within the days of transfer. Sporidia were observed with an optical microscope after 5 days of culture establishment. After establishment of new colonies on PSA media the mycelia were observed microscopically. These strains were re-cultured on shake-cultured method in PS broth and incubated at 28°C (150 rpm) for 8 days and then recorded macroscopic morphological characters. The culture morphology and sporangium, spore morphology are recorded under microscope.

DNA isolation and molecular identification

The young leaf tissues and infected gall of *Z. latifolia* was collected from Loktak freshwater wetlands of Manipur. The infected gall was longitudinally dissected and released spores of *Ustilago esculenta* were collected by tapping the gall on a clean paper. The collected leaf tissues and spores were used for DNA isolation. The DNA was isolated from spores and leaf tissues by CTAB method (Doyle and Doyle, 1990) with slight modifications. The isolated DNA was

checked in a agarose gel and used for downstream work.

For establishment of identity of *Z. latifolia* used in the present study we have searched the public databases for any reported sequences from this species and we were able to find a microsatellite sequence in the gene bank of NCBI reported from China (GenBank accession no. EU798777.1). From this sequence we have designed the possible primers to amplify the same sequence from our fungal species. However, we could design primer pair (Forward- 5'-ACGCGGCCACATAGAAAGAA-3'; Reverse- 5'-TGCGTGTAGACATGGTTGCT-3') which would amplify only a part (356bp) of the reported sequence. Designing primers to amplify the entire reported sequence was not successful due to presence of sequences that did not allow optimum primer design using Primer3 online tool.

For molecular identification of the pathogenic fungus under study we have used universal primers (ITS1F- 5'-CGTAGGTGAACCTGCGGAG-3' and ITS4R- 5'-TCCTCCGCTTATTGATATGC-3') to amplify the ITS region spanning partial ITS1, 5.8S and complete ITS2 region . The amplified product (703bp) was cloned and sequenced. The sequence thus obtained was compared with the sequences reported in the public database of NCBI through BlastN programme for sequence similarity based identification of the fungal species under study. The sequences of reported ITS region of *Ustilago esculenta* showing high similarity with the query sequence were retrieved and aligned (clustalw2) to check the sequence similarity.

Phylogenetic position of isolated fungi within the Genus

The Neucleotide Basic Local Alignment Search Tool (BLASTN) analysis of the query sequence have also shown significant similarity with 22 different fungal species under the genus *Ustilago*. These sequences were retrieved and analysed in MEGA6 (Tamura et al., 2013) to know the evolutionary history using neighbor-Joining method (Saitou et al., 1987). By Neighbouring method to know the phylogenetic and evolutionary position of the isolated *Ustilago* species among the reported species under the genus *Ustilago*.

RESULTS

The *Z. latifolia* found in Manipur, India were about 3-4 m height having distinct rhizome, stolon, culm, leaf, roots and adventitious roots. Leaf has 13 cm long sheathing base and 120 cm long lamina about 20 cm wide. Culms were about 50 cm in length with 7-8 nodes (Figure 1a) covered with sheathing leaf base where thick adventitious roots surround the node in a single row.

Non-gall bearing *Z. latifolia*

The culms, leaf, rhizome were normal in shape and size. Culm internode length in the base and tip were shorter and longer in the middle (Table 1, Figure 1a). The cross section of culm revealed 15-20 numbers of soft velvet textured pinkish lamellar discs arranged parallel to each other in the internodal space (Figure 1c and h). The colour of lamellae observed pinkish from 2nd internodes

above the rhizome till tip of the culm. Whereas in the rhizome region 5-10 numbers of thick and white lamellae were found. Lamellae in the apical intermodal region were thin and soft and arranged in close proximity than at the middle culm region (Figure 1e-f). No sign of infusion of culm internode and internodes were detected. The lamella had large number of micro pores 8-10 µm in sizes that were intermingled with fungal hyphae (Figure 1d).

Gall bearing *Z. latifolia*

Plants were short and stout and at the 3rd or 4th nodes small maize shaped gall covered with leaves was observed (Figure 2a). Galls were 2.5-3 cm wide in the middle and 8-12 cm length tapering at the tip (Figure 2b) and the apical part of gall was spirally coiled (Figure 1g). Rectangular shaped 1 x 10 mm pinkish colour distinct spots were arranged linearly all along the leaf surface (Figure 1b). The cross section of gall in the middle portion revealed about 90-100 numbers of sori irregular in shape ranged between 0.5-2 mm diameters upon removal of teliospores a beautiful network of sori was observed (Figure 2c-d). Each sorus had a thin whitish peridium that holds the dark brown coloured powdery mass of teliospores (Figure 1i). The longitudinal section of gall revealed linearly arranged sori of measuring about 1 mm diameter and 8-16 mm length (Figure 1j).

Teliospore

The teliospores were easily removed by gentle tapping the cut surface of gall. From a gall about 15-20 gm of teliospores were collected. Teleiospores were fine and slippery in textures and dark brown in colour. The teliospores were spherical, oval and ellipsoidal and their surface ornamented with spines around. The diameter of teliospores recorded 6-7 µm having distinct 1 µm thickness wall ornamentation with layers of exsoporium, middle layer and endosporium (Figure 2e). There were 1-4 numbers lobes of greenish pigmentation observed inside the teliospores.

Microbial studies

On the second day, watery white coloured spot on the lamellae was observed and on 3rd day white mycelium was observed. The surface of the single colony developed form lamellar source was smooth, moist and round in shape. The colony size recorded was 2.6- 4.0 cm and whitish in colour in the initial stage and turned yellowish to yellowish brown after 7 -10 days of culture. Maximum colony size was observed within 7 days of culture in PSA

Figure 1. Mosphogenesis of gall in *Z. Latifolia*. a) Normal *Z. latifolia* plant, b) Pigmentation on leaf surface size 1 x 10 mm c) pink lamallae in the internodal space length 9.5 cm d) microporous (dimater 10 µm) lamalla with fungal mycelia e) Apical shoot on non gall forming plants f) Internal view of apical shoot non gall forming g) Apical part of gall. h) Cross section of non gall stem i) Cross section of gall with peridium. j) Cross section and longitudinal section of gall.

media maintained at 28°C. Sporulation developed within 8-12 days of incubation (Figure 2f). The microscopic observation revealed distinct spherical sporangium measuring 28 µm in diameter developed on long sporangiophore measuring diameter 7 µm. Large number of spores measuring 1-1.5 µm in diameters were present in each sporangium (Figure 2g). While the teliospore suspension on PSA the germination observed on very next day. After 3 days of incubation individual colony formation was observed on the surface of the PSA solid culture medium. No difference in colony morphology of lamellar and teliospore source was observed except darker in colour on maturity for the later.

Molecular identification

BLASTN analysis of the isolated microsatellite sequence (356bp) had shown high similarity (98%) with the microsatellite sequence reported in the database (GenBank accession no. EU798777.1) from where the primers were designed for amplification. This high similarity of the isolated sequences with the *Z. latifolia* microsatellite sequence in the database indicated that the host species for the fungal pathogen under study was also a *Z. latifolia* found in Manipur. The sequence was later submitted to GenBank as partial sequence of microsatellite sequences of *Z. latifolia* with accession no. KF944445.1.

The BLASTN analysis of the ITS sequence isolated (703bp) from the pathogenic fungus under study had shown high similarity (99%) with the ITS sequences of *U. esculenta* reported in the public database with 99% query coverage. The alignment of top 12 blast hit sequences of *U. esculenta* showed high similarity (Figure 3) with the query sequence had helped us to infer that the pathogenic fungus under study belonged to genus *Ustilago* and species *esculenta*. The sequence was

Table 1. Features of *Zizania latifolia* gall bearing and non-bearing.

Features	Unsuccessful gall	Successful gall
Apical shoot	Straight	Spirally coiled
Stem nodes	Total culm length 50 cm with 7 nodes	Transformed into whitish swollen soft tissue 8-10 cm long and 2.5-3 cm diameter
Stem internodes length (cm)	From rhizome to tipward 1.8, 2.3, 9.5, 4.3, 5.6, 4.1, 2.6	Not distinction
Internodal lamellae	15-20 arranged parallel Thin pinkish microporous lamellae	Not found
Leaf	Leaf sheath Length 15 cm, leaf lamina length 120 cm, lamina width 19.62 mm green in colour	On leaf rectangular pinkish tinge
Mycelia	Present in the lamellae	Only spore found
Teliospores	Not found	Dark brown powdery mass 6-7 µm size in the linear sori

Figure 2. Illustration of gall a) Gall bearing on tip of *Z. latifolia* plants b) A bunch of gall sold in market c) Cross section of gall 2.5 cm diameter with dark brown coloured powdery teliospores d) A section of gall after removal of teliospores 2.5 cm diameter e) Teliospores under microscope 6-7 µm size f) *U. esculenta* sporulation in culture plate g) *U. escuelnta* sporangium (28 µm) with sporangiophore (7 µm diamater) under culture condition.

Table 2. Features of *Ustilago esculenta* under culture condition.

Features	Observations
Colony shape	Round 2.6-4 cm in diameter
Colony colour	White for lamellar source and white brown for teliospore origin
Sporangium	28 µm diameter red in colour on the top of long sporangiaphore
Sporangiaphore	7 µm wide,
Spore	1-1.5 µm in diameter
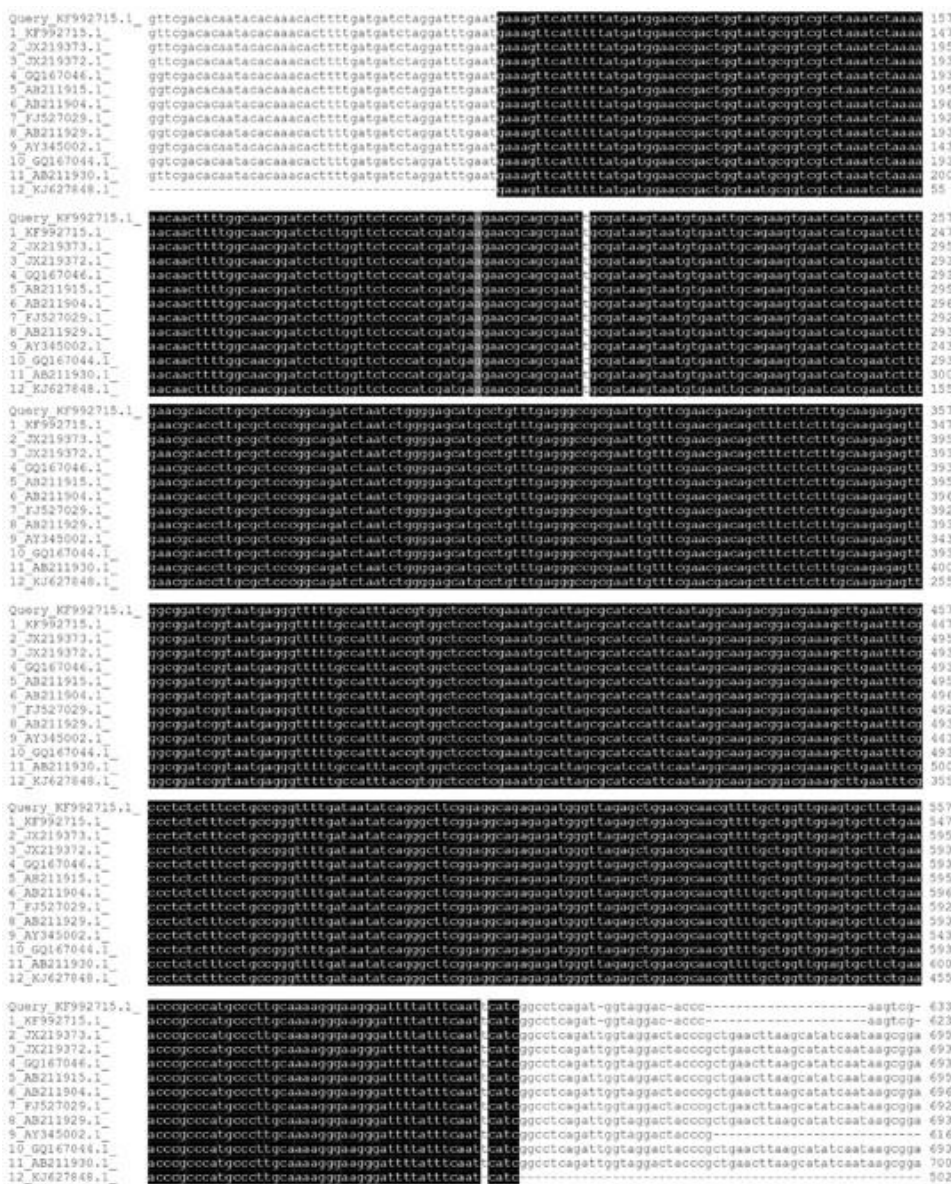
Figure 3. Alignment of isolated ITS region of the pathogenic fungus under study with the top 12 blast hit species of *Ustilago esculenta* showing high sequence similarity with the query sequence. The numbers on the left column of the figures indicates the Gene bank accession numbers of sequences used in the alignment. The bases that are conserved in all sequences under study are indicated by black background while bases not conserved in all sequences are indicated with white background.
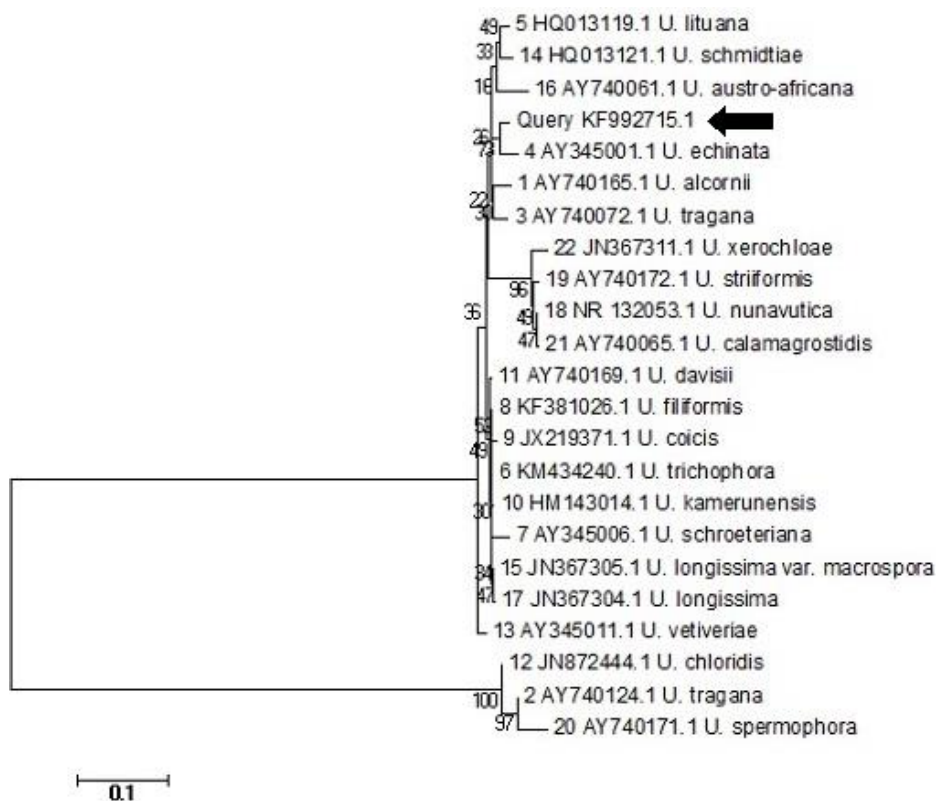
Figure 4. Evolutionary position of isolated fungi based on ITS sequence. The phylogenetic tree constructed in MEGA6 showing the taxonomic position of isolated fungi (indicated by black solid arrow) among the members of the genus *Ustilago*.

submitted to Genbank as ITS region of *U. esculenta* with accession no KF992715.1

The phylogenetic tree in MEGA 6 (Tamura et al., 2013) was constructed to know the taxonomic position of the fungus among other members of the genus *Ustilago*. The optimal tree was constructed with sum of branch length = 1.36901858 (Figure 4). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches (Felsenstein et al., 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and were in the units of the number of base substitutions per site. The isolated fungus had been clustered with *U. echinata* with a bootstrap value of 73 (Figure 4).

DISCUSSION

Gall formation occurs in *Z. latifolia* sporadically due to *U. esculenta* infection however if mycelia present in the main stem fails to invade new tillers during the tillering period the plants remained without infection (called 'male' Jiaobai) during cultivation (Ding et al., 1991). Histological studies revealed that *U. esculenta* are distributed throughout the stem tissue except leaves and root tissues of *Z. latifolia* (Yang and Leu, 1978) and even specifically designed primers for the pathogen failed to amplify the DNA extracted from leaves and healthy plant tissue (Chen and Tzeng, 1999). Present studies illustrates that in Indian *Z. latifolia* population non-gall bearing plants has infection of *U. esculenta* on stem tissues that local people called as "Eishing Kambong Laba" (a Manipuri word of male wateroat) that are not eaten by communities thus get selectively discarded. Cell wall degrading enzymes from *U. esculenta* participates in gall formation of *Z. latifolia* (Nakajima et al., 2012). *U. esculenta* infection interfere inflorescence development and subsequent seed production in *Z. latifolia*, which results in an increase in the size and the number of host cells (Chan and Thrower, 1980; Yang and Leu, 1978). Gall formation includes an increase of the size and number of host cells, suggestive of the involvement of a high IAA content within the swelling tissues. The hypertrophy of tissues also implicates

cytokinin involvement in the gall formation (Chan and Thrower, 1980). The imbalance between IAA and cytokinin due to pathogen infections is important for overgrowth, hypertrophy and tumor formation in many plant-microbe interactions (Surico et al., 1985). Although higher levels of IAA and cytokinins are often found in the gall tissues (Chan and Thrower, 1980), the role of these two plant regulators in gall development has not been yet experimentally demonstrated. The mycelia infested pinkish soft lamellae in the intermodal space of culm is the first report from unsuccessful gall forming culm of *Z. latifolia* (Table 1). There may be several abiotic and biotic factors influencing the gall formation even after infection by *U. esculenta* in to the host plant that needs to be investigated. The gall as vegetable is high in fiber, vitamins, essential amino acids and minerals (Jiang and Cao, 2008). The gall is derived from a hypertrophy of host tissues due to colonization of pathogen and usually located in the stem region of 3-4 nodes (Chan and Thrower, 1980; Yang and Leu, 1978). The gall is edible with unique flavour and tendency. Therefore, the crop is cultivated as a vegetable in southern Asia (Guo et al., 2007). In India *Z. latifolia* is not domesticated yet thus collections are made from wild habitat especially in and around Loktak wetland, Manipur, India. Based on the colour of the outer gall sheath, three cultivars (green, white and red) *Z. latifolia* are commonly identified due to infection by *U. esculenta* in Taiwan (Yang and Leu, 1978). In India only green cultivar is found that are harvested during September-October. Further two unique strains of *U. esculenta* such as sporidial (T) and mycelial (M-T) strains are reported in *Z. latifolia* (Yang et al., 2014). In Indian population both strains of *U. esculenta* are also observed.

Since there is very high similarity of the isolated microsatellite sequence of the host with the *Z. latifolia* reported in the database, we can infer that the host plant under study is a *Z. latifolia* plant collected from Manipur. We have also confirmed the identity of the pathogenic fungi as *U. esculenta* by comparing the ITS region. The high similarity of the isolated ITS region of the pathogenic fungi with the *U. esculenta* sequence in the database helped us to infer that the isolated fungus belongs to the genus *Ustilago* under the species *esculenta*. The taxonomic position of the fungus among other species of the genus *Ustilago* have shown that it is very closely related to *U. echinata* and forming a small cluster with bootstrap value of 73. Inclusions of these two species within a single cluster have indicated their close evolutionary relationship. Further, unlike other species of *Ustilago*, these two species has got similar type of host plant, that is, wet land grasses. *U. esculanta* parasitize *Z. latifolia* while *U. echinata* parasitize *Phalari* sp. or *Glyceria* sp., both are wetland grasses (Matthias et al., 2003). Both species have been reported to infect the stems forcing the plant to enter into a sterile life phase (Vánky, 1987, 1994). These similarities between these two species have justified their close evolutionary relationship as revealed by the phylogenetic analysis. This is first sequence of *Z. latifolia* and *U. esculenta* deposited in the Genbank database from India.

This study authentically identified the *Z. latifolia* host plants and its pathogen *U. esculenta* using partial microsatellite region and Internal Transcribed Spacer (ITS1-4) region of nuclear ribosomal DNA (nrDNA) sequence alignment respectively. Indian *Z. latifolia* population is described as green cultivars with dark brown coloured teliospores. The non-gall bearing *Z. latifolia* stem has unique microporous pink coloured lamellae in the stem internodal space and same is fused into hypertrophic soft edible tissue as gall that are consumed as vegetables. In India *Z. latifolia* is found only in and around Loktak lake, Manipur and is not cultivated yet thus the collections are made from wild. *Z. latifolia* vegetation along with other species form a unique ecosystem called Phoomdi a floating mass distributed in all throughout the lake and serves as habitat for the Sangai the brow-antlered deer (*Cervus eldi eldi*).

Conflict of Interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

In vitro antimicrobial activity of essential oil of *Cymbopogon citratus* (lemon grass) on *Streptococcus mutans* biofilm

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The present study aimed to investigate the effect of essential oil of lemon grass (LGO) on *Streptococcus mutans* biofilm developed in hydroxyapatite discs surface. Initially, the susceptibility of *S. mutans* to the LGO through the inhibition zone test in planktonic suspension and minimum inhibitory concentration (MIC) was investigated. To evaluate the effect of the essential oil in biofilms, hydroxyapatite discs were used to simulate the tooth surface. The biofilms of *S. mutans* were developed on the discs for 5 days and immersed daily in the following groups: G1 - immersion for 5 min in LGO to 0808 mg/ml (test group) and G2 – Brain Heart Infusion (BHI) 1% Sucrose (negative control). Then biofilms were counted for colony forming unit (CFU) and transformed into log^{10}. The data were analyzed by ANOVA test with a P value <0.05. The susceptibility test was positive indicating inhibition of microorganisms and the MIC value was 0.04 mg/mL. As for biofilm results, it decreased the bacterial growth in G2 compared to G1 with a statistically significant difference (P <0.034). Considering the limitations of this study, it was concluded that the essential oil of lemon grass was effective in controlling bacterial growth in biofilms of *Streptococcus mutans*.

Key words: Phytotherapy, *Cymbopogon citratus*, *Streptococcus mutans*, biofilm, dental plaque.

INTRODUCTION

Dental caries is considered an imbalance of re/demineralization with a high incidence in humans (De Lorenzo, 2004). According to the World Health Organization (WHO), the prevalence of caries in school children is 60-90%, and is virtually universal among adults in most countries (Petersen et al., 2005). It is still the leading cause of tooth loss, especially in people younger than 40 years (De Lorenzo, 2004). *Streptococcus mutans* are the microorganisms that best meet all cariogenic requirements, and therefore are considered

the main causative agents of dental caries. They are gram positive, facultative anaerobic bacteria whose cells are arranged singly or in chains of cocci. This microorganism is widely known for its intense acidogenesis due to production of organic acids as byproducts, which cause a carious lesion by dissolving the crystal structure of dental enamel, as well as for its ability to synthesize extracellular glucans, key factors in the development and establishment of a cariogenic biofilm (De Lorenzo, 2004; Yatsuda et al., 2005).

The traditional strategies for the prevention of caries include non-operative measures, such as control of both dental plaque and the individual's diet. With the goal of promoting health, supportive measures such as topical application of fluoride, fluorinated water supply, and the use of chemical substances are the most traditionally used and prescribed methods for high-risk populations. Despite their applicability, there are obstacles to providing these measures, such as the unequal coverage of fluorinated water in different regions of Brazil; mechanical removal being dependent on the maturity and dexterity of manual skill; and the adverse effects resulting from the use of chemical measures, often carried out indiscriminately and without professional supervision and resulting in discoloration, loss of taste, and reversible mucosal desquamation (Cury, 1997).

Research into natural extracts has increased in recent years owing to their potential to be developed into new, commercial pharmacological products for the treatment and prevention of various oral pathologies and that are less toxic, biocompatible, and more affordable (Castilho et al., 2007).

Medicinal plants have been used throughout history owing to their capability of synthesizing a wide variety of chemical compounds with important biological functions in the defense against fungi, bacteria, and various other diseases (Lai and Roy, 2004).

Several plant products have been shown to have antimicrobial potential against cariogenic organisms (Yanagida, 2000). These plant products (mainly essential oils) exert their antimicrobial activity by partitioning the lipids of the bacterial cell membrane, disrupting its structure and function and leading to cell death (Burt, 2004; Ben Arfa et al., 2006). Further, such oils are rich in flavonoid compounds that act on bacterial cells consequently inhibiting enzyme activity (Born et al., 2000), while others function within the cell wall with consequent build-up and rupture of the structure (Dorman and Deans, 2002).

The species *Cymbopogon citratus* belongs to the Poaceae family and is commonly known as lemongrass, barbed wire grass and silky heads among others. It is widely used for its antibacterial and anti-inflammatory properties; although native to India, *C. citratus* has acclimatized in Brazil (Brito et al., 2011). This plant is internationally known as, "lemon grass," for its medicinal characteristics in both humans and animals. It is an herbaceous plant, and its aromatic leaves are long, narrow, sharp, and ragged, with a prominent midrib. It grows in clumps of more than 1 m, and the leaves are rich in essential oils (Martins et al., 2004).

The plant is resistant to variations of soil and weather (Akisue et al., 1996, cited by Santos et al., 2009), but hot and humid weather, with full sun exposure and evenly distributed rainfall are optimum for its development (Ortiz et al., 2002). Owing to its therapeutic properties, it has been included among the herbal medicines regulated by the National Health Surveillance Agency since 2010 (Brazil, 2010).

Since natural extracts have garnered attention due to its promising therapeutic characteristics, the aim of this present study was to evaluate the effects of lemongrass essential oil (LGO) on cariogenic biofilm.

MATERIALS AND METHODS

Research site

This study was conducted in the Oral Microbiology Laboratory at the CEUMA University. The study did not require ethical consent since it comprised use of only a laboratory strain.

Experimental design

Initially, the susceptibility of planktonic cells of *S. mutans* UA 159 (ATCC700610) to LGO was verified through inhibition zone test using the agar diffusion method on solid medium and by evaluating the minimum inhibitory concentration (MIC) through the microdilution technique (microtechnique). These results were the basis for evaluation of the effect of the essential oil on *S. mutans* biofilm formed on hydroxyapatite disks (10x2 mm; Clarkson Chromatography Inc., South Williamsport, PA), created in order to simulate the tooth surface. The biofilm was developed on the discs for 5 days with a daily medium change (Brain Heart Infusion - BHI + 1% sucrose). Biofilms were divided into groups and immersed daily in the following treatments: BHI plus 1% sucrose (negative control) and LGO for 5 min daily.

Reference bacteria and reactivation of microorganism

The *S. mutans* reference strain UA159 was used in this study. The microorganism was reactivated in BHI medium at a final concentration of 1% glucose in a microaerophilic atmosphere at 37°C for 18-24 h. After growth, the suspension was centrifuged at 3000 RPM for 5 min, and the resulting pellet was then washed twice with sterile phosphate buffered saline (PBS). The turbidity of the resultant material was adjusted with the aid of a spectrophotometer until it reached an absorbance similar to a stock suspension of $1x10^6$ bacteria/ mL (Duarte et al., 2008).

Plant material and botanical identification

The plant material used in the study was *Cymbopogon citratus* leaves, commonly known as lemongrass. The leaves were grown in the Attic Seabra Herbarium located in the Federal University of Maranhão, São Luís, Maranhão, Brazil. The plant sample was collected between November 2013 and August 2014.

Table 1. Phytochemical analysis of essential oil of *Cymbopogon citratus.*

Identified compounds	%
6-metil-5-hepten-2-ona	0.88
beta-mircen	12.59
cis-ocimen	0.31
linalol	1.14
exo isocitral	0.58
<Z> isocitral	2.24
Rosefuran epoxide	0.37
<E> isocitral	3.01
neral (beta-citral)	34.16
geraniol	2.62
geranial (alfa-citral)	41.75
Geranil acetate	0.36

Collection and chemical composition of LGO

The leaves were separated and dried in an oven with air circulation at 37°C for 48 hours. The dried leaves were sent to the Chemical, Biological and Agricultural Pluridisciplinary Research Center (CPQBA UNICAMP), Paulínea, SP, for processing and obtaining LGO, followed by phytochemical analysis.

S. mutans sensitivity to LGO

The antimicrobial activity of LGO was determined by diffusion method according to Bauer et al. (1969). The *S. mutans* strain was cultivated in BHI supplemented with 1% glucose, and incubated at 37°C under 5% CO_2 for a period of 18-24 h in a microaerophilic atmosphere. Blood agar plates with 5% defibrinated sheep blood were prepared and inoculated with the microorganism, previously adjusted to a density of 1×10^6 organisms/mL. A cell spreader was used to uniformly inoculate the entire surface of the petri dish, using the surface inoculation technique (NCCLS, 2013). The Petri plates were divided into quarters with wells filled with the tested substances. An aliquot of 50 µL of the essential oil at 0.08 mg/mL was pipetted into one quadrant, and in another quadrant, an aliquot of 50 µL of PBS solution was pipetted to identify possible contamination of the inoculum (negative control). The plates were placed once again at a temperature at 37°C under 5% CO_2 for 48 h. After the incubation period, the antibacterial activity was evaluated by means of inhibition zone measurement.

Minimum inhibitory concentration (MIC) test

The minimum inhibitory concentration (MIC) of LGO was determined using the microdilution methodology (microdilution) (NCCLS, 2003). Ninety six well microplates were used. Each well in the test group received 100 µL of bacterial inoculum and 100 µL LGO. The negative control was established by adding only BHI liquid medium to the last row of wells. The 96-well plate was placed at 37°C under 5% CO_2 for 24 h. Readings to determine the MIC against the strains were performed using the visual method. The MIC value was considered as the lowest concentration capable of producing visible inhibition of the growth of tested bacteria.

S. mutans biofilm susceptibility to LGO

For the formation of *S. mutans* biofilm, the bacteria was reactivated as described above. The biofilms were formed on hydroxyapatite disks on a 24-well plate with each well containing BHI with 1% sucrose, changed daily for 5 days (Usacheva, Teichert and Biel, 2001; Deminova and Hamblin, 2005). Prior to daily changes of the culture medium, discs with biofilms were exposed to the following treatments: G1 - LGO (experimental group) and G2 - BHI + 1% sucrose (negative control).

Quantitative analysis

For the quantitative analysis, the biofilms were disrupted and transferred to tubes containing 5 mL of PBS and subjected to sonication using three 15-second pulses at a power of 6 W (Branson Sonifier 150, Branson Ultrasonics, Danbury, CT), with 15-second intervals (Duarte et al., 2008). A 100µL aliquot of the homogenized suspension was utilized for decimal serial dilution, subsequently plated on blood agar plates, and then incubated at 37°C under 5% CO_2 for 48 hours. The results were expressed as colony forming units (CFU) and transformed to log_{10}. Assays were performed in triplicate for each group, and repeated in two consecutive days (n = 6).

Statistical analysis

Data were analyzed using ANOVA test with a P value at 5% for differences statistically significant.

RESULTS AND DISCUSSION

In recent decades, phytopharmacology has played an important role in determining alternative therapeutic means in dentistry by evaluating the antimicrobial properties of candidate compounds in oral diseases, especially those resulting from the formation of dental biofilm (Gebara et al., 1996; Pereira, 1998).

The present study aimed to evaluate the antimicrobial properties of LGO on *S. mutans*, bacteria essential in the development of caries, corroborating a baseline data for future *in vivo* studies and serving as a supportive alternative to effective oral hygiene associated with mechanical removal of biofilm.

The leaf mass and oil mass, content, and yield were 431.34 g and 3.80 g, 0.88%, and 4 g, respectively. Table 1 shows the chemical composition of LGO.

Neral and geraniol, the major components of LGO, are stereoisomers, and their mixture results in citral, the main element responsible for the antimicrobial effect of *C. citratus*, whose mechanism of action involves increasing the permeability of the cell membrane via hydrophobic interactions with the membrane (Sikkema et al., 1994; Oliveira et al., 2011). Recent studies using chromatography have demonstrated the presence of glycolipid macromolecules in lemongrass. These glycolipids (monogalactosyldiacylglycerol and digalactosyldiacylglycerol) are abundant in photosynthetic tissues, and may be found in the outer portion of the cell

Table 2. Mean values of CFU (log_{10}) after the treatments. Data represent mean values (N = 6) and submitted to one-way ANOVA at P < 0.05.

Groups	CFU log $_{10}$ (± s.d.)	P value
LGO	7.25 ± 0.29	0.034
Control	7.81 ± 0.55	-------

LGO: lemon grass essential oil; Control: BHI + 1% sucrose; s.d: standard deviation.

membrane, mitochondrial membrane, endothelial reticulum, and chloroplasts of the plant foliage. In recent years, various studies have focused on these compounds in terms of their inhibitory activity on DNA polymerase (antitumor activity), P-selectin (anti-inflammatory effects), antiviral activity (anti-HIV), and inhibition of both the receptor membrane and the growth of specific cell lines (Mendes et al., 2006)

The results obtained in the first phase of the experiments demonstrated the inhibitory effect of LGO on *S. mutans* (UA 159) in planktonic suspension through measurement of halo of growth inhibition.

A previous study aimed to evaluate the *in vitro* antimicrobial activity of *C. citratus* on *S. mutans* by using agar diffusion method, and found a 21.5-mm inhibition zone relative to the 27 mm for chlorhexidine, indicating a slight difference (Nogueira et al., 2008). Lucena et al. (2013) showed inhibition halos of 10, 12 and 14 mm in diameter for 5 μL *S. mutans* strain, 10, 14 and 20 mm for 10 μL of the bacterial inoculum, and 10, 14 and 16 mm for 20 μL. Such studies demonstrate the marked susceptibility of *S. mutans* to *C. citratus*.

The minimum concentration required for inhibiting the bacterial species was 0.04 mg/mL. Perazzo et al. (2012) also determined the MIC value of LGO and found a higher value to exhibit an antimicrobial action (0.5625 mg/mL).

The high antimicrobial efficacy of the essential oil on the bacteria *S. mutans* has been demonstrated in a study making it extremely important in the field of dentistry, since microorganisms related to the cariogenic process produce acid from the metabolism of carbohydrates derived from the individual's diet, demineralize enamel, dentin, and cementum, inducing the development of dental caries (Vargas et al., 2010).

The use of dynamic biofilm model includes the daily application of herbal treatment, simulating the daily use of mouthwash. In certain cases, the reduction of the adhesion of the biofilm can occur, interfering with the synthesis of polyglycans, consequently acting on the adhesion mechanism of bacteria on the surfaces of teeth. Because bacterial adherence has been shown to be one of the primary mechanisms involved in initiation of development of the biofilm, the inhibition of this process would certainly lead to its effective control, contributing to the prevention of caries (Argenta et al., 2012). The results obtained for the quantitative analysis are shown in Table 2.

A statistically significant difference was found in the experimental group (LGO) when compared to the control group (p<0.05) with a reduction of 0.56 log. Although it was a non-significant reduction, which cannot be reflected clinically, the aim of the therapy is not to completely eradicate, but disrupt the biofilm associated with traditional therapy, which can be corroborated by the decreasing number of cariogenic microorganisms and the time of onset of caries. Additionally, this result may be explained in part by the presence of the biofilm matrix, which maintains the structural integrity and stability, limiting the diffusion of substances and providing protection to the bacteria against challenges such as those found in the present investigation (Paes Leme et al., 2006).

Regardless of how promising the results are, additional studies are necessary to assess the cytotoxicity of the oil against epithelial cells and keratinocytes, and to determine the antimicrobial effects of lemongrass oil in combination with the oils of other plants, substances, or preservatives contained in mouthwashes used *in vivo*, to evaluate its antimicrobial potential.

Conclusion

Considering the limitations of this study, it is possible to conclude that the immersion of hydroxyapatite discs, simulating the tooth surface in LGO was effective in controlling the growth of *S. mutans* biofilm. Although the mechanical removal of biofilm is the most accepted method for its control, the use of chemical adjuvants is of great value and results in greater control of the biofilm, consequently, reducing its pathogenicity. Thus, herbal therapy is a viable treatment for the prevention of caries. It should be highlighted that caries has multifactorial causes and depend on diet, hygiene, and susceptibility of the host, and virulence of microorganisms.

Conflict of Interests

The authors declare no conflicts of interest to the materials or techniques employed in this present study.

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Full Length Research Paper

Antimicrobial activity of extracts from *Crotalaria bernieri* Baill. (Fabaceae)

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This work was designed to study the antimicrobial activity of *Crotalaria bernieri* Baill. (Fabaceae). Extracts from leaf, root, pod and seed using hexane, ethyl acetate and methanol were tested *in vitro* for their activity against 17 bacteria, 5 fungi (3 yeasts and 2 molds) using disc diffusion and micro dilution methods. At the concentration of 1 mg/disc, all the extracts exhibited antimicrobial activity depending on the plant part and the extraction method used. The most sensitive germs were *Salmonella enteridis*, *Streptococcus pyogenes* and *Candida guilliermondii* with inhibition zone diameter (IZD) of 11 mm, 15 mm and 13 mm respectively. Most of extracts showed, broad activity spectrum varying from one extract to another. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of all extracts were recorded. Ten extracts displayed an excellent effect (MIC < 100 µg/ml), 8 a moderate effect (MIC from 100 to 500 µg/ml), 5 a weak effect (MIC from 500 to 1000 µg/ml) and the others were ineffective (MIC > 1000 µg/ml). Leaf methanol extracts were the most efficient and Gram positive bacteria the most sensitive. All extracts had bactericidal (MBC/MIC ≤ 4) or fungicidal action (MFC/MIC ≤ 4) in certain microorganisms and bacteriostatic (MBC/MIC > 4) or fungistatic action (MFC/MIC > 4) in others. Antimicrobial activity might be due to tannins, polyphenols, steroids, triterpenes and flavonoids that were present in most of the plant organs, but alkaloids in leaf and pod and saponosides in root might also be involved. *C. bernieri* with the effectiveness of all its parts, the variety of its secondary metabolites, the great number of sensitive pathogen microorganisms and its ubiquity make this plant species an interesting source of antimicrobial agents.

Key words: *Crotalaria bernieri*, antimicrobial activity, disc diffusion method, microdilution method, minimum inhibitory concentration, minimum bactericidal concentration, minimum fungicidal concentration.

INTRODUCTION

Antimicrobial resistance is one of the world's most serious public health problems. There is an urgent need

Figure 1. *Crotalaria bernieri* (a) the whole plant; (b) flower; (c) fruits (Source: the authors).

to find new disposable and affordable remedies to face this problem (Zongo et al., 2011). Many studies led to systematic screening of plant extracts as a source of anti-bacterial compounds (Dalmarco et al., 2010; Stefanovic and Comic, 2011). Several *Crotalaria* species have been reported to display antimicrobial properties. For example, *Crotalaria madurensis* is active against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Candida albicans* (Bhakshu et al., 2008), *Crotalaria capensis* against *Salmonella typhimurium* (Dzoyem et al., 2014), *Crotalaria burhia* against *B. subtilis* and *S. aureus* (Sandeep et al., 2010; Mansoor et al., 2011), *Crotalaria juncea* against *S. aureus* (Chouhan and Sushil, 2010), *Crotalaria pallida* against *E. coli* and *Pseudomonas* sp (Pelegrini et al., 2009), and *Cladophora trichotoma* against *Alternaria solani* (Ravikumar and Rajkumar, 2013).

The purpose of this study was to assess the antimicrobial activity of *C. bernieri* by testing plant part's extracts obtained in different methods on pathogen bacteria and molds. *C. bernieri* is one of the 53 *Crotalaria* species growing in Madagascar, an annual herb which is found in open vegetation, grassy places and roadsides in most regions of Madagascar (Peltier, 1959). It flowers on July to October and December to March (Polhill, 1982; Dupuy et al., 2002).

MATERIALS AND METHODS

Plants

C. bernieri (Figure 1) were harvested in Ibity, District of Antsirabe, Region of Vakinankaratra, 200 km from Antananarivo region. The plant was collected in April and July, 2013 and was identified by Polhill R.M. Voucher specimens (Herizo R. 010) of *C. bernieri* were deposited in the herbarium of Plant Biology and Ecology Department of the Faculty of Sciences of the University of Antananarivo.

Microorganisms strains

The microorganisms used in this study consisted of 17 strains of bacteria (10 Gram (-) and 7 Gram (+)), 3 yeasts and 2 molds (Table 1). These strains were obtained from the collections of Laboratoire de Chimie des Substances Naturelles et Sciences des aliments (LCSNSA) of La Réunion University. They were maintained on agar slant at 4°C and cultured on a fresh appropriate agar plate during 24 h prior to antimicrobial tests.

Chemicals for antimicrobial assay

Commonly used pre-impregnated discs, from Bio-Rad F 92430 Marnes-la-Coquette were chosen as antimicrobial references

Table 1. Bacterial, yeast and mold strains used to study antimicrobial activities.

Microorganisms		Strains	References
Bacteria	Gram(-)	*Campylobacter jejuni*	ATCC 29428
		Enterobacter aerogenes	ATCC 13048
		Enterobacter cloacae	ATCC 13047
		Escherichia coli	ATCC 25922
		Pseudomonas aeruginosa	ATCC 10145
		Salmonella enteridis	ATCC 13076
		Shigella flexneri	ATCC 12022
		Vibrio parahaemolyticus	ATCC 17802
		Yersinia enterocolitica	ATCC 23715
		Proteus mirabilis	ATCC 35659
	Gram(+)	*Bacillus cereus*	ATCC 14579
		Clostridium perfringens	ATCC 13124
		Enterococcus faecalis	ATCC 29121
		Listeria monocytogenes	ATCC 19114
		Staphylococcus aureus	ATCC25923
		Streptococcus pneumoniae	ATCC 6305
		Streptococcus pyogenes	ATCC 19615
Fungi	Yeasts	*Candida albicans*	ATCC 10231
		Candida guilliermondii	ATCC 6260
		Candida krusei	ATCC 14243
	Molds	*Aspergillus fumigatus*	ATCC 204305
		Aspergillus niger	ATCC 16888

Table 2. Abbreviations designating the different extracts tested.

Extracts	Hexane Extract	Ethyl acetate Extract	Methanol Extract
Leaves	LHE	LEA	LME
Seeds	SHE	SEA	SME
Pods	PHE	PEA	PME
Roots	RHE	REA	RME

(Camara et al.,2013; Rakholiya et al., 2014): amoxicillin 25 µg, chloramphenicol 30 µg, penicillin 6 µg as antibiotics and miconazole 50 µg as antifungal.

Preparation of extracts

The dried leaves, seeds, seed pods, and roots of the plant were grounded into powder. The resulting powder (100 g) was extracted successively with 4x500 mL of hexane, ethyl acetate and methanol for 24 h under stirring at room temperature. After filtration using a Whatman filter paper, each combined extract was evaporated under reduced pressure to dryness. The dry residues, dissolved in

hexane, ethyl acetate and sterile distilled water, constituted hexane, ethyl acetate and methanol extracts respectively (Table 2).

Phytochemical screening

The reactions for the detection of chemical groups were those developed by Fong et al. (1977) and Marini-Bettolo et al. (1981).

Antimicrobial assays

Antimicrobial activity test

The *in vitro* antimicrobial activity of the extracts was determined using disc diffusion method described by Pyun and Shin (2006) and Ngameni et al. (2009). Two mL of inoculum corresponding to 0.5 MacFarland (10^8 CFU/ml) was uniformly spread on the surface of Columbia Agar medium (for *Streptococcus*); Mueller-Hinton Agar (MHA) for the other bacteria and Potato Dextrose Agar (PDA) for yeasts. Sterilized filter paper discs 6 mm diameter (BioMérieux, REF 54991) were impregnated with 10 µL of each extract to the concentration of 100 mg/mL (1 mg/disc). The soaked discs were then placed on the surface of the agar and incubated at 37°C during 24 h for bacteria, or at 25°C for yeasts. The inhibition zone diameter (IZD) was measured and the results were interpreted by

Table 3. Extraction yields of *C. bernieri* extracts.

Extracts		Yield %
Leaf	LHE	14.6
	LEA	22.5
	LME	12.0
Seed	SHE	18.4
	SEA	12.1
	SME	10.0
Pod	PHE	15.1
	PEA	11.2
	PME	4.2
Root	RHE	13.7
	REA	15.3
	RME	24.1

means of the scale used by Ponce et al. (2003) and Celikel and Kavas, (2008) stating that bacteria are not sensitive for IZD less than 8 mm, sensitive for IZD of 9 to 14 mm, very sensitive for IZD of 15 to 19 mm and extremely sensitive for IZD larger than 20 mm. Antifungal activity was evaluated by a method described by Favel et al. (1994). One ml of each extract was added to 19 ml of medium culture of PDA and maintained at 45°C. The mixture is then poured into Petri dishes and dried for 15 min at 37°C. 10 µl of each tested microorganism corresponding to 0.5 MacFarland were spread on the medium surface. IZD were measured after incubation at 25°C for 72 h. Negative controls were prepared by using the same solvents employed to dissolve the plant extract samples while the reference antibiotics were used as positive controls. All the experiments were performed in triplicate. The results were expressed as mean values ± standard deviations (mm ± SD).

MIC, MBC and MFC determination

For extracts showing antibacterial activity in the disc diffusion method (IZD ≥ 8 mm), MIC (minimum inhibitory concentration), MBC (minimum bactericidal concentration) MFC (minimum fungicidal concentration) were determined by microdilution method (Kuete et al., 2009).

The concentration of each extract was adjusted to 25 mg/ml. This was serially diluted two-fold to obtain concentration ranges of 0.024 to 25 mg/ml. Each concentration was added in a well (96-well microplate) containing 95 µl of Mueller-Hinton broth (MHB) and 5 µl of inoculum (standardized at 0.5 MacFarland). A positive control containing bacterial culture without the extract and a negative control containing only the medium, were also analyzed. The plates were covered with sterilized aluminum foil, and then incubated at 25°C (yeasts and molds) or at 37°C (bacteria) for 24 h. The MIC of each extract was detected following addition 40 µl of 0.2 mg/ml *p*-iodonitrotetrazolium chloride and incubation at 25°C (yeasts and molds) or at 37°C (bacteria) for 30 min (Kuete et al., 2009). Viable bacteria reduced the yellow dye to a pink color. MIC was defined as the lowest sample concentration that prevented this change and exhibited complete inhibition of bacterial growth. For the determination of

MBC and MFC, 5 µl from each well that showed no change in color was transferred on MHA or PDA plate and incubated at 25°C (yeasts and molds) or at 37°C (bacteria) for 24 h. The lowest concentration at which no growth occurred on the agar plates corresponded to the MBC or MFC.

The ratios MBC/MIC and MFC/MIC were calculated for each extract, to determine the nature of the effect. The extract is bactericidal or fungicidal for MBC/MIC or MFC/MIC ≤ 4 and bacteriostatic or fungistatic when these ratios are >4 (Djeussi et al., 2013; Bouharb et al., 2014; Chamandi et al., 2015).

Statistical analyses

Results were expressed as mean values ± standard deviations of three separate determinations. One-way analysis of variance (ANOVA) which was followed by Newman Keuls comparison test with Staticf® software was used for statistical analysis. Statistical estimates were made at confidence interval of 95%.

RESULTS

Extraction yields

The extractive yield of different parts of *C. bernieri* with different solvents varied from 4.2 (PME) to 24.1% (RME) (Table 3).

Qualitative phytochemical analysis

The major secondary metabolites identified in the different organ extracts are presented in Table 4. Tannins, polyphenols, steroids, triterpenes and unsaturated sterols occurred in all the *C. bernieri* organs. Flavonoids were found in all organs except root. Alkaloids were present only in leaf and pod while saponins only in root. Iridoïds, leucoanthocyanins, and quinones were not detected in all parts of *C. bernieri*.

Antimicrobial activity

At 1 mg/disc, a concentration generally used for antimicrobial activity assessment in plants (Sandeep et al., 2010; Govindappa et al., 2011; Linthoingambi and Mutum, 2013; Marimuthu et al., 2014), the large majority of *C. bernieri* extracts (16 of 22) inhibited the microorganism growth with IZD ranging from 8 to 15 mm (Tables 5 to 7). However, activity depended on the microorganism, the plant parts and extraction method used. The most sensitive germs were *S. enteridis* (IZD=11 mm), *S. pyogenes* (IZD=15 mm) and *C. guilliermondii* (IZD=13 mm) in Gram (-) bacteria, Gram (+) bacteria and yeasts, respectively. Gram (-) strains *C. jejuni* and *E. coli*, *E. faecalis,* Gram (+) *L. monocytogenes* and the two molds *A. fumigatus* and *A. niger* were resistant to all the extracts. REA, with an IZD of 15 mm against *S. pyogenes,* displayed the highest antibacterial activity.

Table 4. Phytochemical screening of *C. bernieri* extracts.

Chemical groups	Tests	Leaf			Seed			Pod			Root		
		a	b	c	d	e	f	g	h	i	j	k	l
Alkaloids	Mayer	-	-	+	-	-	-	-	-	+	-	-	-
	Wagner	-	-	+	-	-	-	-	-	+	-	-	-
	Dragendorff	-	-	+	-	-	-	-	-	+	-	-	-
	Confirmatory test (solubility in ethanol)	-	-	+	-	-	-	-	-	+	-	-	-
Saponins	Foam test	-	-	-	-	-	-	-	-	-	-	-	+
	Confirmatory test (hemolytic test)	-	-	-	-	-	-	-	-	-	-	-	+
Flavonoids	Willstätter	-	+	+	-	+	+	-	-	+	-	-	-
Leucoanthocyanins	Bate-Smith	-	-	-	-	-	-	-	-	-	-	-	-
Tannins and Polyphenols	Gelatin 1%	+	-	+	-	+	+	-	-	-	+	+	+
	Gelatin-salt 10%	+	-	+	+	+	+	-	+	-	+	+	+
	FeCl$_3$	-	+	+	-	-	+	-	-	+	-	-	+
Quinones	Borntrager	-	-	-	-	-	-	-	-	-	-	-	-
Steroids	Liebermann-Burchard	+	+	-	+	+	+	+	+	-	+	+	-
Iridoïds	Hot HCl	-	-	-	-	-	-	-	-	-	-	-	-
Triterpenes	Liebermann-Burchard	+	-	-	+	+	+	+	+	-	+	+	-
Unsaturated sterols	Salkowsky	+	-	-	+	+	+	+	+	-	+	+	-

+: positive result; -: negative result a: LHE; b: LEA; c: LME; d: SHE; e: SEA; f: SME; g: PHE; h: PEA; i: PME; j: RHE; k: REA; l: RME.

In yeasts, most of leaf extracts were active against the three *Candida* strains tested, but seed and pod extracts were active only against *C. guilliermondii*. Antibiotics used as references in this study (amoxicillin 25 µg, chloramphenicol 30 µg, penicillin 6 µg and miconazole 50 µg) were more effective than most of *C. bernieri* extracts. MIC, MBC, MFC and MBC or MFC/MIC ratio values are presented in Tables 8 to 10. MIC ranged from 0.048 to 25 mg/ml. MIC maximum values registered was 12.5 mg/mL except for RHE on *S. pyogenes* (MIC=25 mg/ml). Concerning MBC or MFC, maximum values for all extracts were 25 mg/ml except for root extracts on some Gram (+) bacteria and *C. guilliermondii* (MBC>25 mg/ml). The ratio MBC or MFC/MIC varied from 1 to more than 100.

The most sensitive microorganism were *P. mirabilis* in Gram (-) bacteria (MIC=MBC=0.097 mg/ml), *B. cereus* (MIC=0.048 mg/ml, MBC=0.195 mg/ml) and *S. pyogenes* (MIC=MBC=0.048 mg/ml) in Gram (+) bacteria and *C. guilliermondii* (MIC=MFC=0.048 mg/ml) in yeasts.

All methanol extracts were active. This is also the case for ethyl acetate extracts except LEA. As to hexane extracts, PHE and RHE were efficient but not LHE and SHE. Pod extracts had the broadest spectrum of activity

with 10 sensitive microorganisms and seed extracts the narrowest ones with 8 sensitive microorganisms.

DISCUSSION

The present study shows that the *C. bernieri* extracts inhibited the growth of most tested microorganisms, indicating the presence of antimicrobial compounds in all parts of the plant. Phytochemical screening showed the presence of diverse secondary metabolites, reported to have antimicrobial property. At this stage of the work, results did not yet allow to state whether the same or different compounds are involved in the different parts of the plant. However, they suggested that *C. bernieri* antimicrobial activity might be mainly due to tannins, polyphenols, steroids, triterpenes and flavonoids, which were present in all or most of the plant organs. Alkaloids might also be concerned in leaves and pods and saponosides in root.

C. bernieri extracts showed generally a broad antimicrobial spectrum. They were capable of inhibiting the growth of different Gram (-) and Gram (+) bacterial strains as well as some yeasts. However, each extract

Table 5. *In vitro* Antimicrobial Activity (IZD in mm) of extracts (1 mg/disc) on Gram (-) bacteria.

Extracts/controls		Cj	Ea	Ec	Esc	Pa	Se	Sf	Vp	Ye	Pm
Leaf	LHE	-	-	-	-	-	-	-	-	-	-
	LEA	-	-	-	-	-	-	-	-	-	-
	LME	-	10.00±0.01	-	-	9.33±0.47	-	-	7.00±0.01	-	8.33±0.47
Seed	SHE	-	-	-	-	-	-	-	7.00±0.01	-	-
	SEA	-	-	10.00±0.01	-	-	-	-	9.00±0.01	-	8.00±0.01
	SME	-	-	-	-	-	-	-	-	8.00±0.01	-
Pod	PHE	-	9.67±0.47	-	-	-	-	-	-	-	-
	PEA	-	-	9.00±0.01	-	-	-	-	10.67±0.01	-	-
	PME	-	-	-	-	-	11.00±0.82	8.00±0.01	9.00±0.82	8.00±0.01	8.00±0.01
Root	RHE	-	-	-	-	10±0.01	8.00±0.01	-	-	-	-
	REA	-	-	-	-	-	-	-	9.00±0.01	-	-
	RME	-	-	-	-	-	9.00±0.01	-	-	-	9.00±1.41
PC	Amx	45.00	-	-	23.00	10.00	27.00	25.00	-	10.00	-
	Chl	38.00	25.00	25.00	30.00	15.00	32.00	30.00	-	38.00	-
	Pen	40.00	-	-	-	-	-	-	-	-	25.00
NC	Hex	-	-	-	-	-	-	-	-	-	-
	EtOAc	-	-	-	-	-	-	-	-	-	-
	Sdw	-	-	-	-	-	-	-	-	-	-

Cj: *C. jejuni;* Ea: *E. aerogenes;* Ec: *E. cloacae;* Esc: *E. coli;* Pa: *P. aeruginosa;* Se: *S. enteridis;* Sf: *S. flexneri;* Vp: *V. parahaemolyticus;* Ye: *Y. enterocolitica;* Pm: *P. mirabilis* PC: Positive control (Amx: Amoxicillin 25µg; Chlor: Chloramphenicol 30µg; Pen: Penicillin 6µg); NC: Negative control (Hex: Hexane; EtOAc: Ethyl acetate; Sdw: sterile distilled water); −: No activity.

displayed a specific activity spectrum that could be due to difference between the chemical nature and concentration of bioactive compounds in extracts. The results obtained with microdilution method were more reliable than those with disc diffusion. That might be due to the fact that bioactive compounds were in direct contact with germs in liquid medium whereas they diffused little or not at all in solid medium.

There was no consensus on the acceptable level of inhibition for natural products (Benko and Crovella, 2010). For Dalmarco et al. (2010), for crude extracts and fractions, a MIC lower than 100 µg/mL was considered as an excellent effect, from 100 to 500 µg/ml as moderate, from 500 to 1000 µg/mL as weak, and over 1000 µg/ml as inactive. According to Kouitcheu et al. (2013), when a crude extract was used, the MIC values of 8 mg/mL or below against any microorganism tested was considered as active.

If the scale adopted by Dalmarco et al. (2010) was used as a reference, 10 extracts displayed an excellent effect, 8 a moderate effect, 5 a weak effect then the remaining extracts were inactive. Excellent effects were observed on *P. mirabilis* (RME), *S. enteridis* (PME)*, B.* *cereus* (LME, PEA, REA), *S. pneumoniae* (LME), *S. pyogenes* (REA)*, C. albicans* (LME) and *C. guilliermondii* (LME, SEA). Moderate effects, were found against *E. aerogenes* (SEA)*, P. mirabilis* (LME), *P. aeruginosa* (LME)*, C. perfringens* (LME)*, S. aureus* (LME)*, S. pyogenes* (RME, PEA, LME) and *C. guilliermondii* (SME). Weak effects were observed on *E. aerogenes* (SEA), *P. aeruginosa* (LME), *S. aureus* (REA) and *S. pneumoniae* (REA, RME).

The most efficient extracts were RME (MIC=MBC= 0.097 mg/ml) against *Y. enterolytica* in Gram (-) bacteria, REA (MIC=MBC=0.048 mg/ml) against *S. pyogenes* in Gram (+) bacteria and LME (MIC=MFC=0.048 mg/ml) against *C. guilliermondii.* Some of the extracts were very effective against some organisms (LME against *B. cereus, S. pneumoniae, C. albicans* and *C. guilliermondii,* REA against *B. cereus* and *S. pyogenes*) while others were totally inactive (SME against *S. pneumoniae* and *S. pyogenes*).

However, if the interpretation of Kouitcheu et al., (2013) was taken into account, only nine extracts had MIC higher than 8 mg/mL on some germs, which means that all the other extracts of *C. bernieri* used showed

Table 6. *In vitro* Antimicrobial Activity (inhibition zone diameter in mm) of extracts (1 mg/disc) on Gram (+) bacteria.

Plant parts/controls	Extracts	*Bc*	*Cp*	*Ef*	*Lm*	*Sa*	*Spn*	*Spy*
Leaf	LHE	-	-	-	-	7.00±0.01	-	-
	LEA	-	-	-	-	-	-	-
	LME	9.00±0.01	8.00±0.01	-	-	10.00±0.01	12.67±1.25	12.33±1.70
Seed	SHE	-	-	-	-	7.00±0.01	-	-
	SEA	-	-	-	-	9.00±0.82	11.33±0.47	9.00±0.01
	SME	-	-	-	-	-	8.33±1.25	8.00±0.01
Pod	PHE	-	9.67±1.25	-	-	7.00±0.01	-	-
	PEA	11.33±0.47	8.33±0.47	-	-	10.33±0.47	13.00±0.82	12.00±1.41
	PME	-	-	-	-	7.00±0.01	8.00±0.01	7.00±0.01
Root	RHE	-	-	-	-	-	-	8.00±0.82
	REA	11.33±0.94	7.00±0.01	-	-	11.00±0.01	13.00±0.82	**15**.00**±0.01**
	RME	9.00±1.41	10.00±0.01	-	-	8.00±0.01	13.00±0.83	12.00±1.41
PC	Amx	15.00	27.00	-	-	37.00	26.00	32.00
	Chl	38.00	30.00	-	30.00	30.00	25.00	22.00
	Pen	15.00	-	-	-	35.00	23.00	25.00
NC	Hex	-	-	-	-	-	-	-
	EtOAc	-	-	-	-	-	-	-
	Sdw	-	-	-	-	-	-	-

Bc: *B. cereus;* Cp: *C. perfringens;* Ef: *E. faecalis;* Lm: *L. monocytogenes;* Sa: *S. aureus;* Spn: *S. pneumoniae;* Spy: *S. pyogenes* Amx: Amoxicillin 25 µg; PC: Positive control (Amx: Amoxicillin 25 µg; Chlor: Chloramphenicol 30 µg; Pen: Penicillin 6 µg; NC: Negative control (Hex: Hexane; EtOAc: Ethyl acetate; Sdw: sterile distilled water); −: No activity.

Table 7. *In vitro* Antimicrobial Activity (inhibition zone diameter in mm) of extracts (1mg/disc) on yeasts and molds

Plant part	Extract	Yeast			Mold	
		Ca	*Cg*	*Ck*	*Af*	*An*
Leaf	LHE	-	7.00±0.01	-	-	-
	LEA	-	7.00±0.01	7.00±0.01	-	-
	LME	8.00±0.01	8,67± 0.94	7.00±0.01	-	-
Seed	SHE	-	7.00±0.01	-	-	-
	SEA	-	13.00±0.82	-	-	-
	SME	-	11.00±0.01	-	-	-
Pod	PHE	-	7.00±0.01	-	-	-
	PEA	-	7.00±0.01	-	-	-
	PME	-	7.00±0.01	-	-	-
Root	RHE	-	8.00±0.01	-	-	-
	REA	-	7.00±0.01	-	-	-
	RME	-	8.00±0.82	-	-	-
PC	Mic	18.00	30.00	33.00	28.00	23.00
NC	Hex	-	-	-	-	-
	EtOAc	-	-	-	-	-
	Sdw	-	-	-	-	-

Ca: *C. albicans;* Cg: *C. guilliermondii;* Ck: *C. krusei;* Af: *A. fumigatus;* An: *A. niger* PC: Positive control (Mic: Miconazole 50µg); NC: Negative control (Hex: Hexane; EtOAc: Ethyl acetate; Sdw: sterile distilled water); −: No activity.

Table 8. MIC and MBC values (mg/mL) of *C. bernieri* extracts (1mg/disc) on Gram(-) bacteria

Gram(-) Bacteria	Extracts	MIC (mg/ml)	MBC (mg/ml)	MBC/MIC
Enterobacter aerogenes	LME	0.195	25	128.21
Enterobacter cloacae	SEA	0.781	25	32.01
	PEA	6.25	25	4.00
Pseudomonas aeruginosa	LME	0.781	25	32.01
	RHE	0.195	25	128.21
Salmonella enteridis	PME	0.097	12.5	128.87
	RHE	12.5	12.5	1.00
	RME	12.5	25	2.00
Shigella flexneri	PME	3.125	25	8.00
Vibrio parahaemolyticus	SEA	1.562	6.25	4.00
	PEA	1.562	25	16.01
	PME	3.125	6.25	2.00
	REA	1.562	1.562	1.00
Yersinia enterolitica	SME	1.562	12.5	8.00
	PME	1.562	12.5	8.00
Proteus mirabilis	LME	0.195	0.781	4.01
	SEA	1.562	3.125	2.00
	PME	1.562	0.781	0.50
	RME	0.097	0.097	1.00

antimicrobial activities.

All the extracts had bactericidal action (MBC/MIC ≤ 4) in certain bacteria and bacteriostatic action (MBC/MIC > 4) in other ones. For example LME was bactericidal against *B. cereus* and *C. perfringens* but bacteriostatic against *S. aureus* and *S. pneumoniae*. The comparison of *A. bernieri* extract activities to foreign *Crotalaria* species was not easy because antimicrobial activity was assessed under different conditions (other microorganism strains and extract doses used).

Compared to available data, the IZD of *C. bernieri* extracts were generally of the same order of magnitude as those of leaf ethyl acetate extract from *C. madurensis* against *B. subtilis* and *S. aureus* (IZD=14 mm), *M. luteus* (IZD=12 mm), *E. coli* and *C. albicans* (IZD=10 mm (Bhakshu et al., 2008) and leaf ethanol extract from *C. pallida* against *X. axanopodis* (IZD=16 mm), *E. coli* (IZD=14 mm) and *C. michiganensis* (IZD=13 mm) (Govindappa et al., 2011). Root methanol extract from *C. burhia* was more efficient with an IZD of 18 mm against *B. subtilis* and *P. aeruginosa* (Sandeep et al., 2010).

If comparison was based on antimicrobial indexes, LME (MIC=0.781 mg/ml, MBC=25 mg/ml) and REA (MIC=0.195 mg/ml, MBC=25 mg/ml) were more efficient against *P. aeruginosa* than the leaf methanol extract from *C. quartiniana* (MIC=MBC=37.5 mg/ml) (Omori et al., 2011). The leaf hexane extract from *C. retusa* (MIC=0.125 mg/ml, MBC=37.5 mg/ml) (Maregesi et al., 2008) was less active against *B. cereus* than LME (MIC=0.097 mg/ml, MBC=0.195 mg/ml), PEA and REA (MIC=0.048 mg/ml, MBC=0.195 mg/ml). By contrast, *C. bernieri* extracts were less active on *P. mirabilis* (MIC between 0.097 and 1.56 mg/ml) than a peptide isolated from *C. pallida* seeds (MIC=0.030 mg/ml) (Pelegrini et al., 2009). Compared to the antibacterial activities from other plant extracts, several *C. bernieri* extracts were more efficient than methanolic aerial part extracts of *Inula viscosa* against *B. subtilis* (MIC=25 mg/ml, MBC=50 mg/ml) and *S. aureus* (MIC=12.5 mg/ml, MBC=50 mg/ml) (Larbi et al., 2016). By contrast, tuber ethyl acetate extract of *Tropaeolum pentaphyllum* against *E. coli* (MIC=0.02 mg/ml, MBC=0.64 mg/ml), *P. aeruginosa* (MIC=0.04 mg/ml, MBC=0.64 mg/ml) (da Cruz et al., 2016) and organic extract (aerial parts) of *Rapanea parvifolia* against *E. faecalis* (MIC=0.03 mg/ml, MBC=0.06 mg/ml) (Suffredini et al., 2006) were more efficient.

Table 9. MIC and MBC values (mg/ml) of *C. bernieri* extracts (1mg/disc) on Gram(+) bacteria.

Gram (+) Bacteria	Extract	MIC (mg/ml)	MBC (mg/mL)	MBC/MIC
Bacillus cereus	LME	0.097	0.195	2.01
	PEA	0.048	0.195	4.06
	REA	0.048	0.195	4.06
	RME	1.562	1.562	1.00
Clostridium perfringens	LME	0.195	0.390	2.00
	PHE	12.5	25	2.00
	PEA	6.25	12.5	2.00
	RME	6.25	>25	-
Staphylococcus aureus	LME	0.195	6.25	32.05
	SEA	6.25	12.5	2.00
	PEA	3.125	25	8.00
	REA	0.781	25	32.01
	RME	12.5	25	2.00
Streptococcus pneumoniae	LME	0.097	3.125	32.22
	SEA	3.125	12.5	4.00
	SME	12.5	25	2.00
	PME	1.562	6.25	4.00
	REA	0.781	>25	-
	RME	0.781	>25	-
Streptococcus pyogenes	LME	0.195	1.562	8.01
	SEA	1.562	12.5	8.00
	SME	12.5	25	2.00
	PEA	0.195	0.781	4.01
	RHE	25	>25	-
	REA	0.048	0.048	1.00
	RME	0.195	12.5	64.10

Table 10. MIC and MBC values (mg/ml) of *C. bernieri* extracts (1mg/disc) on yeasts.

Yeasts	Extraits	MIC (mg/ml)	MFC (mg/ml)	MFC/MIC
Candida albicans	LME	0.097	0.195	2.01
	LME	0.048	0.048	1.00
	SEA	0.048	1.562	32.54
Candida guilliermondii	SME	0.195	25	128.21
	RHE	12.5	>25	-
	RME	12.5	>25	-

On fungi, LME (MIC=0.097 mg/ml, MFC=0.195 mg/ml) was more efficient than leaf methanolic extract of *Myrtus nivellei* against *C. albicans* (MIC=4.5 mg/ml) (Touaibia and Chaouch, 2015) whereas LME, SEA and SME against *C. guilliermondii* (MIC=0.08 mg/ml, MFC=0.32 mg/ml) were less efficient than ethyl acetate extract of *T. pentaphyllum* (da Cruz et al., 2016).

Conclusion

This study clearly demonstrates the potential of *C. bernieri*

as a source of interesting natural wide spectrum antimicrobial molecules. All its parts were efficient and could be easily found in significant amounts for the plant grows in fields, in the vicinity of homes, on roadsides and can be cultivated. Furthermore, according to our survey of local populations, *C. bernieri* is consumed by zebus but no cases of poisoning have yet been reported. At present, our works are concerned with the isolation of pure compounds from different extracts of *C. bernieri* and the elucidation of their structures in order to better evaluate their pharmacological activity.

In view of later therapeutic use of *C. bernieri*, study on various experimental models of animals is also on going to assess the harmful effects it might have.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENT

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Full Length Research Paper

Role of probiotics in prevention of hospital acquired pneumonia in Egyptian children admitted to Pediatric Intensive Care Unit of Mansoura University Children's Hospital

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The objective of the study is to evaluate the effect of probiotics to reduce the risk of hospital-acquired pneumonia in Pediatric Intensive Care Unit. The study is a double-blinded randomized placebo-controlled study. Patients were evenly and randomly assigned in two groups. The first group was 50 randomly selected patients who formed the placebo group, while the second group was 70 patients selected randomly to form the intervention group that received the probiotic capsules; *Lactobacillus rhamnosus* strain GG, once a day. The study site was Pediatric Intensive Care Unit in a university-affiliated children's hospital. Fifteen (30%) patients in the placebo population developed hospital-acquired pneumonia. Of the 15 patients, infections in 22% were caused by Gram-negative organisms. In the intervention group, 10% developed hospital-acquired pneumonia. The causative agents were predominantly Gram-negative organisms (22% Gram-negative vs. 7% Gram-positive; *P*–value; 0.01). After 72 h of study, significantly higher oral and gastric colonization rates were observed in patients who were given placebo treatment, compared with those given *Lactobacilli*. The current study is the first to report these higher significant variations. The current study found an influence of probiotic supplementation on the rate of hospital-acquired pneumonia.

Key words: Peiatric intensive care unit, probiotics, hospital-acquired, pneumonia.

INTRODUCTION

Health care-associated infections (HCAIs), hospital- acquired urinary tract infection, blood stream infection,

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pneumonia, and meningitis in Critical Care Units are correlated with increased mortality and morbidity (Sohn et al., 2001). Blood stream infection, with the potential cerebro-spinal fluid infection, lung and other sterile sites in the body may result from transfer of enteric pathogenic organisms through the intestinal wall (Duffy, 2000).

Probiotics may prevent or reduce infections in a variety of ways by altering intestinal permeability and interacting with pathogenic and commensal microorganisms in the gut mucosal system. Their administration may stimulate the host immune system in cases of infectious diarrhea. Furthermore, probiotics may compete with potential pathogens for nutrients or binding sites and subsequently inhibit growth and invasion of pathogenic organisms (Wang et al., 2016).

The use of probiotics like *Lactobacillus rhamnosus* GG (LGG) and *Saccharomyces boulardii* (strong recommendation, low quality of evidence), may be considered in the treatment of children with acute gastroenteritis besides rehydration therapy. Less imperative evidence is available for *Lactobacillus reuteri* DSM 17938 (weak recommendation, low quality of evidence) (Szajewska and van Goudoever, 2014). A 2011-meta-analysis of three randomized clinical trials comprising, 1092 children, reported that, there were significantly lower rates of diarrhea in placebo management compared with LGG administration (Szajewska, 2011). Children attending daycare centers are at high exposure of respiratory infections (Hao et al., 2011). Therefore, a systematic review evaluated the impact of probiotics on preventing upper respiratory tract infections (URTIs). Many outcome measures were reported in different trials on children (Rio et al., 2002; Sanz et al., 2006; Rautava et al., 2009; Hojsak et al., 2010; Merenstein et al., 2010; Rerksuppaphol and Rerksuppaphol, 2012). Honeycutt et al. (2007) conducted a randomized, placebo-controlled trial, using *L. rhamnosus* strain GG that was not shown to be effective in reducing the incidence of nosocomial infections (Honeycutt et al., 2007).

Prevention of life-threatening infections can be achieved through gastrointestinal (GI) tract colonization with probiotics (anaerobic, nonpathogenic bacteria) which competitively inhibits the binding of bacterial pathogens, reducing their possibility for colonization and translocation (Honeycutt et al., 2007). Hospital-acquired pneumonia (HAP) is the leading risk of death due to hospital-acquired infections in the United States with an incidence of 5 to 10 cases per 1000 hospital admissions. Hospital-acquired pneumonia, determined as pneumonia occurs 48 h or more after hospital admittance, and has not been incubating at the time of admission. Hospital-acquired pneumonia adds excess medical costs ranging from $12,000 to $40,000 per patient and prolongs hospital stays for an average of 7 to 9 days. Mortality attributed to HAP is estimated to be between 33 and 50% with the highest mortality occurring in patients with bacteremia or infections with drug resistant *Acinetobacter or Pseudomonas aeruginosa* species (Griffiths et al., 2004). Although, there is theoretical possibility, there is insufficient evidence that probiotic products decrease the incidence of HAP.

The aim of this study is to evaluate the clinical impact of probiotics in decreasing the rates of HAP compared with standard conventional therapy alone in cases admitted to Pediatric Intensive Care Unit (PICU) of Mansoura University Children's Hospital (MUCH).

STUDY DESIGN AND METHODS

A total of 2,050 patients were admitted to PICU of MUCH, Mansoura, Egypt, between October, 2012 and December, 2015. The study is a parallel assignment, placebo controlled, randomized (on PICU admission, according to computer generated randomized numbers list) and double blind study (microbiology laboratory subject, investigator, primary care clinicians, outcomes assessor, bedside nurses, were blinded to group assignments). The patients were considered eligible on admission to PICU.

Patients were excluded if they had suspicion or evidence of perforated intestine, mechanical GI obstruction, absolute neutrophil count $< 0.5 \times 10^9$ cells/L; been admitted to the PICU for more than 72 h; had use probiotics in the week before study accession; had no parental presence, or consent.

All the 2,050 patients were screened. Of the total number, 1,900 were excluded due to one exclusion criteria or the other and unavailability of parental consent on the first day of admission. There were 150 patients in this prospective, double-blinded, randomized, placebo-controlled trial. The 150 cases were equally and randomly assigned into two groups (75 cases in each of placebo and the intervention group). Then, the first placebo and the second intervention groups were reduced to 50 and 70 cases respectively due to further exclusions at randomization due to death, discharge from the hospital (transferred to another hospital) or the patient's parents no longer wished to participate (Figure 1).

The intervention group received one *L. rhamnosus* strain GG capsule once a day (Culturelle, 10×10^9 cells/capsule, ConAgra Foods, Omaha, NE) for the duration of hospitalization. Probiotic capsules were prepared in a suspension of (5 ml) of 5% dextrose. They were administered by oro-gastric, naso-gastric tube or by mouth in the patients who could be fed orally. More than two doses of *Lactobacillus* GG missing by any patient, excluded the patient from probiotic regimen. Daily clinical and demographic data were obtained prospectively by the study investigators (Honeycutt et al., 2007; Banupriya et al., 2015).

Information was collected on a daily basis from all participants. This information was then, entered into a Microsoft Excel database for subsequent statistical analysis. Patients received all routine care, including antibiotic therapy as deemed necessary, HAP preventive measures as per hospital protocols, under their physician direction.

All the study participants were subjected to careful history taking, clinical examination, other demographic information, pediatric risk of mortality; (PRISM) score (Taori et al., 2010), laboratory investigations including complete blood count and C-reactive protein, monitoring for HAP, blood cultures, tracheal aspirates and quantitative broncho-alveolar lavage cultures. Tracheal aspirate cultures, blood cultures, and complete hemogram, were emitted every three day. Outcome variables studied in both groups included the mechanical ventilation use, ICU stay duration, HAP duration,
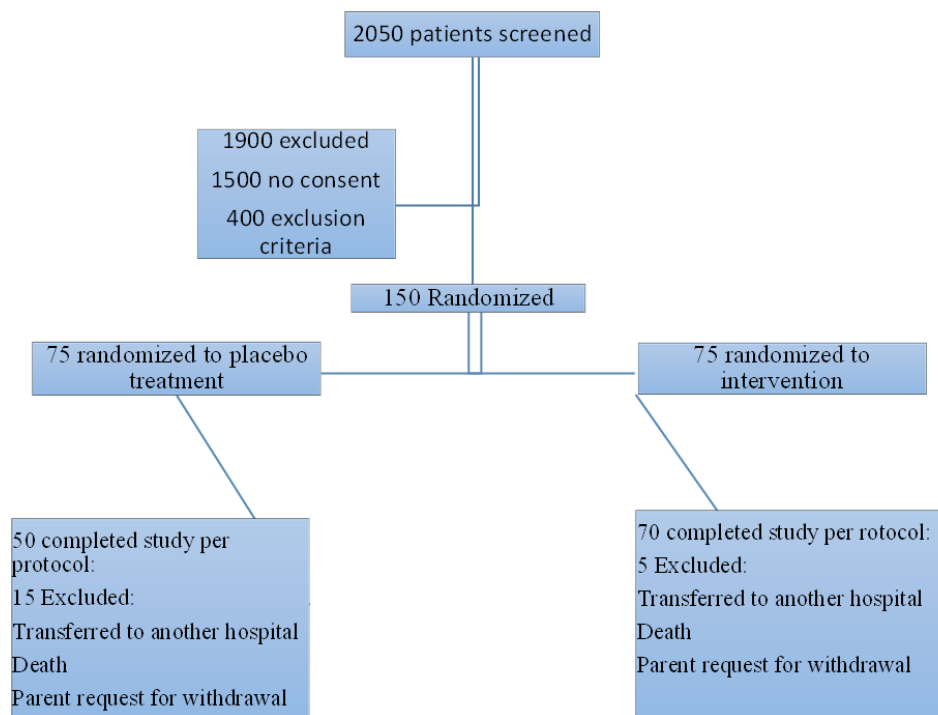
Figure 1. Participants of the study.

duration of antibiotic use and mortality.

The diagnostic criteria for HAP were determined as any pneumonia contracted by a patient in the hospital, within at least 48 to 72 h, after admission with x-ray evidence of consolidation, a new infiltrate, or cavitations (Cernada et al., 2014).

In HAP diagnosis, the patient also should have either, increased difficulty in breathing or tachypnea, new onset or increased purulent sputum, isolation of a known respiratory pathogen on blood culture, isolation of known respiratory virus by antigen detection, or evidence of pneumonia histopathologically (which were done in the MUCH laboratory) (Foglia et al., 2007).

Study protocol was approved by Institutional Review Board (IRB) of Mansoura University. Approval was taken from the management of hospital in which the study was conducted. Informed verbal consent was obtained from each participant sharing in the study. Confidentiality and personal privacy were respected in all levels of the study.

Statistical analysis

All statistical tests were conducted using the IBM Statistical Package for Social Sciences; version 23 (IBM SPSS, Chicago, IL). Fisher's exact test (2-tailed) was used for comparison between the categorical variables. The Mann-Whitney U test was used to compare continuous variables between the two groups. Chi square was used for comparison between the categorical variables. Significance was set at P-value < 0.05.

RESULTS

All the 2,050 patients were screened (Figure 1); 1,900

were not enrolled due to one exclusion criteria or the other; or informed consents could not be obtained on the first day of admission. One hundred and fifty patients were evenly distributed between two groups (75 patient per each group) based on baseline and demographic characteristics (Table 1).

The study is a prospective, randomized, double-blinded, placebo-controlled trial. Fifty patients (children) were randomly selected to the placebo group and 70 to the intervention group. The clinical features of the infected patients in both the placebo and intervention groups are recorded in Table 2. There were no cases of bacteremia caused by *Lactobacilli* found in the intervention group. There were no known serious adverse effects observed in any subject during the study period.

Out of the 75 patients of the placebo and intervention groups; 43 (57.3%) and 38 (50.6%) were male, respectively. The mean age was 5.3 ± 2.7 (1-12) years in placebo group and 5.7± 4.9 (1-10) years in the randomized intervention group (p-value < 0.05); a statistical significance without any clinical significance since the mean age in both groups was 5.3 and 5.7. In PRISM score, the mean range was 33.9 ±13.9 and 34.2 ±15.6 in randomized placebo and intervention groups respectively (68.3% Confidence level). Also, the reasons for PICU admission were mainly respiratory problems. Fifteen (30%) patients in the placebo population developed HAP. Overall, of the 15 infections, 11 (22%) were caused by Gram-negative organisms (Table 2).

Table 1. Demographic and study population characteristics.

Parameter	Placebo (*n* = 75)	Intervention (*n* = 75)	*P*-value
Male sex	43(57.3%)	38(50.6%)	0.41
Age, mean ± SD (range), years	5.3± 2.7 (1-12)	5.7±4.9 (1-10)	0.00
PRISM score, mean ± SD (range)	33.9±13.9 68.3%Confidence level	34.2±15.6 63.3%confidence level	0.08
Reasons for ICU admission			
Respiratory	30	32	
Cardiovascular (shock, resuscitation, arrhythmias)	5	4	
Gastrointestinal	20	19	
Neurologic	4	4	
Hematology/Oncology	8	7	
Endocrine/Metabolic	3	4	
Renal	3	3	
Multisystem	2	2	

SD, Standard deviation; PRISM, pediatric risk of mortality; ICU, intensive Care Unit.

Table 2. Incidence and microbiology of HAP.

Microbiology	Placebo	Intervention	Chi- square	P- value
	15/50	7/70	7.79	0.005
Subjects with Gram-positive HAP	3/50(6%)	2/70(2.9%)	0.72	0.39
Subjects with Gram-negative HAP	11/50(22%)	5/70(7%)	5.57	0.01
Gram-positive pathogens isolated	3	2		
MSSA	1	1		
MRSA	1	0		
Streptococcus species	1	1		
Gram-negative pathogens isolated	11	5		
Pseudomonas	3	1		
Haemophilus influenza	2	1		
Acinetobacter	1	1		
Klebsiella	1	1		
Proteus	1	1		
Escherichia coli	1	0		
Serratia	1	0		
Citrobacter	1	0		
Yeast	1	0		

HAP, Hospital-acquired pneumonia; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus.*

Gram-positive bacteria accounted for 3 (6%) of HAP. Candida species were responsible for one (2%) of the infections. Of the 70 patients of the intervention group, 7(10%) developed HAP. Overall of the 7 infections, 5 (7%) were caused by Gram negative organisms. Gram positive bacteria accounted for 2 (2.9%) of HAP. There was statistically significant variation between the studied arms regarding the microbiology of the HAP (*P*-value; 0.005), and the causative agents were predominantly Gram negative organisms (22 vs 7%; *P*- value; 0.01). There was no difference in case of Gram positive organisms (6 vs 2.9%; *p*-value; 0.39). The relative risk of developing HAP in the placebo group was 3.3 (confidence interval; CI, 1.44 to 7.51(95%), *p*-value; 0.004). Thus, patients of placebo group would be 3.3 times as likely as the intervention group to develop HAP.

In this study, death rate significantly varied between both groups (24% in the placebo group and 14.3% in the

Table 3. Secondary outcomes.

Parameter	Placebo (n = 50)	Intervention (n=70)	Chi-square	*P*-value
Death	15 (24%)	10 (14.3 %)	4.36	0.03
Days of ICU-associated pneumonia, mean ± SD	6.9 ± 3.8	4.1 ± 3.7	2.4	0.12
Total antibiotic-days, mean ± SD	17.3 ± 14.4	13.3 ± 10.4	3.7	0.05
ICU length of stay in days, mean ± SD	15.6 ± 11.6	14.8 ± 11.8	1.7	0.19
No of patients who needed MV	20 (40%)	10 (14.3%)	10.28	0.001

ICU, intensive Care Unit; SD, standard deviation; MV, mechanical ventilation.

Table 4. Surveillance of culture.

Parameter	Non*	Rare	Few	Moderate	Many	Chi-squre	*P* Value
Oral swab pathogen+ (density at baseline)							
Placebo (50)	17 / 34%	2	2	10 / 66%	19	0.2	0.65
Intervention (70)	22 / 31.4%	19	8	12 / 68.6%	9		
Gastric aspirate pathogen+ (density at baseline)							
Placebo (50)	18 / 36%	2	2	10 / 64%	18	0.56	0.45
Intervention (70)	22 / 31.4%	16	10	13 / 68.6%	9		
Oral swab (pathogen density at 72 h)							
Placebo (50)	10 / 20%	2	2	10 / 80%	27	39.7	0.00001
Intervention (70)	45 / 64.3%	9	2	5 / 35.7%	9		
Gastric aspirate (pathogen density at 72 h)							
Placebo (50)	10 / 20%	6	5	12 / 80%	17	43.16	0.00001
Intervention (70)	46 / 65.7%	7	7	5 / 34.3%	5		

*None if no growth is seen; rare if growth is restricted to only the first quadrant; few if growth extends into the second quadrant; moderate if growth extends into the third quadrant; many if growth extends into the fourth quadrant. [+] Pathogens from oral swabbing and gastric as pirates included Enterobacteriaceae, non-fermenting Gram-negative bacteria and Staphylococcus aureus (including methicillin-resistant strains).

probiotic group; *P*-value*;* 0.03). Also, there was a statistical variation between 2 groups regarding the mean duration of antibiotic use (17.3 ± 14.4 vs. 13.3 ± 10.4; *P*-value*;* 0.05). On the contrary, probiotics administration resulted in non-significant difference in mean duration of PICU associated pneumonia; in addition, the mean duration of PICU was not statistically variable between the mentioned groups. Moreover, patients in placebo group showed a strong trend toward increased probability of using mechanical ventilation, compared with patients in intervention group (40 vs. 14.3% *P*-value; 0.001) (Table 3). No cases of pneumonia or bacteremia caused by

Lactobacillus in the patients of the intervention group were observed.

The oral colonization, rates of microbial species, was not significantly variable at baseline between the 2 groups, (66% for placebo vs. 68.6% for *Lactobacillus*; *P*–value; 0.66) (Table 4). The gastric colonization rates at baseline were also non-significantly variable (64% for placebo vs. 68.6% for *Lactobacillus*; *P*–value; 0.45). Patients given placebo had significantly higher rates of oral colonization of pathogenic bacteria after 72 h of study compared with those who were given *Lactobacillus* (80.0% for placebo vs. 35.7% for *Lactobacillus*; *P*–value;

0.00001).

The rates of gastric colonization with pathogenic bacteria were also increased after 72 h in placebo patients (80.0% for placebo vs. 34.3% for *Lactobacillus*; *P*-value; 0.00001).

DISCUSSION

Evaluation of the use of probiotics in preterm infants and children indicates a valuable influence for enterocolitis prevention and mortality, but less suggestion for HCAIs (Alfaleh et al., 2014). The present study aims to check the clinical impact of probiotics in decreasing HAP rates compared with standard conventional therapy alone, in cases admitted to PICU of MUCH, Mansoura, Egypt.
In this study, probiotic treatment showed a statistically significant decline in HAP rate depending on strict diagnostic criteria that require microbiological confirmation using respiratory samples beside the other mentioned diagnostic criteria. Probiotic treatment also exhibited less duration of antibiotic prescribed for therapy. Generally, these data considered that *Lactobacillus* may represent a unique, cheap, and non-antibiotic intervention to prevent hospital-acquired infections in properly selected PICU patients.

Previous studies demonstrated in voluntary humans reported that *Lactobacillus rhamnosus* colonized the bowel tract when 10^8 CFUs/day were administered [Griffith et al., 2004); therefore, a dosage of 10^9 CFUs/12 h was chosen.

150 patients were used in this prospective, which was a double-blinded, randomized, placebo-controlled study distributed evenly between 2 groups (75 each). Out of the 75 patients that were randomly selected to the placebo treatment, 25 were excluded thus leaving 50 placebo patients. From those who were randomly selected to the intervention treatment, 5 patients were excluded, leaving 70 (that is, 70 intervention patients). These secondary exclusions (that is, occurred after randomization of both placebo and intervention groups) was due to death, transference to another hospital or parental request to withdraw from the study. In this study, death before fulfilling adequate duration in the PICU was an exclusion since adequate time space to assess the secondary outcomes (days of ICU-associated pneumonia, total antibiotic days, ICU length of stay, number of patients needed MV, and finally death was required (Table 3). There were statistically significant variations regarding child age, yet, no clinically significant difference between both groups (Age range in randomly selected placebo group from 1-10 and in intervention group from 1-12). Likewise, statistically significant variations were observed between groups regarding the microbiology of the HAP (*P*-value; 0.005), and the causative agents were predominantly Gram negative organisms (*P*-value; 0.01).

This was not in accordance with the study of Honeycutt et al. (2007) who evaluated the impact of probiotics in decreasing the rates of HCAIs in PICU. Sixty-one patients were randomly selected, 31 in the *Lactobacillus* treatment group and 30 in the placebo group. In the control group of the same study, three patients had 4 infections and 6 patients in the treatment group had 11 infections with a trend towards increased infections rate in the group receiving probiotics (Honeycutt et al., 2007).
In another study, Mihatsch et al. (2010), investigated whether *Bifidobacterium lactis* decreases the incidence of HCAIs in infants with very low birth weight (VLBW; <1,500 g) of <30 weeks of gestation. In that randomized controlled trial, there were 90 in the placebo group and 93 infants in the *B. lactis* group that showed no significant variation with regard to the incidence of HCAIs (Mihatsch et al., 2010).

Moreover, Rojas et al. (2012) designed a large double-blinded placebo-controlled trial using *Lactobacillus reuteri* to test the effect of probiotics on infant HCAIs and death rate. However, primary outcome, death, or HCAI frequency, were similar in the probiotic and placebo groups (Rojas et al., 2012).

On the contrary, the study of Hojsak et al. (2010) conducted a double-blind, randomized placebo-controlled trial of hospitalized children receiving *Lactobacillus* GG (n = 376) and placebo (the same post-pasteurized milk, deprived of *Lactobacillus* GG, placebo group, n = 366). They found a significantly reduced risk for respiratory tract and GI infections, in *Lactobacillus* GG group, compared with the placebo group (Hojsak et al., 2010).
Most recently, the study of Hojsak et al. (2015) aimed to investigate the role of *Bifidobacterium animalis*; subsp. lactis in preventing HCAIs. They organized a randomized, double-blind, placebo-controlled trial in 727 hospitalized children. The children were randomly assigned to receive placebo therapy (n = 365) or *Bifidobacterium animalis* subsp. lactis in a dose of 10^9 CFU, once daily for the entire duration of the hospital stay (intervention group, n = 362). There was no statistical difference in primary outcome or incidence of common hospital acquired GI and respiratory tract infections between both groups and no statistical variation regarding the duration of HCAIs, the secondary outcomes.

Many trials were conducted in children and found that probiotics showed benefit in decreasing the number of children that experienced URTI episodes (Rio et al., 2002; Sanz et al., 2006; Rautava et al., 2009; Hojsak et al., 2010; Merenstein et al., 2010; Rerksuppaphol and Rerksuppaphol, 2012; Caceres et al., 2010).

These mentioned previous studies had different results from the present one and this may be due to the significant difference in their criteria of inclusion, the probiotic administration rout, dosing, probiotic agent(s) used, populations studied, the HAP diagnostic criteria and probiotic treatment mechanisms that are, inherently,

based on capability to alter the microbial flora (Klarin et al., 2008). Significant variations were also seen in respiratory colonization with Gram negative bacteria and the duration of ICU stay. Also, there was significant reduction in oropharyngeal and gastric colonization, in this study, after probiotic administration. Changes in colonization were significantly parallel to the HAP development that was confirmed microbiologically in the current study.

Probiotic intake had dramatical effects on decreasing the rates of microbiologically confirmed HAP caused by Gram-negative pathogens (22 vs. 7%; P-value; 0.01). On the contrary, HAP caused by gram-positive organisms was not variable between groups (6 vs. 2.9%; p-value; 0.39). The inadequacy of realizing probiotic mechanisms, made this observation difficult to be explained. However, the changes in the host flora were consistent based on the information on microbial colonization from the current study (Forestier et al., 2008; McNabb and Isakow, 2008)

The oral colonization rates of microbial species were not significantly variable at baseline between 2 groups (66% for placebo vs. 68.6% for *Lactobacillus*; P–value; 0.66) (Table 4). The gastric colonization rates at baseline were also non-significantly variable (64 for placebo vs. 68.6% for *Lactobacillus*; P–value; 0.45).

Patients given placebo had significantly higher rates of oral colonization of pathogenic bacteria after 72 h of study compared with those given *Lactobacillus* (80.0% for placebo vs. 35.7% for *Lactobacillus*; P-value; 0.00001). The rates of gastric colonization with pathogenic bacteria were also increased after 72 h in placebo patients (80.0% for placebo vs. 34.3% for *Lactobacillus*; P–value; 0.00001). Oral colonization changes were correlated significantly with the existence of microbiologically confirmed HAP.

The pathogenesis of HAP is complicated, but, the involvement of aerodigestive tract colonization with pathogenic bacteria is one of the pathogenesis mechanisms. Enteric bacteria colonize the GI tract in increasing numbers from the stomach to the colon. There is a debate whether or not colonization and adherence of the GI tract is necessary for probiotics to provide biological activity (Klingberg and Budde, 2006).

Respiratory sampling with quantitative cultures to reach the microbiologic HAP diagnosis was used in this study (Caceres et al., 2010). This study also differ from others, in that, high-risk patients were chosen as subjects, as recognized by the high PRISM score. A particular probiotic agent (*L. rhamnosus* GG) which was a different type was also selected. This agent was chosen because it had little information indicating that it may have favorable activity in the upper airways and strong safety data (Brandtzaeg, 1992; Gluck, 2003). It remains unknown whether other agents would have the same or remarkable results because of the short plan of comparative data in this field.

These data should be viewed as introductory and cannot be generalized to the general PICU population given the prolonged period of enrolment, the large number of exclusion criteria, the small number of patients included, and the strict inclusion criteria. Moreover, the current study carries multiple defects. That is because, these data were collected from one hospital and has inherent biases related to the patients served and the habits of local practice.

Mansoura University Children's Hospital has a patient population with many risk factors for colonization with hospital-acquired pathogens and serves an urban community with limited resources. This explains the high colonization rate seen in the baseline cultures.

Furthermore, like most other existing HAP preventive strategies, probiotic treatment requires compliance (Pitsouni et al., 2009; Wolvers et al., 2010). Such compliance is difficult to achieve in routine practice. Eventually, the delivery of probiotics to the oro/naso-pharynx and the stomach (that is, anatomically different sites), the delivery site is not known. Additionally, the differences in antibiotic use information are restricted by the methods used to calculate antibiotic prescription (antibiotic-days).

Contrary to the iatrogenic infection safety concern, the high death rate seen in the probiotic side of PROPATRIA was attributable to intestinal ischemia (Besselink et al., 2008).

Moreover, their effects may vary in disease and health, in distinct disease states, and in various age groups. Thus, this clinical trial in this certain population that results from specific probiotic strain cannot be generalized to other populations or strains.

Additional work is required to clear up the relative significance of strain-specific effects in different scenarios and the nature of interactions between probiotics.

Conclusion:

The current study found an influence of probiotic supplementation on the rate of HCAIs, including HAP. Further studies are necessary to display further mechanistic concerns and probiotic interactions. Clinicians have to be aware of the benefits and risks of these treatments, because of the increasing use of probiotics as therapeutic agents and health supplements.

Conflict of Interests

The authors have not declared any conflict of interests.

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