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Full Length Research Paper

In vitro efficacy of *Trichoderma asperellum* and detached leaflet assay on late blight pathogen: *Phytophthora infestans*

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Late blight is highly variable adapting to new fungicides and overcoming host resistance. The objective of the study was to determine efficacy of *Trichoderma asperellum* against *Phytophthora infestans* and its compatibility with fungicides. *T. asperellum* at 33% (3×10^6), 66% (7×10^6) and 100% (1×10^7 CFU/mL; g/L), Ridomil® (Metalaxyl 4% + Mancozeb 64%) and Mistress 72® (Cynamoxil 4% + Mancozeb 64%) were plated alongside *P. infestans in vitro* and detached leaflets assay. Results indicated that Ridomil® and Mistress 72® completely inhibited mycelial growth of *P. infestans in vitro* and in detached leaves. The 33% *T. asperellum* concentration had the lowest inhibitory power (38.0%) while 66% (91.10%) and 100% (91.30%) *T. asperellum* concentrations were not significantly different (*P*=0.05). Lesion sizes were not significantly different in 66% *T. asperellum* (1.91 cm^2) and 100% (1.89 cm^2) concentration while 33% concentration (3.5 cm^2) and untreated (3.55 cm^2) did not differ significantly. Ridomil® and Mistress 72® had no significant effect on *T. asperellum* mycelial growth. The results suggest that *T. asperellum* at 66% was effective in managing late blight. Results further indicate that *T. asperellum* could be used in combination with fungicides for effective and economical option.

Key words: *Trichoderma asperellum*, *Phytophthora infestans,* detached leaf assay, *in vitro*.

INTRODUCTION

Potato has high potential to address the food insecurity and low income due to its high yield per unit area and relatively high nutritional value as compared to cereal grains (Azimuddin et al., 2009). The global and Africa annual potato production is estimated to be 377 and 25 metric tonnes, respectively. Kenya is ranked fifth in Africa with production of 1.35 metric tonnes annually (FAOSTAT, 2018). Potato demand is projected to increase by 250% by 2020 with an annual demand increase of about 3.1% (Scott et al., 2010). In Kenya, potato yield per hectare ranges between 8 and 10 tonnes compared to a potential of 40 tonnes. This low yield is attributed to diseases as well as poor seed quality (Muthoni et al., 2013) with late blight being the major that reduce yield (Were et al., 2014).

Potato late blight caused by *Phytophthora infestans*

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can cause up to 100% yield loss depending on weather conditions and variety susceptibility (Mariita et al., 2016). Globally, late blight is responsible for 6.7 billion USD worth of potato yield loss annually (Nowicki and Majid, 2012) and therefore threatens food security globally (Cooke et al., 2012). Late blight epidemics is accelerated by shortage of seed experienced in most sub Saharan countries that has led to adoption of farm saved seed by farmers (Okello et al., 2017). Potato blight occurs in all potato production regions worldwide and is considered as the world's costly disease because it is managed by extensive use of fungicides that cost about 1 billion USD (Haverkort et al., 2008). Late blight can rapidly defoliate a whole field within a week if unchecked resulting to tuber infection that lowers both tuber quality and quantity (Gigot et al., 2009). The oomycete survives well in potato seed (Johnson and Cummings, 2009) and serves as source of primary inoculum to new crops resulting into early late blight epidemics (Runno-Paurson et al., 2013). Seed tubers available to farmers may have latent infection that produce viable sporangia that cause disease epidemics in the new crop (Johnson and Cummings, 2013).

Ridomil® and Mistress 72® are among the most widely used fungicides in Kenya to manage late blight due to their curative, preventive and systemic modes of action (Nyankanga et al., 2007). However, dependence on chemical application has raised environmental and human health concerns and emergence of fungicide insensitive strains including metalaxyl insensitive isolates threatening the efficacy of fungicides (Matson et al., 2015). In addition, emergence of new strains of *P. infestans* that adapt to new chemical fungicides and host resistance has led to reduced spray intervals (three to five days interval) resulting into 10 to 15 sprays per cropping season (Njoroge et al*.*, 2019). Use of biocontrols could offer the best sustainable and ecofriendly alternative to chemical application (Yao et al., 2016). Biological agents including *Trichoderma* species*, Bacillus* species and *Pseudomonas fluorescents* and plant extracts have been explored in managing late blight on Solanaceae plants (An et al., 2010; Chowdappa et al., 2013; Kabir et al., 2013). *Trichoderma* spp. is one of the most studied fungi that is widely used in management of diseases. The fungus is known to induce systemic disease resistance in plants as well as offer prior protection by activating enzymes that degrade cell walls in the pathogen (Yao et al., 2016). Saravanakumar et al. (2016) reported that suppression mechanisms of *Trichoderma harzianum* on *P. infestans* through competition, antibiosis, promotion of crop growth and mycoparasitism while Wu et al. (2017) reported enzymatic activities against plant and soil borne pathogens. Various studies have shown that *Trichoderma asperellum* could manage a number of plant diseases. Patel and Saraf (2017), Carrero-carr et al. (2016), and Kipngeno et al. (2015) reported that *T. asperellum* could manage *Fusarium* wilt in tomato, *Verticillium* wilt in olive and *Pythium* in tomato. However, there is limited information on the efficacy and potential of *T. asperellum* to manage late blight on potato.

The use of biocontrols is yet to be fully exploited in managing late blight, because synthetic fungicides act faster than biocontrols against disease causing agents (Xu et al., 2011). The quick action of fungicides against plant pathogens can be effectively tapped and combined with biocontrols to reduce overuse that has raised economical, human and environmental concerns. However, little is known on possibility of combining the two in managing *P. infestans* on potato that could result to reduced chemical applications. Therefore, the objective of the study was to determine the antifungal activity and efficacy of *T. asperellum* against *P. infestans in vitro* and detached leaflets and to assess the compatibility of Ridomil® and Mistress 72® with *T. asperellum*.

MATERIALS AND METHODS

Isolation, culturing and bulking of *P. infestans* inoculum

Thirty freshly blighted potato leaves samples were collected randomly from Kenya Agricultural Livestock and Research Organization (KALRO) Tigoni fields. The centre is located at longitude 36 4' 72'' east and latitude 10° 9′ 22″ south and located at an altitude of 2300 m above sea level (Jaetzold et al., 2006). Isolation of associated fungi and pure culture on Pea Agar amended with rifampicin antibiotics (50 µg/mL) was obtained according to Rhouma et al. (2016). Mycelial plug of about 80 mm in diameter of the pure culture from Pea Agar plates was obtained using a sterilized spatula and put in 20 mL Eppendorf tube containing 10 mL of sterilized distilled water. The suspension was vortexed for 2 min using electric vortex model VM-1000 of MRC Laboratory Equipment Company at 3000 revolution per minutes (rpm), filtered through sterilized four layered muslin cloth and incubated in refrigerator for 4 h at 4°C to enhance sporangia and zoospore formation. Identification and pathogenicity test was done on healthy test potato seedling and tuber slices of *Asante* varieties using Koch's postulates (Forbes, 1997). This was used for the detached leaf assay.

Antifungal bioassay through co-inoculation of *T. asperellum* and *P. infestans*

Dual culture method was employed at the Tigoni laboratories of Kenya Agricultural Livestock and Research Organization (KALRO) to determine the inhibition of *P. infestans* caused by the biocontrol agent as described by Fatima et al. (2015). *T. asperellum* pure spores were obtained from Real IPM Company, Kenya and their viability confirmed on Potato Dextrose Agar (PDA). The PDA in 9 cm Petri dishes were inoculated with 0.5 × 0.5 cm *P. infestans* mycelial plug cut using sterilized surgical blades and incubated at 18°C for 48 h. This was followed by introduction of the biocontrol suspensions prepared as follows: 0.1 g of *T. asperellum* spores powder was weighed and placed in falcon tube containing 10 mL of distilled water and was adjusted to 1×10^7 CFU/mL (100%) using hemocytometer. 66% (7×10^6 CFU/mL) and 33% (3×10^5 CFU/mL) concentrations were achieved by varying the 0.1 g of *T. asperellum* that formed 100% concentration by 66 and 33%, respectively followed by adjustment using hemocytometer. This was mixed with sorghum coarse grains and incubated at room temperatures for 3

days to initiate sporulation. Around 20 µL of the suspension was pipetted into PDA plates bearing *P. infestans* culture about 1 cm from the point of inoculation of the pathogen in the Petri Dishes. To prepare positive control plates, 20 µL droplet of positive controls (Ridomil® (2.5 g/L) and Mistress 72® (2 g/L)) were separately inoculated in similar way as the *T. asperellum* concentrations mentioned earlier. Negative control plates were inoculated with *P. infestans* only. The plates were incubated at room temperature (18 ± 2°C) under alternating lighting of 12-h light and 12-h darkness for 7 days (Goufo et al., 2017). The treatments were laid in completely randomized design with three replications. The experiment was repeated two times and observations were made using optical microscope on 3rd, 5th and 7th days after inoculation. Inhibition of the test phytopathogenic fungus and the control were determined by the percentage of mycelial growth inhibition in centimetres (cm) calculated by the formula of Yao et al. (2016):

$$I=(R1-R2/R1)\times 100$$

Where I represents the percentage reduction of growth (inhibition) of the fungi, R1 diameter of radial growth of pathogenic fungus in control plates and R2 diameter of radial growth of *P. infestans* in the presence of *T. asperellum* concentrations.

Antagonistic effects of *T. asperellum* on *P. infestans* in detached leaf assay

Approximately equal in size (6 cm long × 4.5 cm wide) healthy leaflets from middle canopy were detached from apical cuttings (6-7 weeks old) in glasshouse using sterilized office scissors. The leaflets were washed with sterilized distilled water and their bases covered with moist cotton to reduce desiccation (Goufo et al., 2008). *T. asperellum* concentrations and positive controls (Mistress 72® and Ridomil®) suspensions were prepared and applied by dipping the leaves for 1 s on the abaxial side only in the suspensions under study in a shallow dish. The leaves were then placed upside down (abaxial surface up) in 20 cm (length) × 18 cm (width) × 6 cm (depth) plastic dishes lined with a wet serviette paper to create humidity (6 leaflets per dish). Pure culture of *P. infestans* mycelial plug from the incubator was scrubbed using a sterilized spatula and put in Eppendorf tube containing 10 mL of sterilized distilled water. The suspension was vortexed for 2 min using electric vortex and filtered through four layered cheese cloth. The suspension was incubated in the refrigerator for 4 h at 4°C to enhance sporangia and zoospore formation. The suspension was adjusted to 1×10^4 zoospores/mL using hemocytometer and 40 µL was applied on the abaxial side of leaf using a micropipette. The negative control included leaves inoculated with *P. infestans* alone.

The plastic dishes while open were placed in laminar flow hood for about 5 min to air dry the wet leaves and then incubated at room temperatures (20 ± 2°C) for 24 h. The treatments were laid in completely randomized design with three replications and measurements on lesion size taken after 3 days and then once after every two days for two weeks. The lesion size was measured using the formula of Fontem et al. (2005):

$$S = \pi (L + W)\frac{2}{4}$$

Where S, L and W represents the area, length and width of the lesion for each detached leaflet, respectively. π = 3.14

T. asperellum compatibility with Ridomil® and Mistress 72®

The mycelial plug of *T. asperellum* from PDA plate was scrubbed using a sterile spatula and placed in 10 ml of distilled sterilized water in falcon tube. About 40 µL (1×10^7 sporangia/mL) of the suspension was drawn and inoculated on fresh PDA plate and incubated for 48 h. About 40 µL of Ridomil® (2.5g/L) and Mistress 72® (0.5 g/L) was applied on the developing *T. asperellum* mycelia in PDA plates. Mycelial growth was observed daily under optical microscope for 7 days.

Data analysis

Data on percentage inhibition of *P. infestans* induced by *T. asperellum* and lesion size on detached leaf assay (first transformed using the square root (x + 0.5) (Goufo et al., 2008) was subjected to analysis of variance (ANOVA) using Statistical Analysis System (SAS) version 8.2. Treatment means were separated using Tukey's Honest Significant Difference (HSD) whenever ANOVA showed significant difference ($p < 0.05$) among the treatment means.

RESULTS

Antifungal bioassay

The antagonistic activity of *T. asperellum* against *P. infestans* was observed in dual culture but depended on the biocontrol inoculum concentration. *T. asperellum* at 66 and 100%, positive control at manufacturer recommended rates (MRR), Ridomil® and Mistress 72® significantly (p=0.05) inhibited mycelial growth of *P. infestans in vitro* (Figure 1). *P. infestans* mycelial growth inhibition was clearly observed on the third day reaching 89.7 and 89.3% but showed no observable change in growth after the fifth day on plates treated with Ridomil® and Mistress 72®, respectively. Higher mycelial growth rate of *T. asperellum* at 66% (64 mm) and 100% (65 mm) than *P. infestans* (42 mm) in the pure culture was observed from third day after inoculation (Figure 2). There was no significant difference ($p<0.05$) in rate of growth of *P. infestans* treated with *T. asperellum* concentrations at 66 and 100%, Ridomil® and Mistress 72® by third day after inoculation. *T. asperellum* concentration at 33% gave the lowest inhibition (37.3, 46.0 and 38.0 mm) in co-culture across all days, respectively after inoculation (Table 1). Figure 2 shows *P. infestans* mycelial growth continues even in the presence of *T. asperellum* at 33% concentration. *P. infestans* and 33% *T. asperellum* in the dual culture had a lower mycelial growth rate than any other treatment. This figure also shows that pure cultures of 33% *T. asperellum* concentration growth rate was similar to that of *P. infestans* in separate plates and indeed in 33% *T. asperellum* and *P. infestans* dual culture, *P. infestans* mycelial radius was higher than dual culture associated with 66 and 100% *T. asperellum* whose mycelial growth was higher than the pathogen in separate pure cultures. In addition, 66 and 100% *T. asperellum* co-culture shows a plateau at day three indicating successful inhibition of the pathogen while for 33% dual culture showed

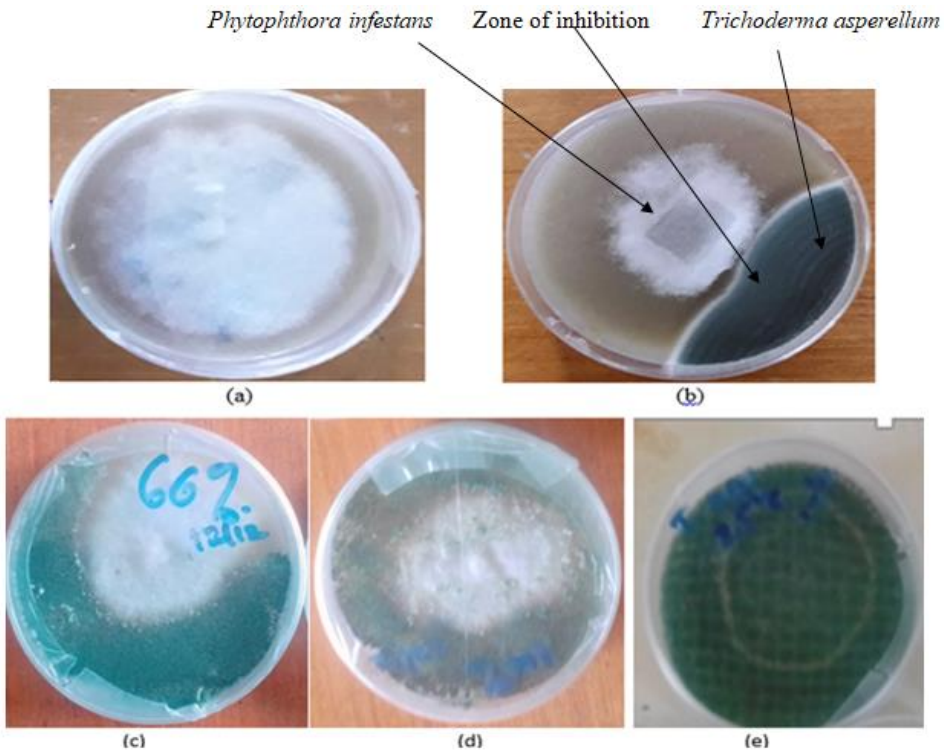

Figure 1. The antifungal effects of *T. asperellum* on *P. infestans* in dual culture; pure culture of *P. infestans* (a), *T. asperellum* concentrations at 33% (b), 66% (c) and 100% (d) and pure culture of *T. asperellum* (e) after 5 days.

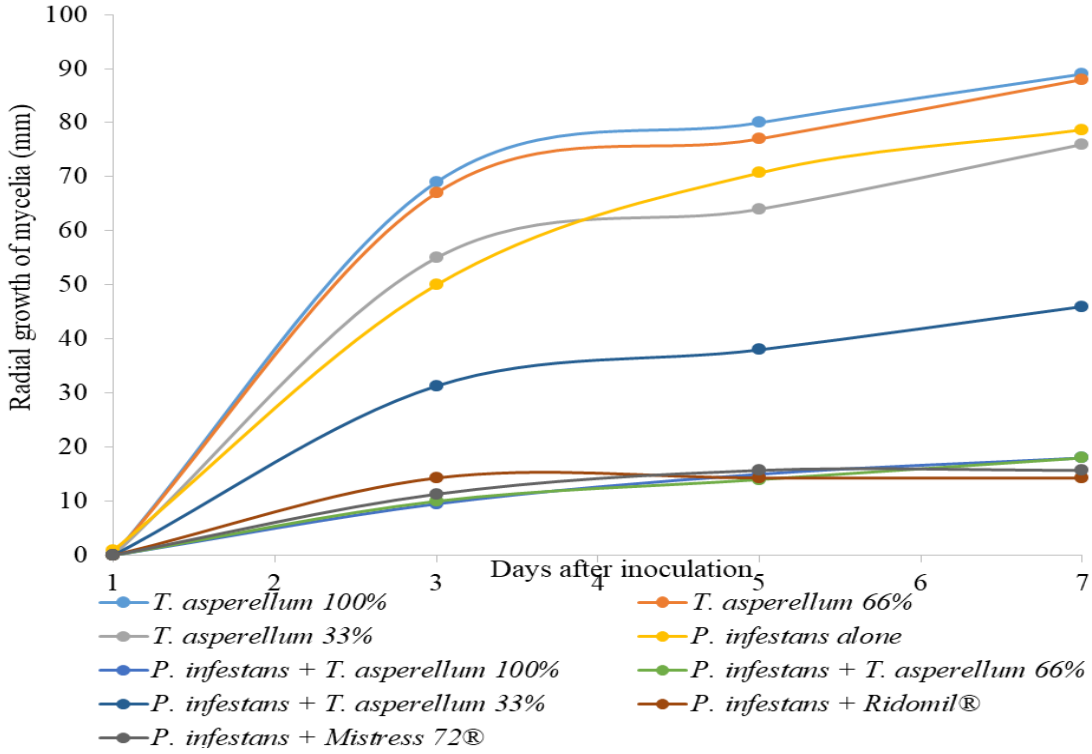

Figure 2. Growth trend of *Trichoderma asperellum* concentrations, *P. infestans* and *P. infestans* in the presence of *T. asperellum* concentrations, Ridomil® and Mistress 72® on PDA.

Table 1. Effect of *P. infestans* growth inhibition (percentage) induced by *T. asperellum*.

Treatment	Radial growth inhibition (mm) across days after inoculation		
	3	5	7
Ridomil®	80.30[a]	89.7[a]	100.0[a]
Mistress 72®	79.50[a]	89.3[a]	100.0[a]
T. asperellum 100%	80.00[a]	88.30[b]	91.30[b]
T. asperellum 66%	80.0[a]	88.00[b]	91.10[b]
T. asperellum 33%	37.3[b]	46.0[c]	38.0[c]
$HSD_{(0.05)}$	11.12	7.24	7.97
CV%	6.63	4.24	4.82

Means followed by same letter in each column are not significantly different (p=0.05). HSD represents Tukeys honest significant difference at 95% confidence interval.

Table 2. Effect of *T. asperellum* applied at different concentrations on lesion size (cm^2) of potato late blight (*P. infestans*) on detached leaf assay.

Treatment	Lesion size (cm^2) across days after inoculation				
	3	5	7	9	11
P. infestans	1.73[b]	2.23[b]	2.817[b]	3.05[b]	3.55[b]
T. asperellum 33%	1.66[ba]	2.12[b]	2.617[b]	2.94[b]	3.50[b]
T. asperellum 66%	1.49[ba]	1.66[c]	1.78[c]	1.89[c]	1.91[c]
T. asperellum 100%	1.46[c]	1.62[c]	1.72[c]	1.86[c]	1.89[c]
Mistress 72®	0[a]	0[a]	0[a]	0[a]	0[a]
Ridomil®	0[a]	0[a]	0[a]	0[a]	0[a]
$HSD_{0.05}$	0.249	0.251	0.206	0.287	0.215
CV%	5.55	4.65	3.25	4.22	2.892

Means followed by the same letter within the same column are not significantly different at p<0.05. HSD represents Tukeys honest significant difference at 95% confidence interval.

continuous growth. An increase in *T. asperellum* inoculum concentration (from 33 to 100%) resulted to enhanced growth restriction of pathogen from 3rd to 7th day after inoculation (Table 1).

Determination of antagonistic effects of *T. asperellum* against *P. infestans* in detached leaflet assay

T. asperellum and the fungicides influenced late blight lesion size on the detached leaf assay. Lesion size was progressively reduced from day 3 by 4, 14 and 16% to 11th day by 1, 47 and 49%) after inoculation when *T. asperellum* at 33, 66 and 100% concentrations were applied, respectively relative to *P. infestans* (Table 2). There was no significant difference (p<0.05) in lesion size between *T. asperellum* at 66% (1.91 cm^2) and 100% (1.89 cm^2) concentrations. Lesion expansion was curtailed in detached leaflets treated with Mistress 72® and Ridomil® (Figure 3). Initially (first 3 days after inoculation), *T. asperellum* at 33% concentration was able to manage lesion increase thereafter it was not significantly different with the negative control (*P. infestans* alone) indicating its inability to halt the pathogen growth and multiplication.

T. asperellum compatibility with Ridomil® and Mistress 72®

T. asperellum pure culture colony grew in Potato Dextrose Agar (PDA) forming a white concentric ring that changed to green as it matured after 5 days (Figure 4). Establishment and development of *T. asperellum* was not inhibited in anyway by Ridomil® nor by Mistress 72® *in vitro*. *T. asperellum* mycelia continued to grow in PDA over the incubation period neither in Ridomil® nor by Mistress 72®. Radial mycelial growth for pure *T. asperellum* culture, *T. asperellum* + Ridomil® and *T. asperellum* + Mistress 72® were not significant different. However, Mistress 72® showed more *T. asperellum* mycelial growth suppression than Ridomil® across all days after inoculation (Figure 5).

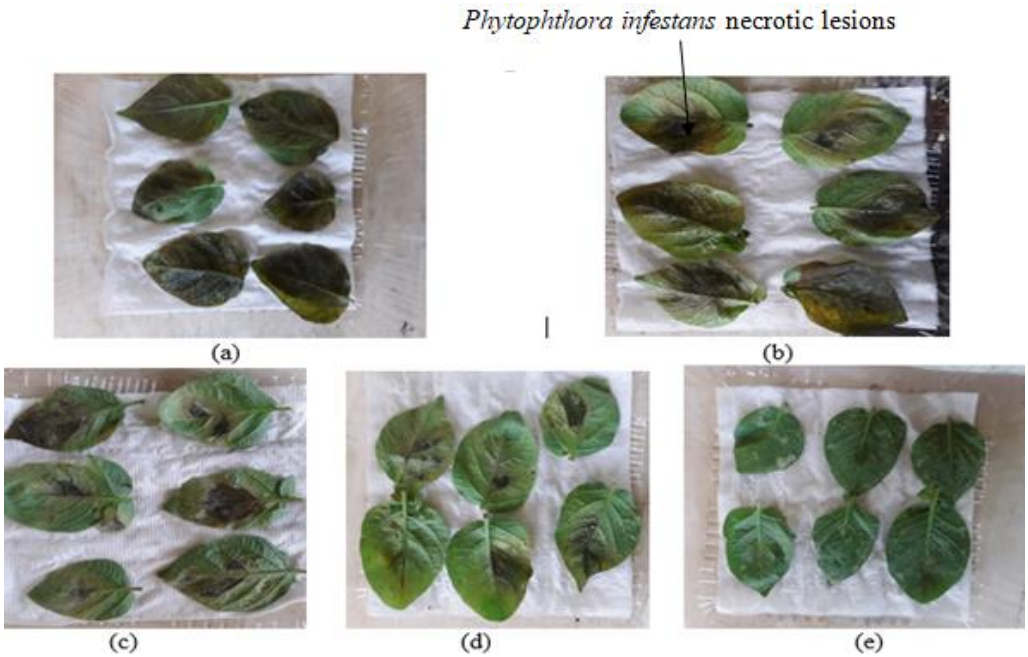

Figure 3. Detached leaf assay at day five after inoculation *P. infestans* (negative control) (a), *T. asperellum* at 33% (b), 66% (c) and (d) 100% concentration and Ridomil® (positive control) (e) inoculated on healthy detached leaves.

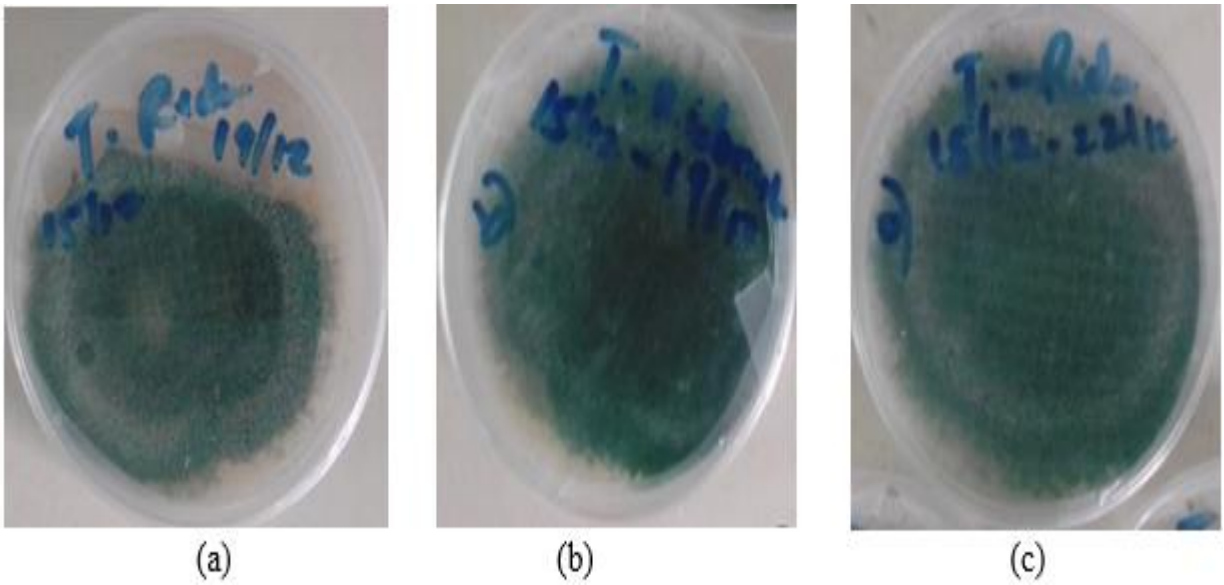

Figure 4. Effects of Ridomil® (a) and Mistress 72® (b) on *T. asperellum* (c).

DISCUSSION

The use of biological agents to suppress plant diseases has been demonstrated in previous studies to inhibit *P. infestans* (Miles et al., 2012; Fatima et al., 2015; Yao et al., 2016; Syed et al., 2018). The results of the present study indicate that *T. asperellum* is pathogenic and aggressive against *P. infestans* but this inhibitory action is underpinned by the initial biocontrol inoculum concentration. These findings are consistent with previous study of Kipngeno et al. (2015) who reported the efficacy of *Bacillus subtilis* and *T. asperellum* on *Pythium*

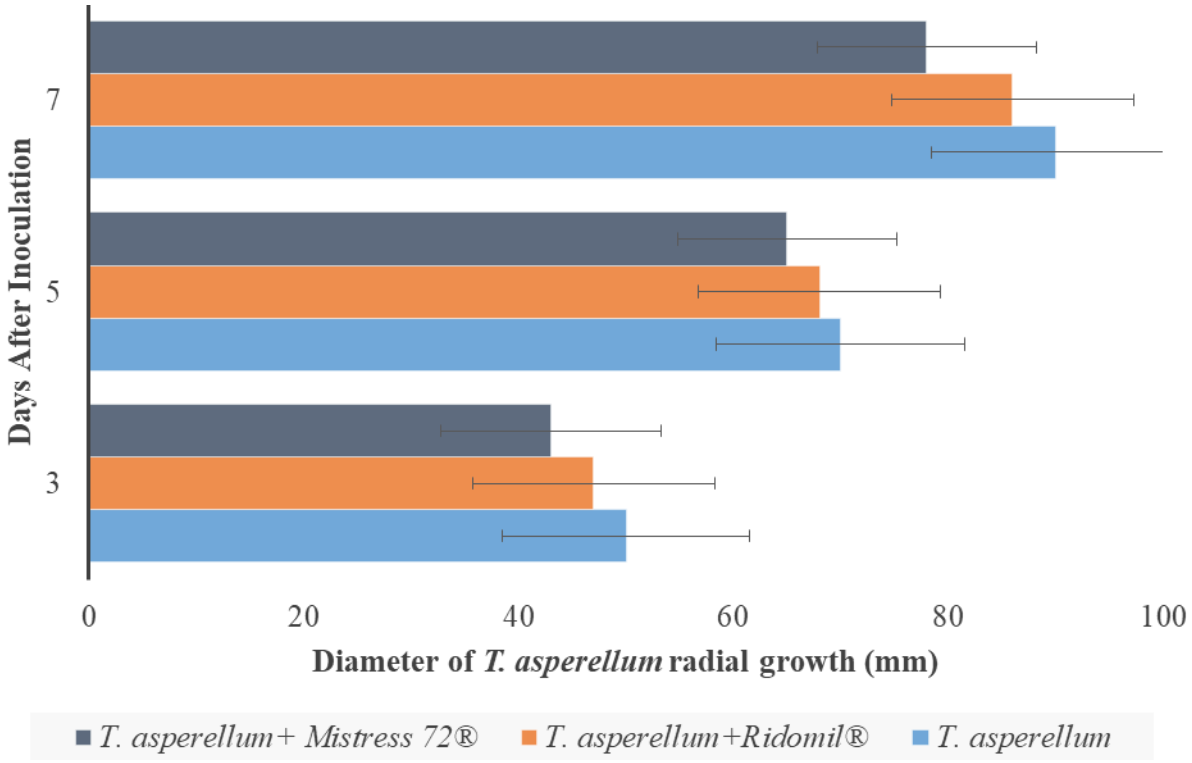

Figure 5. Effect of Ridomil® and Mistress 72® growth inhibition on *Trichoderma asperellum in vitro*. Error bars show SE on means of *T. asperellum* mycelial growth and where the bars overlap indicate the treatments are not significantly different.

aphanindermatum in tomato. Positive results were also reported by Istv (2014), Widmer (2014), Fatima et al. (2015), Yao et al. (2016), and Bahramisharif and Rose (2018) during their studies in effort to manage potato diseases using biocontrols. Agbeniyi et al. (2014) reported that *T. asperellum* was able to reduce fungicide Cacao pod rot severity when combined with mancozeb fungicide. However, there is need for new strains of biological agents from the vicinity of host plant that may have a better biocontrol activity against phytopathogens to be explored to enhance effective disease control. Therefore, this study attempts to explore the effects of *T. asperellum* on *P. infestans* to widen the biocontrol's action spectrum against pathogens when combined with reduce fungicides application frequency.

Antifungal bioassay

In dual culture, *T. asperellum* at 66 and 100% concentration which were not significantly different had the highest inhibitory action *in vitro.* The treatments had a high sporulation and competing capacity filling up the PDA plates faster than *P. infestans.* This overwhelmed the pathogen and indeed the *Trichoderma* mycelia grew over the *P. infestans*. High rate of sporulation and colonization are the key important traits for excellent biocontrol (Xu et al., 2011), zone of inhibition was observed between the two fungi which could be attributed to the effect of diffusible products released by the *T. asperellum* which suppressed further growth of the *P. infestans*. The continual growth of *P. infestans* mycelial in the presence of *T. asperellum* at 33% concentration (Figure 2) indicated that concentration of biocontrols is an important factor in their action against disease causing microorganisms. Similar results were reported by Patel and Saraf (2017) on efficacy of *T. asperellum* against *Fusarium oxysporium* on tomato (*Lycospercum esculenta*) that caused 85% wilting severity decrease. The mycoparasitic action of the *T. asperellum* suggests that the biocontrol has a high potential of managing late blight of potato. Similar mycoparasitism and competition by *Trichoderma* spp. against phytopathogens were reported by Sharma et al. (2017). The inhibition zone observed (white mycelial growth) indicated release of metabolite products by the biocontrol which was also reported by Widmer (2014). The absence of inhibition zone observed in dual culture associated with 33% *T. asperellum-P. infestans* interaction suggests that the biocontrol concentration was low and therefore unable to release sufficient metabolites to overcome similar products released by the pathogen. This was consistent with Sharma (2011) study who reported *Fusarium* wilt-*Trichoderma* chemical signal interactions. Further, the

two increase in mycelial growth was low compared to other treatments in co-culture possibly due to 'tag of war' involving metabolites that have to be secreted first and in sufficient amounts before being released by the two fungi against each other where the strong one overcome the other. Leonetti et al. (2017) and Naglot et al. (2015) reported SA signaling pathway and enzymatic activities, respectively have to be started before mycelial growth. This shows that for a biocontrol to be effective it should have high reproduction and strong in releasing metabolites that cause antibiosis, cell wall degradation and mycoparasitism as reported by Wu et al. (2017). *T. asperellum* mycelia growth was directed towards the *P. infestans* indicating chemotropism towards the pathogen as observed by Sharma (2011). However, the present study reports the antifungal activities by *T. asperellum* being slow allowing the pathogen to partially grow compared to Ridomil® and Mistress 72® that were effective in inhibiting *P. infestans* growth in 33% *T. asperellum* concentration suggesting fast growth and inhibitory is affected by concentration of the biocontrol. The effectiveness of Ridomil® and Mistress 72® could be attributed to the fact that their active ingredients act by targeting specific region of the pathogen as reported by Sharma and Saikia (2013).

Antagonistic effects of *T. asperellum* against *P. infestans* in detached leaflet assay

The trend in slowed increase in lesion size from day three to eleven (Table 2) after inoculation indicate that *T. asperellum* are slow acting as reported by Lal (2017). The 66 and 100% *T. asperellum* inhibited late blight lesion increase while Ridomil® and Mistress 72® did not allow lesion establishment at all. The 33% *T. asperellum* lesion size was similar to that of *P. infestans* alone (control) providing further evidence that concentration of the biocontrol is a key trait. Even though their lesion development was observed in 66 and 100% *T. asperellum*, the treatments in the long run managed the disease lesion preventing further increase showing biocontrols have slow action against phytopathogens. The biocontrol required pathogen signal to secrete enzymes, pathogenesis related proteins and metabolites that may take some time giving the pathogen a chance to establish. The observed antifungal activity could be attributed to a faster growth of the *T. asperellum* compared to *P. infestans* (competition) and secondary metabolites (defense mechanisms) released by the biocontrol that have antagonistic activity against the pathogen (Amin et al., 2010). Schuster et al. (2010) reported the presence of cell wall degrading enzymes including the glucanases that degrade *P. infestans* cells affecting their growth. Further, the antagonistic activity of *T. asperellum* on *P. infestans* revealed mycoparasitism of *P. infestans* as indicated presence of white mycelial between the two fungi that confirms the report of Itachi et al. (2007). Similar mycoparasitic actions were observed in *Trichoderma viride* antagonistic activities against *P. infestans in vitro* (Ephrem et al., 2011). This study provides evidence that a biocontrol concentration is an important characteristic in biological agents for them to be effective and this is observed missing in the literatures.

At 66 and 100% concentrations, *T. asperellum* provided excellent control of late blight. Thus, it appearance at 66% of *T. asperellum* concentration could be used to manage the *P. infestans* under field conditions. The implication is the slowing of the rate of evolution of new strains that adapt to new fungicides formulations and resistant varieties. Newly emerging strains tend to be aggressive and require increased fungicide application (Childers et al., 2014) to control. This poses a threat to environment, human population and increased cost of production (Cooke et al., 2011). The present work also reports unhindered mycelial growth of *T. asperellum* in the presence of Ridomil® and Mistress 72® indicating possibility of combining the two in integrated disease management program. This information could lead to adoption of effective rate of *T. asperellum* which offer safer option as they are eco-friendly and can be combined with fungicides in effort to reduce overuse of synthetic fungicides.

***T. asperellum* compatibility with Ridomil® and Mistress 72®**

In Ridomil® and Mistress 72®-*T. asperellum* compatibility experiment, the biocontrol radial mycelial growth was not inhibited by Ridomil® and Mistress 72®. These results are in agreement with Aparecida et al. (2018) who reported that *Trichoderma asperelloides* reduced *Sclerotinia minor* growth more when combined with azoxystrobin. Thus, the results indicated possibility of combining *T. asperellum* with either of these two synthetic fungicides in alternation to effectively control late blight while reducing human, economic and environmental concerns. However, Mistress 72® had more suppression on *T. asperellum* mycelial than Ridomil®. The two fungicides have mancozeb in similar concentration but in addition Ridomil® has metalaxyl while Mistress 72® has cynamoxil. This suggests cynamoxil may have inhibition aspects if its concentration was increased while *T. asperellum* could tolerate metalaxyl better. Co-formulation of fungicides with metalaxyl aims at lower metalaxyl dose to reduce chances of resistance development by *P. infestans* strains (Muchiri et al., 2017).

Conclusion

This study aimed to ascertain effectiveness of *T.*

asperellum in managing late blight and possibility of combining the biocontrol with fungicides under controlled conditions. Concentration of biocontrol is one of the most key characteristics in enhancing their effectiveness. Late blight development was influenced by change in concentration of the *T. asperellum*. *In vitro* and detached leaflet assay experiments demonstrate *T. asperellum* concentrations against *P. infestans* are key. However, the study did not establish mechanisms and defence metabolites expressed by *T. asperellum* which could be explored in the future studies. Although this biological agent was effective in controlling *P. infestans in vitro* and detached leaf assay conditions, their adoption could offer safer and sustainable alternative to synthetic fungicides and become a key component of Integrated Disease Management (IDM) system under field conditions that will ultimately reduce fungicides usage and their negative impacts, thereby contributing to increased national potato production. However, field trials of the biocontrol are required to determine consistency as well as possibility of managing other diseases of potato. Cynamoxil is one of the proposed fungicide molecules to replace metalaxyl due to emergence of metalaxyl resistant *P. infestans* strains, therefore further studies need to focus on increased cynamoxil dosage on *T. asperellum* mycelial growth.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Assessing the polymorphism of DHFR gene from *Plasmodium falciparum* in the south of Côte d'Ivoire

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Since 2005, Côte d'Ivoire has adopted new strategies of malaria management including free provision of Artemisinin-based Combination Therapy (ACT) to children less than five years of age and sulfadoxine-pyrimethamine (SP) as Intermittent Preventive Treatment (IPT) for pregnant women. However, introduction of ATCs raises concerns about the extensive use of cheap SP which could increase *Plasmodium falciparum* resistance level to SP. Therefore, this study aimed to determine the prevalence of the Asn-108 marker in three sites in Southern Côte d'Ivoire. After obtaining consent, blood samples were collected in Anonkoua-kouté, Port-Bouët, and Ayamé sites from 180 patients over 2 years of age and having simple *P. falciparum* malaria. *P. falciparum* genomic DNA extracted from these samples was amplified by nested-PCR with pfdhfr specific primers. The amplification products were revealed by electrophoresis on 1.5% agarose gel and then sequenced according to Sanger method. After sequencing, the prevalence of mutation points associated with *P. falciparum* resistance to pyrimethamine was determined. For the three study sites, 180 DNA fragments, of which 165 (165/180 or 91.66%) were successfully sequenced. Analysis of the 165 sequences indicated a prevalence of 61.29% (76/124) of the Asn-108 mutant allele versus 17.41% (27/155) of the wild type Ser-108 allele. Results also indicated that the prevalence of Ser-108-Asn mutation were 69.07, 69.04 and 82.75% for Anonkoua-Kouté, Port-Bouët and Ayamé, respectively. More than a decade after the adoption of SP as IPT for pregnant women, the prevalence of the marker Asn-108 was relatively high in Anonkoua-kouté, Port-bouët and Ayamé.

Key words: Pfdhfr, Asn-108, Côte d'Ivoire, sulfadoxine-pyrimethamine, resistance, antimalarial drug.

INTRODUCTION

Malaria remains a major cause of morbidity worldwide. According to the World Health Organization (WHO), 219 million cases of malaria were recorded in 2017, of which 345,000 led to death with 93% occurrence in Africa (WHO, 2018). For children under five years of age, the deaths were estimated to 61% in 2017 (WHO, 2018).

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The treatment of this disease involves antimalarial drugs, because effective vaccine is not yet available. However, malaria control is limited by *Plasmodium falciparum* resistance to most antimalarial drugs. Indeed, high levels of chloroquine resistance have forced some countries to abandon chloroquine as first-line treatment in favor of sulfadoxine-pyrimethamine (SP). However, resistance to this drug has emerged regarding treatment failures reported in Africa, Asia, Indonesia and South America (Adnan et al., 2018; Ratcliff et al., 2007; Ravi (2016); Shannon and Miriam (2015); Vladimir et al., 2010).

Pyrimethamine and sulfadoxine act synergistically to inhibit two important enzymes in the pathway of parasite folate biosynthesis namely dihydrofolate reductase (DHFR) and dihydropteroate synthetase (DHPS) (Kasturi et al., 2018; Yaro, 2009). Mutations in *pfdhfr* and *pfdhps* genes confer resistance to pyrimethamine and sulfadoxine respectively, with an *in vitro* decrease in *P. falciparum* sensitivity related to the number of mutations in each gene (Ingrid and John, 2010; Vladimir et al., 2010). These mutations are correlated with treatment failure in clinical trials (Ratcliff et al., 2007; Yaro, 2009).

The presence of mutations in *pfdhfr* gene appears to be more important in treatment failure than mutations in *pfdhps* gene (Sankar et al., 2010). Indeed, the triple mutation in codons 108, 51 and 59 of *pfdhfr* gene increases the risk of *in vivo* resistance to SP by 4.3 (OR; 95% CI: 3.0-6.3, meta-analysis of 16 studies) (Picot et al., 2009). Detection of Ser-108-Asn mutation is predictive of the presence of the other two mutations.

In Côte d'Ivoire, since 2005, SP has been used as intermittent preventive treatment (IPT) in pregnant women and children as recommended by the WHO (WHO, 2016). However, introduction of ATCs raises concerns about the extensive use of cheap (SP) which could increase *P. falciparum* resistance level to SP. This study, conducted in three sites in Southern Côte d'Ivoire, aims to determine the prevalence of key mutations associated with *P. falciparum* resistance to pyrimethamine (*dhfr* codons N51, C59, S108) in patients with uncomplicated malaria.

MATERIALS AND METHODS

Study site

This study was carried out in three localities of Côte d'Ivoire (malaria endemic zone) as part of a monitoring study of antimalarial drug resistance. The study included a standard questionnaire to collect socio-demographic data from participants and blood collection for molecular testing. The study was conducted from February to August, 2015 at the Anonkoua Kouté health center and the general hospitals of Port-Bouët and Ayamé. All these sites (Anonkoua Kouté 5°25'55.90" N ; 4 02'45.27" W, Port-Bouët 5°15'20" N et 3°57'52" W and Ayamé 5°36'12,43" N ; 3°09'19,36" W) are located in the Southern region of Côte d'Ivoire where climate is equatorial, with annual rainfall exceeding 1700 mm and temperature varies between 27 and 33°C. Malaria is seasonal, predominating in the rainy season from June to September with prevalence peaks in October-November. *P. falciparum* is the dominant specie with more than 90% of identified malaria parasites (Adja et al., 2011). Anonkoua-kouté Health Centre, Port-Bouet and Ayamé General Hospitals were selected based on high annual incidences of malaria cases.

Study population and blood sample collection

All suspected cases of malaria at Anonkoua-kouté health center, Port-Bouët and Ayamé general hospitals were randomly selected for the study. After informed consent, patients' socio-demographic data were recorded from the questionnaire; then blood samples were collected from participants over 2 years of age and suffering from uncomplicated *P. falciparum* malaria detected after microscopy test. Approximately 2-5 ml of venous blood was drawn and collected in Ethylene Diamine TetraAcetic (EDTA (BD Vacutainer®-367844)) containing tube and 50 µl of whole blood was placed on Whatman 3 MM (Whatman Inc., Maidstone, United Kingdom) filter paper using a micropipette. Blood spots on filter paper were dried for approximately 60 to 120 min at room temperature. Unused blood in EDTA tube was stored in at − 20°C for further analysis.

Extraction of *P. falciparum* genomic deoxyribonucleic acid (DNA)

Plasmodial DNA was extracted from filter paper blood spots with methanol (Miguel et al., 2013). Indeed, fine cuts of spots were immersed in 1 ml of Wash Buffer (950 µL PBS 1X and 50 µl 10% saponin) and then incubated at 4°C overnight. The wash buffer was removed and 150 µl of methanol were added. After 20 min incubation at 4°C, the methanol was gently removed and the samples were dried at room temperature for 2 h before adding 300 µl of sterilized water. Samples were then heated at 99°C in a thermo-mixer for 30 min to extract the DNA. The DNA extracts were aliquoted into a 1.5 ml Eppendorf tube and stored at − 20°C.

Amplification of the pfdhfr gene

The *pfdhfr* gene was amplified by nested PCR using specific pair of primers and commercial DNA polymerase kit (5X FIREPol® Blend Master Mix (Solis Biodyne)) with mM $MgCl_2$. This kit is a pre-mix (for the reaction mixture) ready to use composed of DNA polymerase (FIREPol® DNA polymerase), buffer (5× Blend Master Mix Buffer), MgCl2 (7.5 mM $MgCl_2$) and dNTPs (2 mM dNTPs of each).

For primary PCR, the primer pairs used for amplification of the *pfdhfr* gene were dhfr_M1 (5'TTTATGATGGAACAAGTCTGC) / dhfr_M7 (CTAGTATATACATCGCTAACA). The primary PCR (25 µL) reaction contained 0.625 µl of each primer, 3 µl of plasmodial DNA, 5 µl of Taq DNA polymerase and 15.75 µl of milliQ water. The cycling parameters used were as follow: Initial denaturation at 95°C for 15 min followed by 30 denaturation cycles at 95°C for 30 s, annealing at 58°C for 2 min and extension at 72°C for 2 min. Terminal extension step was set at 72°C for 10 min.

The second PCR was carried out on amplification products (amplicon) of the primary PCR in a reaction volume of 50 µL containing: 1.25 µl of each primer, 5 µl of amplification product (amplicon), 5 µl of Taq DNA polymerase and 37.5 µl of milliQ water. The primer pairs used for the secondary Polymerase chain reaction (PCR) were dhfr_M9 (5'CTGGAAAAA AATACATCAC ATTCATATG) /dhfr_M3 (5'T GATGGAACAAGTCTGC GACGTT). The secondary PCR cycling operation was performed as follow: Initial denaturation at 95°C for 15 min followed by 30 denaturation cycles at 95°C for 30 s, annealing at 60°C for one min and extension at 72°C for 1 min.

Table 1. Study population profile.

Site	Gender	
	Female n (%)	Male n (%)
Anonkoua-Kouté	37 (59,67)	25 (40,33)
Port-Bouët	39 (66,10)	20 (33,9)
Ayaamé	35 (59,32)	24 (40,68)
Total	**111 (61,66)**	**69 (38,34)**

Table 2. Blood samples used for molecular analysis of pyrimethamine chemoresistance.

Sites	Sampling period (2015)	Age groups (years)	Average age (years)± SD	Blood samples
Anonkoua-kouté	February - March	2 to 53	16.60 ±14.30	62
Port - Bouët	April - May - July	2 to 62	16.69±13.24	59
Ayamé	June - July - August	2 to 55	15.84±14.87	59
	Total			**180**

Terminal extension step was set at 72°C for 10 min.

Detection and analysis of PCR products

The amplification products were transferred on a 1.5% agarose gel containing ethidium bromide (EtBr). After migration, the gel was visualized under UV lamp using the UV transilluminator (Gel DocTM EZ Imager (Bio-Rad)).

Sequencing amplification

Amplified DNA fragments (*pfdhfr* gene) of *Plasmodium falciparum* were subjected to sequencing according to the Sanger method by the Company Eurofins MWG opéron (Cochin sequencing platform). Samples were dropped in a microplate (Greiner Bio-one-652270B) that was sent to the platform for sequencing. The DNA sequences received after sequencing reaction were recovered fast. The software BioEdit was used to analyze the sequences in order to search for possible mutations.

Statistical analysis of data

Data were collected based on standard questionnaire that was tested and validated. They were analyzed using the statistical software R; version 3.2.2 (Core Team R, 2013). The $\chi 2$ comparison test of three mean values was used to compare the prevalence of the molecular marker of pyrimethamine resistance (*pfdhfr* S108N). The $\chi 2$ test was used to determine whether the molecular marker prevalence can be considered to be all equal (hypothesis H0) or if two or more prevalence are different (alternative hypothesis Ha). A difference and/or statistical association was considered significant if p-value < 0.05.

RESULTS

Profile of selected patients

A total of 180 persons with uncomplicated malaria were selected for this study, including 111 (61.66%) females and 69 (38.34%) males (Table 1). Patients' ages ranged from 2 to 62 years, with mean ages in Anonkoua-kouté, Port-bouët and Ayamé of 16.60, 16.69 and 15.84 years respectively. Thus, 180 blood samples were collected in the study sites (Table 2).

DNA sequencing assessment

For all the study sites, 180 DNA fragments were isolated, of which 165 (165/180, or 91.66%) were successfully sequenced. Molecular analysis of these fragments showed that the number of sequenced DNA fragments with success varied according to the presence of interest codons. Thus, 124 (75.15%), 126 (76.63%) and 155 (93.93%) DNA fragments were successfully sequenced for the nucleotides position 153, 177 and 324 corresponding to the amino acids Asn-51-Ile, Cys-59-Arg and Ser-108-Asn where mutations were observed (Table 3). Sequencing of the DNA region leading to the Ser-108-Asn mutation was more successfully performed (155/165; 93.93%).

Polymorphism of the *pfdhfr* gene

Prevalence of individual alleles of the pfdhfr gene and molecular analysis of corresponding genotypes

For the three study sites, our results indicated that the prevalence of isolates carrying the Ile-51 (61.29%), Arg-59 (54.76%), Asn-108 (74.19%) mutations were higher than those of wild isolates Asn-51 (15.32%), Cys-59 (15.07%), Ser-108 (17.41%) of *pfdhfr* gene (Table 4). Molecular analysis of the genotypes corresponding to

Table 3. Mutation status of sequenced DNA extracted from patients.

Sequenced fragments	Mutations	Success	Failure
Pfdhfr (n = 165)	Asn-51-Ile	124	11
	Cys-59-Arg	126	39
	Ser-108-Asn	**155**	**10**

Table 4. Prevalence of individual alleles of *pfdhfr* gene.

Codons	Alleles		Study sites (N=165)	
			Staff	(%)
			Blood (n=124)	
	Wild Type (N)	Asn-51	19	15.32
		Ile-51	**76**	**61.29**
Dhfr_51		Phe-51	**13**	**10.40**
	Mutants	Lys-51	2	1.61
		Leu-51	6	4.83
		Pro-51	6	4.83
		Ser-51	2	1.61
			Blood (n=126)	
	Wild type (C)	Cys-59	19	15.07
		Arg-59	**69**	**54.76**
		Ala-59	2	1.58
dhfr_59		Gly-59	**21**	**16.66**
	Mutants	Leu-59	2	1.58
		Ser-59	**9**	**7.14**
		Trp-59	4	3.17
			Blood (n=155)	
	Wild type (S)	Ser-108	27	17.41
		Asn-108	**115**	**74.19**
		Ala-108	4	2.58
dhfr_108	Mutants	Phe-108	2	1.29
		His-108	3	1.93
		Thr-108	2	1.29
		Val-108	2	1.29

"N" represents the total number of isolates successfully sequenced. "n" represents the number of isolates for which the codons of interest (51, 59, 108) or nucleotides (153, 177, 324), of the sequence.

pfdhfr gene showed a predominance of triple mutant (75/165, or 45.45%) and double mutant (50/165, or 30.30%) genotypes. The results also indicated that isolates carrying the IRN (triple mutant), NRN (double mutant) and ICN (double mutant) genotypes were observed with prevalence of 31.51, 9.09 and 7.87%, respectively, compared to 13.93% for isolates carrying the NCS (wild type) genotype (Table 5). Single mutant genotypes were also observed with a prevalence of 10.30%.

Prevalence of the Asn-108 mutation of the pfdhfr gene polypeptide in Anonkoua-Kouté, Port-Bouët and Ayamé

Our results showed that the Ser-108-Asn mutation was observed at 69.09%, 69.04% and 82.75% respectively for Anonkoua-Kouté, Port-Bouët and Ayamé (Table 6). For the same mutation (Ser-108-Asn), the highest prevalence was observed in Ayame (82.75%). Analysis also revealed no significant difference between the prevalence of the

Table 5. Prevalence of genotypes corresponding to *pfdhfr* in the three sites.

Haplotype	N51I	C59R	S108N	Blood (N=165)	
				n	Proportion
Wild types	**N**	**C**	**S**	**23**	**13.93**
				17	**10.30**
	N	C	T	2	1.21
	I	C	S	4	2.42
	N	C	F	2	1.21
Single mutant	N	C	N	4	2.42
	N	C	V	2	1.21
	L	C	S	2	1.21
	P	C	S	1	0.60
				50	**30.30**
	N	G	A	2	1.21
	N	S	N	4	2.42
	L	G	S	4	2.42
	F	C	N	4	2.42
Double mutant	**N**	**R**	**N**	**15**	**9.09**
	I	**C**	**N**	**13**	**7.87**
	N	**G**	**N**	4	2.42
	N	**A**	**A**	2	1.21
	P	**W**	S	2	1.21
				75	**45.45**
	S	G	N	2	1.21
	F	L	N	2	1.21
	I	G	H	2	1.21
Triple mutant	F	S	N	4	2.42
	I	**R**	**N**	**52**	**31.51**
	I	R	H	2	1.21
	K	G	N	1	0.60
	I	W	N	2	1.21
	I	S	N	2	1.21
	F	G	N	4	2.42
	P	G	N	2	1.21

An uppercase letter in the "genotypes" column represents the code for an amino acid. Amino acids resulting from the mutation are underlined and in bold. The prevalence correspond to the number of observations on the number of success per gene.

Ser-108-Asn mutation determined from isolates for Anonkoua-kouté, Port-Bouët and Ayamé (p = 0.344).

DISCUSSION

This study indicated that in 2015, the prevalence of the Ser-108-Asn mutation (Asn-108) was observed at the same level of prevalence in Anonkoua-kouté (69.09%), Port-Bouët (69.04%) and Ayamé (82.75%). These data could be explained by the presence of *P. falciparum* potentially pyrimethamino-resistant isolates. The prevalence of this mutation was higher than those obtained in 2008 at Anonkoua-Kouté in Abidjan (49%) and Ayamé (54%) in blood isolates from individuals with malaria symptoms (Ako et al., 2014). Lower proportions were obtained by other authors in 2001 (50%) and 2006 (46.4%) at Yopougon in Abidjan (Djaman et al., 2002, 2010) and at Adzopé (35.4%) in 2010 (Ouattara et al., 2010).

In addition, a study of marker dynamics indicated that the prevalence of the Ser-108-Asn mutation increased significantly in Anonkoua-kouté between 2002 and 2008, with an average of 43% (Ako et al., 2012, 2014). In view of all these results, the prevalence of Asn-108 mutation has increased significantly in this part of the country.

This finding is also important because the sulfadoxine-pyrimethamine combination is recommended in intermittent

Table 6. Frequencies of the wild Ser-108 and Asn-108 mutant alleles of the pfdhfr gene at Anonkoua-Kouté, Port-Bouët and Ayamé.

Codon	Alleles		Anonkoua-Kouté (N=55)		Port-Bouët (N=42)		Ayamé (N=58)		p-value of the test
			n	%	n	%	n	%	χ^2
dhfr_108	Wild	Ser-108	6	10.90	11	26.19	10	17.24	0.682
	Mutants	**Asn**-108	**38**	**69.09**	**29**	**69.04**	**48**	**82.75**	0.344

"N" represents the total number of successfully sequenced isolates per study site. "n" represents the number of successfully sequenced isolates for codon. dhfr_108. The list of other mutants is given in Table 3.

preventive treatment of pregnant women in Côte d'Ivoire (MSHP, 2013). Despite its prohibition in the curative treatment of malaria attacks, SP could be used by some population in Anonkoua-kouté, Port-Bouët and Ayamé and perhaps in other towns (Granado et al., 2009, 2011). The data obtained could also be explained by the increased use of SP (Tinto et al., 2007) in unofficial markets because of withdrawal of chloroquine. This increased use of SP could be explained by non-recommended therapeutic practices such as self-medication (Gokpeya et al., 2013; Kouadio et al*.,* 2006) encouraged by easy access to the molecule already available in the country before 1996 (Henry et al., 1996, 2002). Indeed, Min (2012) mentioned that poor populations prefer to turn to unofficial markets to obtain SP and CQ, which remain inexpensive antimalarial molecules. According to Granado et al. (2009, 2011) and Orostegui et al. (2011), unofficial markets are found in large cities such as Abidjan or San-Pedro in Côte d'Ivoire (Granado et al., 2009, 2011; Orostegui et al., 2011). About 45 illicit sales outlets for pharmaceutical products, including various antimalarial drugs, were counted in such areas in Abidjan in 2005 (Granado et al., 2011). Populations with low purchasing power may explain the recourse to unofficial retailers (Kizito et al., 2012). This uncontrolled use of SP could promote the development of high drug pressure, which could lead to the selection of resistant parasites at Anonkoua-kouté, Port-bouët and Ayamé.

In addition to drug pressure, pyrimethamine resistance in these three localities could be explained by the use of poor quality antimalarial drug. Indeed, the use of poor-quality antimalarial drug can have multiple consequences, including an increased risk of developing drug resistance, as sub-therapeutic doses of drugs will be ineffective in destroying all parasites (Newton et al., 2010; Shunmay et al., 2015).

Elsewhere in sub-Saharan Africa, high rates of *P. falciparum* resistance have been found. Indeed, results of monitoring for *P. falciparum* chemo resistance have shown the following results: in Burkina Faso, the reported rate of Asn-108 mutation was 63.8% (Somé et al., 2016); 92% in Gabon (Ghyslain et al., 2011); 93% in Senegal (Daouda et al., 2013). As the Asn-108 mutation, additional mutations (Asn-51-Ile and Cys-59-Arg) have also been identified. All mutations at codons 51 and 59 were associated with that of codon 108. Parasites carrying these additional Asn-51-Ile and Cys-59-Arg mutations associated with the Ser-108-Asn mutation have higher pyriméthamine resistance than those carrying the Ser-108-Asn mutation alone (Mathieu et al., 2007; Gregson and Plowe, 2005). Compared to the prevalence of 17.33 and 27.27% reported by Ako respectively for Dabakala, Anonkoua-Kouté, Ayamé sites (Ako et al., 2012, 2014) and Bonoua and Samo sites (Ako et al., 2012), the prevalence of the triple mutant IRN (31.51%) increased compared to the sensitive strain (NCS). A high prevalence of triple-mutant *P. falciparum* parasites reduces the efficacy of sulfadoxine-pyrimethamine as an intermittent preventive treatment against malaria in infants and children (Gosling et al*.,* 2009; Nankabirwa et al*.,* 2010), undermines the ability of sulfadoxine-pyrimethamine to clear existing *P. falciparum* infections in asymptomatic pregnant women, and shortens the post-treatment prophylactic period, following Intermittent Preventive Treatment during pregnancy (Desai et al*.,* 2016).

Conclusion

The study indicates that the prevalence of alleles associated with pyrimethamine chemoresistance represented by the *dhfr* Asn-108 mutation has increased in Anonkoua-kouté, Port-Bouët and Ayamé. It also indicates an increase in the prevalence of the genotypes that confer pyrimethamine resistance. The study revealed an increase in potentially pyrimethamino-resistant isolates despite the withdrawal of SP as a first-line antimalarial treatment. These high proportions of known mutations in the *pfdhfr* gene could be in favour of a decrease in the SP efficacy in Côte d'Ivoire.

ETHICAL CLEARANCE AND INFORMED CONSENT

The study was conducted in accordance with the Declaration of Helsinki and approval was received from the National Committee for Ethics and Research (CNER) of the Ministry of Health and AIDS of Côte d'Ivoire. After appropriate information and explanation, the adult participants, parents or legal guardians of all children who wished to participate in the study gave their written consent prior to sampling.

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Naftifine inhibits pigmentation through down-regulation on expression of phytoene desaturase gene *CAR1* in *Rhodotorula mucilaginosa*

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Naftifine, an antifungal drug, inhibits pigmentation in *Rhodotorula mucilaginosa*. However, the relative mechanism is minutely understood. In this study, regulation of gene expression by naftifine was investigated to elucidate mechanism of yeast de-pigmentation. RNA-sequencing (RNA-seq) was used to screen differentially expressed genes (DEGs), followed by quantitative PCR (qPCR). The qPCR results showed that mRNA expression of phytoene desaturase gene *CAR1* was reduced to 37% of its original level, after one day's naftifine treatment. Since CAR1 acts at the immediate upstream of carotenoid biosynthesis pathway, it was concluded that naftifine involves in the process to inhibit the activity of phytoene desaturase, and that the down-regulation of gene *CAR1* by naftifine contributes to de-pigmentation in *R. mucilaginosa*.

Key word: Naftifine, carotenoid, *Rhodotorula mucilaginosa*, phytoene desaturase.

INTRODUCTION

Naftifine is a topical allylamine antifungal drug that is commonly used to treat dermatophytes infections (Carrillo-Munoz et al., 1999; Gupta et al., 2008; Ghannoum et al., 2013). Previously, it was known that naftifine increased the level of squalene and decreased that of ergosterol through inhibiting the activity of squalene epoxidase in fungi. Changes in the above mentioned steroid levels at opposite directions might increase permeability of fungal cells, thus triggered death of their cells (Paltauf et al., 1982). In *Staphylococcus aureus*, naftifine at low concentrations inhibited production of the virulence factor Staphyloxanthin, a carotenoid pigment, with a IC_{50} = 0.088 mg/L and had no effect to inhibit bacterial growth. This inhibitory effect was not through regulating the expression of operon crtOPQMN or by inhibiting isoprenoid biosynthetic pathway, but by inhibiting CrtN enzyme directly (Chen et al., 2016).

Rhodotorula mucilaginosa is a common species of environmental yeasts existing in soil, water and air. Although rarely infecting humans as a conditional pathogen, *R. mucilaginosa* is known to cause diseases under special situations (Mot et al., 2017; Peretz et al.,

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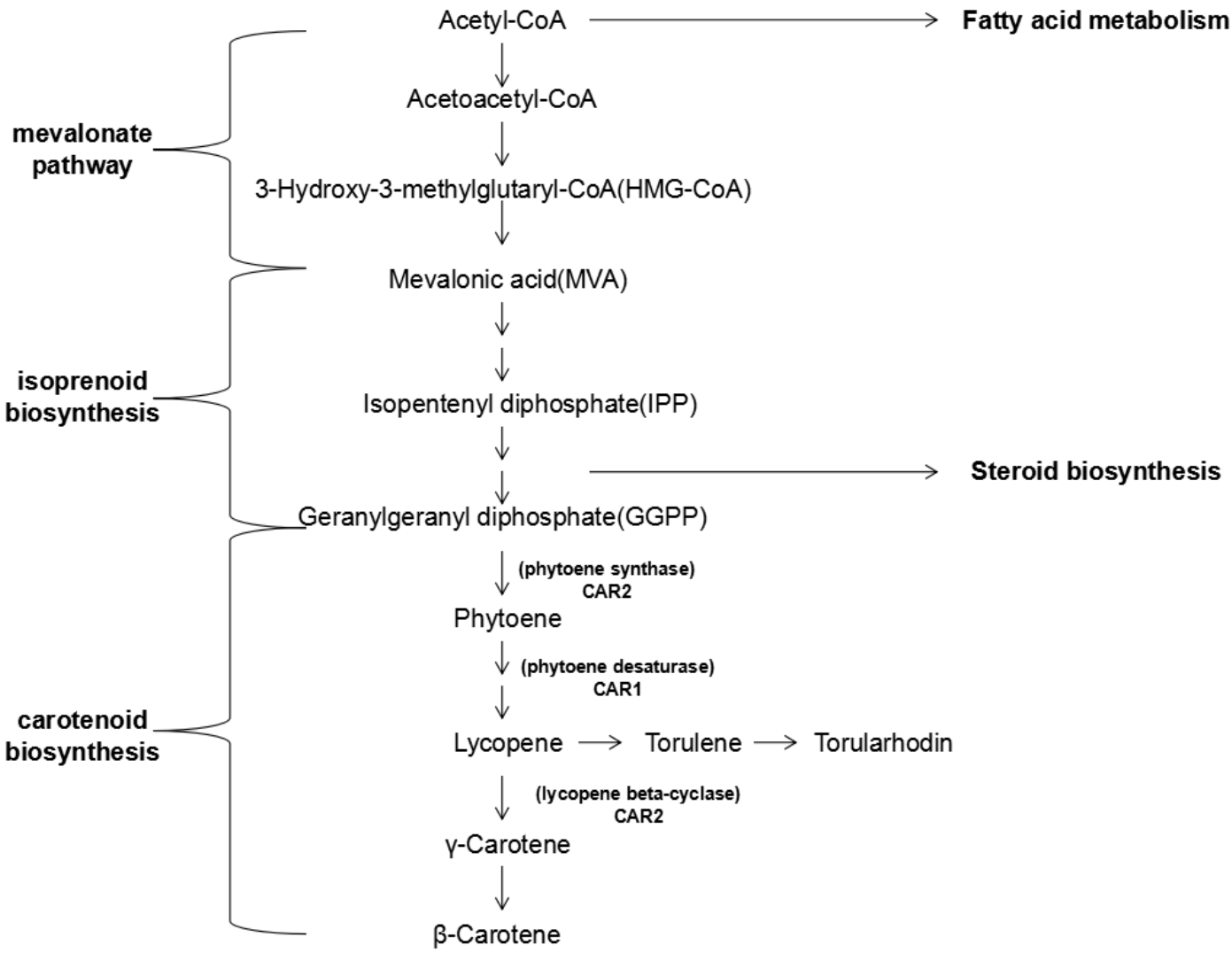

Figure 1. Diagram of the carotenoid biosynthetic pathway.

2018; Idris et al., 2019). Phospholipase was found to be a possible virulence factor in Rhodotorula genus. Rhodotorula showed more phospholipase activity than *Candida albicans* (Mayser et al., 1996). Phospholipase may increase the adhesion capacity of pathogenic microorganisms and increase the mortality of laboratory animals. Some strains of Rhodotorula have significant aspartyl peptide kinase activity (Krzyściak and Macura, 2010) which has been proposed as a virulence factor in opportunistic pathogens of Candida (Schaller et al., 2005). *R. mucilaginosa* is not sensitive to conventional antifungal drugs such as naftifine. However, its pigmentation is inhibited by naftifine at low concentrations (IC_{50} < 0.1 mg/L) as demonstrated by the decoloration of yeast. Decoloration is a reversible process (Mot et al., 2017). Carotenoids are natural apolar pigments, most of them are C40 terpenoids and some of them have oxygen-containing functional groups (Mata-Gómez et al., 2014). Carotenoids and steroids are produced in parallel pathways downstream to isoprenoid biosynthesis (Figure 1). Carotenoids are widely found in plants, fungi, and bacteria. Biosynthesis of carotenoids begins at acetyl-CoA. In *R. mucilaginosa*, acetyl-CoA sequentially converts into mevalonic acid, isopentenyl pyrophosphate, and the carotenoid precursor geranylgeranyl pyrophosphate (GGPP) (Buzzini et al., 2007; Moliné et al., 2012; Mata-Gómez et al., 2014; Kot et al., 2018; Landolfo et al., 2018). Subsequently, two molecules of GGPP are coupled by phytoene synthesase ([EC:2.5.1.32], a function of *CAR2* product) to form phytoene, a C40 carotene (Schmidhauser et al., 1994; Díaz-Sánchez et al., 2011). Phytoene thereafter produces lycopene and 3.4 dehydrolycopene by phytoene desaturase ([EC:1.3.99.30], CAR1) (Schmidhauser et al., 1990; Hausmann and

Sandmann, 2000). Finally, lycopene beta-cyclase (EC: 5.5.1.19], the other function of *CAR2* product) catalyzes production of cyclic carotenoids such as β-carotene, γ-carotene, Torulene (Figure 1) (Schmidhauser et al., 1994; Díaz-Sánchez et al., 2011).

The research aimed at identifying the targets of naftifine and understanding the mechanism of yeast decoloration through naftifine activations. Up to now, the effect of any antifungal drugs at gene expression level was rarely reported. In order to explore gene candidates that are regulated by naftifine as an antifungal drug, RNA-seq was used to screen the DEGs with focus on DNA replication and pigment synthesis pathways, as well as to quantify the mRNA levels of selected genes in qPCR assay.

MATERIALS AND METHODS

Yeast strain

The *R. mucilaginosa* strain was isolated from the nails of a healthy 41-year-old Chinese man (Idris et al., 2019).

Culture of *R. mucilaginosa*

Culture media: YPD (2% glucose, 2% peptone, 1% yeast extract); SDA agar (4% glucose, 1% peptone, 2% agar). *R. mucilaginosa* was inoculated on SDA plates and incubated at 28°C. Single colonies were picked into YPD medium and incubated 160 rpm at 28°C overnight. Log growth phase yeast was then transferred into 10 ml YPD medium in 50 ml flasks with different concentrations of naftifine. The liquid cultured *R. mucilaginosa* was in the log phase before 36 h, and entered the stationary phase after 40 h (Landolfo et al., 2018). The culture was exposed to lab lights.

Pigment extraction

1.5 ml culture, 10,000 ×g 1 min, mixed with 500 μL 2 mol/L hydrochloric acid, 60 min, boiling water 5 min, 4000 ×g 5 min. The pellet was washed, resuspended in 1 mL acetone and vortexed well for 30 min, 10,000 ×g 1 min (Michelon et al., 2012). The supernatant was used for absorbance measurement in Thermo Scientific™ Multiskan™FC.

RNA-seq

R. mucilaginosa was streaked on SDA agar and cultured at 28°C. There were three samples: "Rh_ctrl" grown for 3 days, "Rh_+naftifine" grown with 200 mg/L of naftifine for 3 days, sample "Rh_-naftifine" grown with 200 mg/L of naftifine for 3 days, followed by naftifine-free for 3 days. The samples were crushed with liquid nitrogen. Total RNA was extracted using ESscience Tissue RNA Purification Kit (ESscience, Shanghai, China) according to the manufacturer's instructions. Nano Drop ND2000® spectrophotometry was used to measure RNA purity. RNA was enriched by oligo (dT) beads, fragmented and reverse-transcribed into cDNA, purified with QiaQuick PCR extraction kit, end repaired, poly (A) added, and ligated to Illumina sequencing adapters. The products were selected by size using agarose gel electrophoresis; PCR amplified, and sequenced using Illumina HiSeqTM2500 by Gene Denovo Biotechnology Co.

Bioinformatics analysis

RNA-seq data was submitted to the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA) database with the accession number: PRJNA590855. Alignment tool, Bowtie2, was used for mapping (rRNA reads were removed). The reconstruction of transcripts was carried out with software Cufflinks together with TopHat2 (version 2.0.3.12). To identify new gene transcripts, all reconstructed transcripts were aligned to reference genome and were divided into twelve categories by using Cuff compare (a method of cufflinks, version 2.2.1). Genes with class code “u” (the transcripts was either unknown or in intergenic spacer region) were defined as novel genes. Novel genes were then aligned to the Nr and Kyoto Encyclopedia of Genes and Genomes (KEGG) database to obtain protein functional annotation. Gene abundances were quantified by software RSEM. The gene expression level was normalized by using FPKM (Fragments Per Kilobase of transcript per Million mapped reads) method. To identify differentially expressed genes (DEGs) across samples, the edgeR package (http://www.r-project.org/) was used. Genes with a fold change ≥ 2 and a false discovery rate (FDR) < 0.05 in a comparison as significant DEGs were identified. DEGs were then subjected to enrichment analysis of Gene Ontology (GO) functions and KEGG pathways.

Real-time qPCR

The qPCR primers were designed using the NCBI primer designing tool (Table 1). Two internal controls were selected, ltv1 (a cytoplasmic protein) and DLD2 (D-lactate dehydrogenase), since their fluctuation in expression level between different samples of RNA-seq was insignificant and their FPKM values were moderate (Van et al., 2017). The qPCR results showed that the relative expression of the internal control genes was stable between the naftifine-treated and control groups.

Yeast was cultured in liquid medium and treated with 4 mg/L naftifine for various days. Total RNA was extracted the same way as in RNA-seq. The RNA samples were reverse-transcribed into cDNA by PrimeScript™ RT reagent Kit (Takara, Dalian, China), and qPCR was performed using SYBR Premix Ex Taq GC kit (Takara, Dalian, China). The cycles were: 95°C 2 min, 95°C 20 s, 60°C 20 s, 72°C 15 s, 39 cycles. Dissolution curve conditions were: 65°C 5 s, 95°C 5 s, 4°C 30 s. Each sample was processed in triplicates using the CFX-96 TouchTM Real-Time PCR Detection System (BioRad, USA). In calculating the relative expression level of *CAR1* and *CAR2* genes, the two internal controls were used to calculate their ΔΔct values, and average with $2^{-\Delta\Delta Ct}$ method.

Data analysis software

GraphPadPrism7 was used to calculate IC_{50}. Bio-Rad CFX Manager and GraphPadPrism7 software were used for qPCR analysis and plotting.

RESULTS

Decoloration of *R. mucilaginosa* was induced by low concentration of naftifine

The drug sensitivity of the selected *R. mucilaginosa* strain was first examined. The 50% inhibiting concentration (IC_{50})

Table 1. RT-qPCR primer design.

Gene name	Sequence (5' - 3')	PCR product size (bp)	Annealing temp (℃)
DLD (internal control 1)	Forward primer : GGCTACTCCAAGACGGAACC Reverse primer : TCACTCTGGAACGGCAACTC	125	60
ltv1 (internal control 2)	Forward primer : CCGGAACACCACGATGAAGA Reverse primer : AGTCGCCGGTTGTGAGATTT	149	60
CAR1	Forward primer :CGTGACGGTCCTCGAAAAGA Reverse primer : GTCCTTGAACGCCTCCTCAA	136	60
CAR2	Forward primer : TCCGAGTTCCTCCCGATTCT Reverse primer : CTCTTCGAATGCGCCGAAAG	104	60

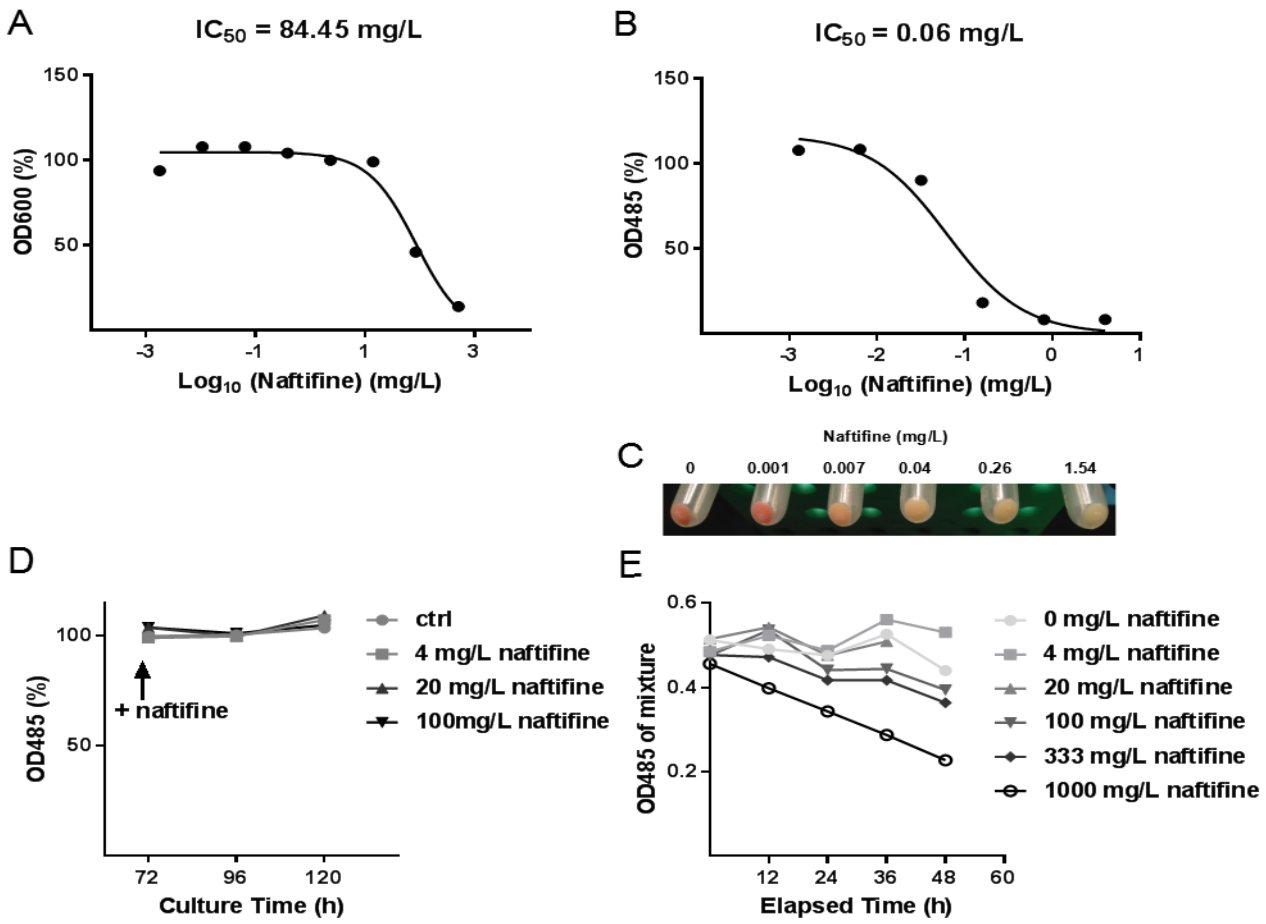

Figure 2. (A) 50% inhibitory concentration (IC_{50}) of naftifine in inhibiting yeast growth. (B) 50% inhibitory concentration of naftifine in inhibiting pigmentation. (C) Decoloration with different concentrations of naftifine. (D) Absorbance of pigment extract with naftifine added at stationary phase (day 3) for 1-2 day treatment. (E) Time-elapsed absorbance of pigment extract mixed with different concentrations of naftifine.

of allylamine antifungals for growth was: naftifine (IC_{50} = 84.45 mg/L) (Figure 2A), butenaphthol (IC_{50} = 16.75 mg/L), terbinaphthol (IC_{50} = 6.38 mg/L). The concentration of naftifine in inhibiting pigmentation was IC_{50} = 0.06 mg/L (Figure 2B and C), which was far lower than its growth inhibitory concentration (about 1, 400-fold lower). Remarkably, butenaphthol and terbinafine, the naftifine analogs, did not decolorize *R. mucilaginosa* at the same

Table 2. Differentially expressed genes between three groups.

Sample comparison	Number of genes up-regulated	Number of genes down-regulated	Total
Rh_+naftifine vs Rh_ctrl	975	279	1254
Rh_-naftifine vs Rh_ctrl	268	150	418
Rh_-naftifine vs Rh_+naftifine	417	885	1302

or even higher mass concentrations even though their growth inhibitory IC_{50} is lower than naftifine's. Based on the dramatic difference in IC_{50}, the mechanisms of growth inhibition and decoloration through naftifine treatments were likely independent. In addition, decoloration depends on the yeast growth phase. No decoloration was observed when 4 mg/mL of naftifine was added to the yeasts that already grew in liquid medium for 3 days, not even with much higher concentrations (Figure 2D).

Naftifine did not accelerate pigment degradation *in vitro*

Next, the mechanism of decoloration induced by naftifine was studied through assessing the possibility that naftifine accelerated pigment degradation. The hypothesis is that whether pigments are prone to degradation when they interact with naftifine. Pigment extract from yeast was mixed with various concentrations of naftifine and monitored for 48 h for light absorbance by a spectrophotometer. Results showed that only at extremely high concentration of 1, 000 mg/L, naftifine could reduce light absorbance at a significant level (Figure 2E). There was no significant acceleration of pigment degradation with concentration up to 100 mg/L, that is > 1, 000-fold of the decoloration IC_{50}. The results suggested that the major yeast decoloration was not due to naftifine-facilitated pigment degradation.

RNA-seq identified the candidate genes in carotenoid biosynthesis pathway

It is reasonable to hypothesize that decoloration might be due to the decrease of carotenoid synthesis. In order to screen candidate DEGs, *R. mucilaginosa* was cultured on SDA plates for different treatment scenarios for 3 days, which yielded three samples for RNA-seq analysis (Rh_ctrl, Rh_+naftifine, and Rh_-naftifine, see Materials and Methods). By aligning with the reference (JGI Rhomuc1_GeneCatalog_20160519), a total of 7, 618 known genes and 98 new genes were identified from these three samples (Table 2). Results indicated that none of the new genes was among carotenoid, isoprene, and steroid biosynthesis pathways based on annotation.

Gene Ontology function analysis showed that DEGs were significantly enriched in terms of both DNA replication and protein-DNA complex (Figure 3A). KEGG pathway analysis showed that DEGs between Rh_ctrl and Rh_+naftifine were enriched in DNA replication, material metabolism (especially fatty acid metabolism), and oxidation pathways (Figure 3B). Out of the expectation, no DEGs were found in the steroid biosynthetic pathway. However, two genes of carotenoid synthesis, *CAR1* and *CAR2*, were marginally at the higher expression levels after 3 days' naftifine treatments. Since *CAR1* and *CAR2* were found to play important roles in carotenoid synthesis in *R. mucilaginosa* (Figure 1) (Landolfo et al., 2018), their expressional regulations were further studied in qPCR assay.

Real-time qPCR showed down-regulation of *CAR1* gene expression after naftifine treatment

The above RNA-seq analysis was from solid culture and the concentration of nafitifine was higher than growth inhibitory IC_{50}. To focus on studying the effect of naftifine on decoloration and to investigate the effect at a time-dependent manner, we switched to liquid culture and used a much lower concentration (4 mg/ml vs 200 mg/ml) for real-time qPCR assay. At 4 mg/ml, nafitifine decolorized yeast, but had little effect on growth. After treatment for 1 day, the expression level of *CAR1* gene decreased to 37% when compared to control (Figure 4A, B). The t-test of three experiments showed the reduction was significant ($p = 0.007$). The expression was also reduced after treatment for 3 days, but not statistically significant. Similarly, the reduction of expression for CAR2 was also not statistically significance after treatment for 1 or 3 days. After treatment for 5 days, both genes showed no change in relative expression level. Since decoloration was not detectable anymore if naftifine was not introduced until the yeast had grown for 3 days and afterward (Figure 2D), whether the *CAR1* down-regulation was also dependent of yeast growth phase in a similar way of decoloration dynamics was further analyzed. Results of real-time qPCR assay indicated that expression levels of both *CAR1* and *CAR2* did not change after 1-day's naftifine treatment when yeast had reached stationary phase (Figure 4C). This result further supported that

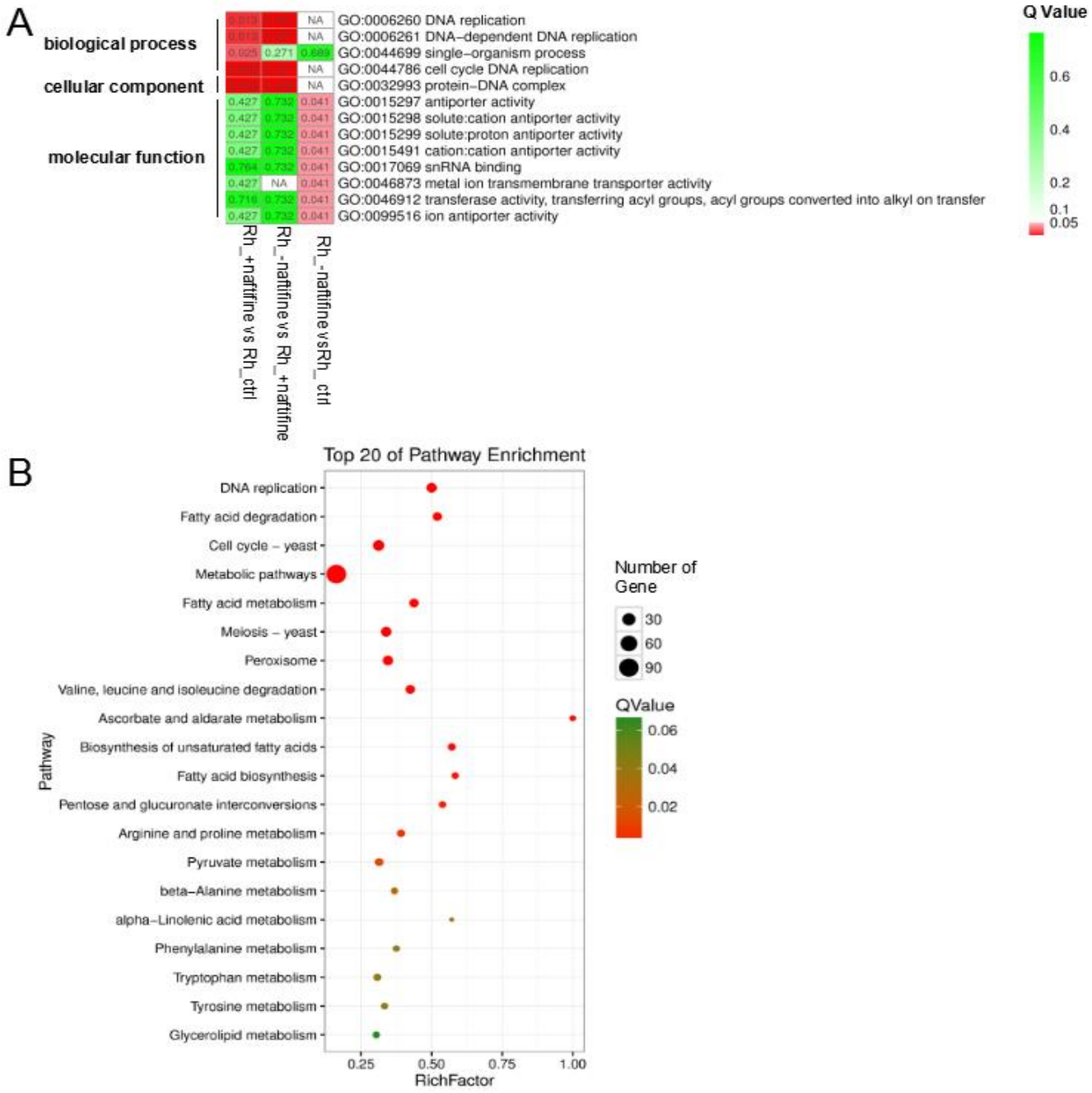

Figure 3. Enrichment analysis of differentially expressed genes: (A) Most significant Gene Ontology terms in molecular function, cellular component and biological process, evaluated by Qvalue. (B) Top 20 biological functions of KEGG between ctrl and + naftifine, evaluated by Qvalue.

decoloration correlated to *CAR1* down-regulation.

Bioinformatical analysis on inhibition of phytoene desaturase activity by naftifine

In addition to analysis of the effect on gene expressions, further evaluation was done to know whether naftifine acted on their protein levels. Since naftifine was a potent inhibitor of CrtN (diapophytoene desaturase) in *S. aureus*, homologous proteins of CrtN in *R. mucilaginosa* was searched. Interestingly, phytoene desaturase encoded by *CAR1* had the highest similarity with CrtN (Figure 5). Functionally, both of them are a membrane-peripheral and

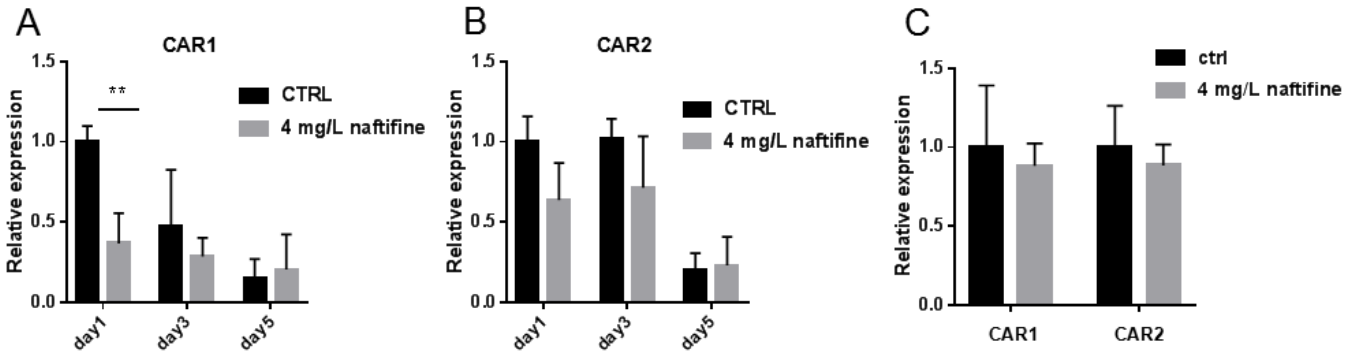

Figure 4. (A), (B) Relative expression levels of *CAR1* and *CAR2* by qPCR with naftifine treatment for 1, 3, 5 days starting at day 0. (C) Relative expression levels of *CAR1* and *CAR2* by qPCR with naftifine added at stationary phase for 1 day treatment.

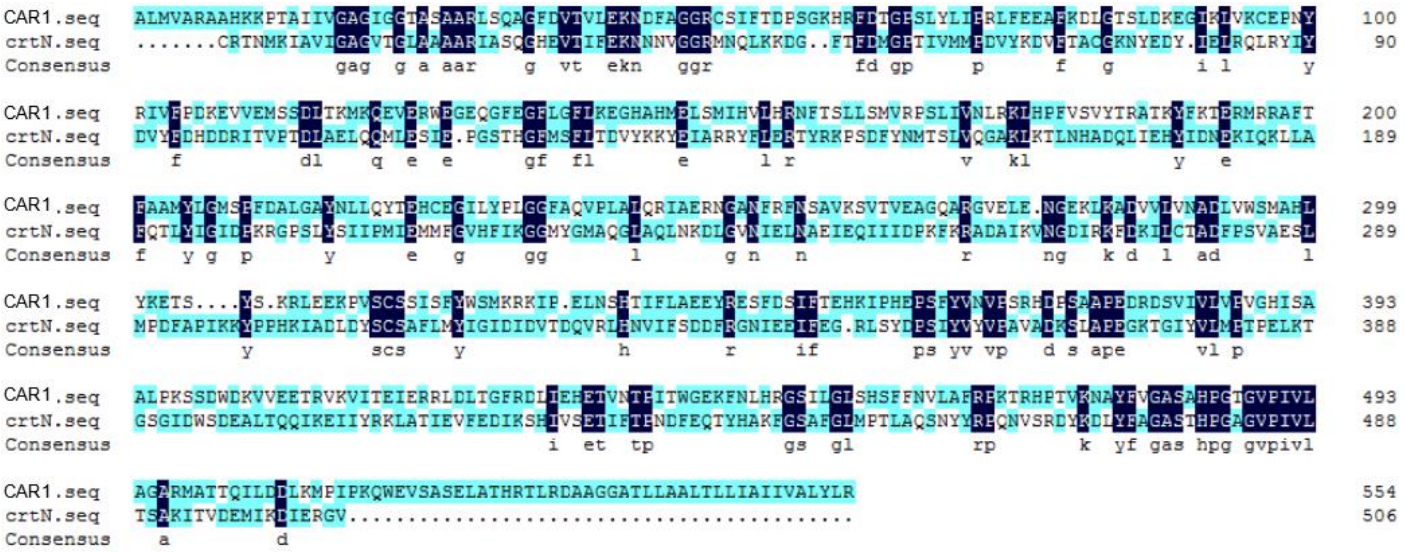

Figure 5. Sequence alignment of yeast lycopene desaturase and *S.aureus* CrtN protein.

FAD-dependent oxidase/isomerase that catalyzes the formation of multiple unsaturated double bonds of carotenoids (Schaub et al., 2012). Based on their high sequence homology, the results suggested that naftifine involved into the process of inhibiting the activity of phytoene desaturase in yeast.

DISCUSSION

Many non-phototrophic bacteria and fungi rely on carotenoids for protection from harmful radicals (Chi et al., 2015; Llansola et al., 2017). In humans, carotenoids are precursors of vitamin A, an effective antioxidant supplied from food (Bohn et al., 2017). As a non-photosynthetic fungus, *R. mucilaginosa* is a carotenoid producer and is protected by carotenoids against oxidative damage from UVB (Moliné et al., 2009, 2010). This study focused on understanding the mechanism of yeast decoloration by naftifine. Pigmentation was reduced when low concentration of naftifine was added to early phase yeast (Figure 2B). For the first time, it was shown further that the reduction was not due to faster degradation in the presence of naftifine. No significant change happened in degradation rate when pigment extract was mixed with up to 333 mg/L naftifine, > 1, 000 fold higher than IC_{50}.

For decolorization in *R. mucilaginosa*, naftifine is much more potent (IC_{50} = 0.30 umol/L) than drug diphenylamine (IC_{50} = 20 umol/L) (Raisig and Sandmann, 2001; Ghannoum et al., 2013; Mot et al., 2017). Diphenylamine reduces carotenoid accumulation by inhibiting desaturation of phytoene. Naftifine may reduce carotenoid levels partially by inhibiting phytoene desaturase in similarity to diphenylamine. Among annotated proteins of

R. mucilaginosa, the phytoene desaturase has the highest homology in sequence with bacterial CrtN, a desaturase inhibited directly by naftifine in bacteria. However, the change of carotenoid level under naftifine treatment was not measured directly in this study. The results suggested that de-pigmentation by naftifine was largely due to regulation at gene expression level of *CAR1*. It was expected that diphenylamine is unlikely down-regulating *CAR1* gene expression since it is much less potent in decoloration.

The study indicated that RNA-seq was an effective method to successfully screen DEGs. Naftifine regulated the expression of genes related to DNA replication and metabolism (Figure 3A and B). Interestingly, *CAR1* and *CAR2* had a slightly higher FPKM values, suggesting that naftifine might regulate gene expression in carotenoid synthesis pathway. To further study naftifine regulation in gene expression, real-time qPCR was used to specifically quantify relative gene expression levels of *CAR1* and *CAR2* with treatment of lower naftifine concentration in liquid culture. Naftifine down-regulated the relative expression of *CAR1* to 37% of control level after one-day treatment. Apparently, the *CAR1* down-regulation was not related to the effect of naftifine in yeast growth. We further suspect that the other antifungal drugs without inducing de-pigmentation have no regulating effect in *CAR1* expression. In conclusion, yeast decoloration by naftifine might be large through down-regulation of *CAR1* expression.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Antibacterial resistance patterns of bacteria isolated from clinical specimens at Uttara IbnSina Diagnostic Centre, Dhaka

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Nowadays, antibiotic resistance is a global public health threat. Bangladesh is accelerated to this owing to its sub-standards healthcare along with the self-medication and overuse of antibiotics. The study aimed to assess patterns of antibacterial resistance in the clinical samples. The study was carried out at Ibn Sina Diagnostic and Consultation Center Uttara, Dhaka, from January to December 2019. All cultures and antimicrobial susceptibility test results of patients were extracted from laboratory records, using a semi-structured checklist. Data were analyzed using Microsoft Excel and SPSS version 20.0. To ensure confidentiality coding was used instead of the patient's identity. A total of 925 culture-positive results were analyzed, of which blood 620(65.0%) and urine 297(32.1%) samples were commonly diagnosed. The most frequently isolated bacterial were *Salmonella* spp. [601(65%)], *Escherichia coli* [244(26.4%)] and *Klebsiella spp.* [57(6.16%)]. The majority of the patients were females [540(58.4%)]. *E. coli* was found to be highly sensitive (>80%) to nitrofurantoin, meropenem, amikacin, amoxiclav, and imipenem; simultaneously, resistant (>45%) to cefixime, cephalexin, piperacillin, aztreonam, ampicillin, cefuroxime, and ciprofloxacin. *S. typhi* and *S. paratyphi* were sensitive (>80%) for cefepime, ceftriaxone, imipenem, tetracycline, cefixime, ceftazidime, cephalexin, cotrimoxazole, aztreonam, cefuroxime, and amoxiclav; concurrently, above 80% resistance for ciprofloxacin, azithromycin, gentamycin, and ampicillin. Overall, most of the isolates showed a significant rising rate of microbial resistance to ciprofloxacin, azithromycin, piperacillin, cephalexin, gentamycin, and ampicillin. The study findings revealed gradually rising rates of antibiotic resistance to commonly prescribed antibiotics. The study suggested the prescribers should be avoided overuse and irrational use of drugs to reduce antimicrobial resistance.

Key words: Bacteriology, antibiotic susceptibility, clinical samples, Dhaka.

INTRODUCTION

Antibiotic resistance is a well-known public health concern at the community, national and global levels (Nordberg et al., 2004). Decreasing the effectiveness of antibiotics in treating bacterial common infections and a decline in the new drug development rate is a concerning issue (Kandelaki et al., 2015; Luepke and Mohr, 2017; Spellberg et al., 2004). Antibiotic resistance poses a significant risk of mortality and economic burden

worldwide (Ahmed et al., 2019). The causes of antibiotic resistance are complex which include enzymatic degradation of antibacterial drugs, alteration of bacterial proteins that are antimicrobial targets, and changes in membrane permeability to antibiotics (Kandelaki et al., 2015). The low- and middle-income countries are more affected because of extensive misuse of antibiotics, non-human antibiotic use, poor quality of drugs, insufficient surveillance, and other factors associated with poor healthcare standards, malnutrition, chronic and repeated infection, unaffordability of more effective and costly drugs (Ayukekbong et al., 2017; Sosa et al., 2010). In 2014, the World Health Organization (WHO) reported on global surveillance of antimicrobial resistance, significant gaps prevail in surveillance, absence of standards methodology, data sharing and coordination. WHO identified the major gaps in the South-East Asia Region, the African Region, and the Eastern Mediterranean region (WHO, 2014).

Bangladesh is one of the South-East Asian developing countries and has a high rate of antibiotic resistance which poses a regional and global concern (Rahman and Huda, 2014). Enteric fever caused by salmonella spp. has been detected among children aged <5 years of age than the age group ≥5 years in the South-East Asian especially in India and Bangladesh. Though, there are no valid data regarding paratyphoid fever in Bangladesh (Naheed et al., 2010). Therapeutic failures in Bangladesh are not uncommon. Multiple studies have demonstrated irrational antibiotic prescribing by physicians, self-medication habits of patients, and indiscriminate use of antibiotics in agriculture and farming in different segments of the country (Biswas et al., 2014a; Biswas et al., 2014b; Sutradhar et al., 2014). Therefore, the prevalence of antibiotic resistance in Bangladesh is high, but no attempts have been undertaken to alleviate it. This study aims to serve as a reference for future works and to guide policymakers and prescribers to adopt the best strategy to lower the extent of antibiotic resistance as well as combat the problems following the expanding resistance.

METHODOLOGY

Study design and setting

This retrospective cross-sectional study was executed from January to December 2019 at IbnSina Diagnostic and Consultation Center Uttara, Dhaka. The sample was collected by using a sterile ascetic technique. A total of 925 culture-positive test result samples were analyzed. All cultures and antimicrobial susceptibility test results of patients were extracted from laboratory records notebook by using a semi-structured checklist. The sample-set included blood, urine, stool and sputum samples as well as wound swabs.

Bacterial isolates and identification

All of the received clinical specimens were initially cultured and subcultures into brain heart infusion, blood agar, Salmonella-Shigella agar, Chocolate agar and Mac-Conkey agar as per need, and after overnight incubation at 37°C, the bacteria identification was completed by gram staining as well as standard biochemical tests (catalase, coagulase, oxidase). This was done by sub-culturing on mediums such as triple sugar iron agar (TSI), SIM medium, and Simmons' citrate agar.

Antimicrobial susceptibility tests

The antimicrobial sensitivity tests of the isolated bacteria were performed by using the Kibry Bauer disk diffusion test on Mueller-Hinton agar (Bauer et al., 1966). The antibiotics agents used were: tetracycline (30 µg), nitrofurantoin (300 µg), azithromycin (15 µg), gentamicin (10 µg), ciprofloxacin (5 µg), doxycycline (30 µg), cotrimoxazole (25 µg), imipenem (10 µg), ceftriaxone (30 µg), ceftazidime (30µg), cefepime (30µg), meropenem (10µg), ampicillin (10 µg), penicillin (g) (10 µg). cefixime (5µg), cephalexin (30µg), piperacillin (75 µg), aztreonam (30 µg), cefuroxime (30 µg) amikacin (30µg), amoxiclav (30µg) vancomycin (30µg) fusidic acid (10µg) and cloxacillin (30 µg). The pattern of sensitivity and resistance was interpreted according to the guideline of the National Committee for Clinical Laboratory Standards (NCCLS) (National Committee for Clinical Laboratory Standards and Barry, 1999).

Statistical analysis

The data were entered into Microsoft Excel and analyzed by SPSS version 20. The results were presented as descriptive statistics in terms of relative frequency, percentage, mean ± standard deviation (SD) and to summarize patients' attributes and other related information.

Ethical considerations

Ethical approval was obtained from the Institutional Review Board. Administrative authorization for this study was obtained from the Branch Manager of the Diagnostic Center. The researchers highly consider the human right of the participants. To ensure the confidentiality coding method was used instead of other identifiers of the patients.

RESULTS

A total of 925 samples were analyzed; 32.1% presented urine, followed by stool (0.5%), blood (67%), sputum (0.2%) and wound swab (0.1%) (Figure 1). Among them males were 385 (41.6%) and females were 540 (58.4%). The ages of the patients ranged from 3 months to 90 years

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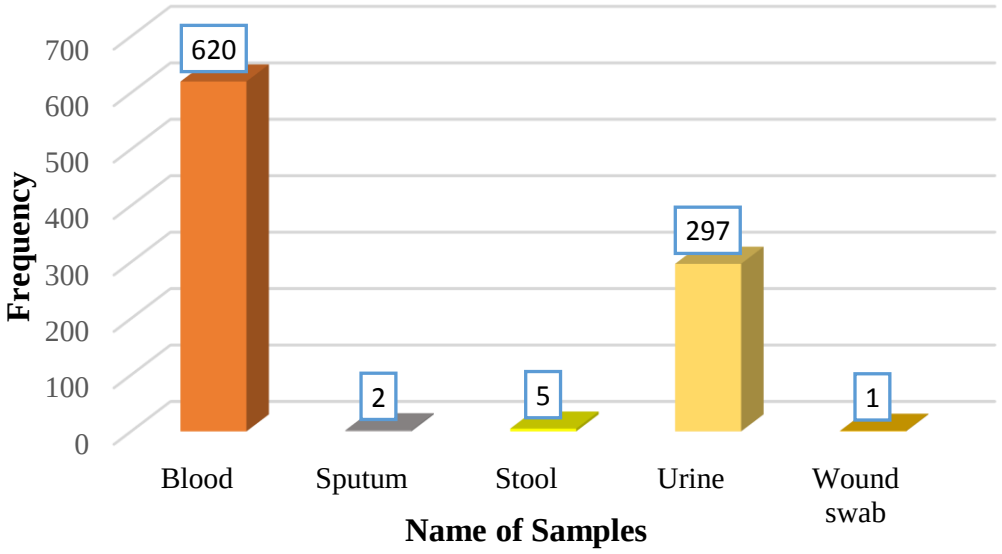

Figure 1. Types of samples.

Table 1. Age and sex status of patients.

Age and sex status of patients	Frequency	Percentage
Age of the patients (years)		
<10	193	20.9
10-19	201	21.7
20-29	209	22.6
30-39	115	12.4
>40	207	22.4
Sex of the patients		
Male	385	41.6
Female	540	58.4
Total	925	100.0

with a mean age of 27 years. The infection was most common among age groups of above >40 years (22.4%) followed by age groups of 20-29 years (22.6%) (Table 1). The set of most frequently isolated organisms included Salmonella spp., 601(65.0%); *Escherichia coli* were,244(26.4%) and *Klebsiella spp*. and *Staphylococcus aureus*, 57(6.16%) and 15(1.62%) respectively (Figure 2 and Table 2).

The sensitivity pattern of *E. coli* shows that the microbes were highly (>80%) sensitive for imipenem, nitrofurantoin, gentamycin, meropenem, amikacin, and amoxiclav. *E. coli* is booming developed resistance (>45%) for some antibiotics such as cefixime, cephalexin, piperacillin, aztreonam, ampicillin, cefuroxime, and ciprofloxacin. The microbes *Salmonella* spp. (*S. typhi* and *S. paratyphi)* were more than 80% sensitive tocefepime, ceftriaxone, imipenem, tetracycline, cefixime, ceftazidime, cephalexin, gentamycin, cotrimoxazole, aztreonam, ampicillin, cefuroxime, amikacin, and amoxiclav. At the same time above 80% had developed resistance to ciprofloxacin and azithromycin. *S. typhi* has developed (>20%) resistant to several antibiotics like ampicillin, piperacillin, and cotrimoxazole.

Klebsiella spp. was above 80% sensitive tocefepime, ceftriaxone, imipenem, gentamycin, meropenem, amikacin, and amoxiclav. This bacteriumhas developed significant resistance (>40%) to some antibiotics such as ciprofloxacin, piperacillin, cephalexin, and cefepime. *Staphylococcus aureus* was highly (>80%) sensitive tocotrimoxazole, gentamycin, amikacin, doxycycline, vancomycin, fusidic acid, cloxacillin. *S. aureus* developed resistance for ciprofloxacin, penicillin, and azithromycin. *Pseudomonas* spp. was substantially sensitive to cefepime, imipenem, ceftazidime, piperacillin,

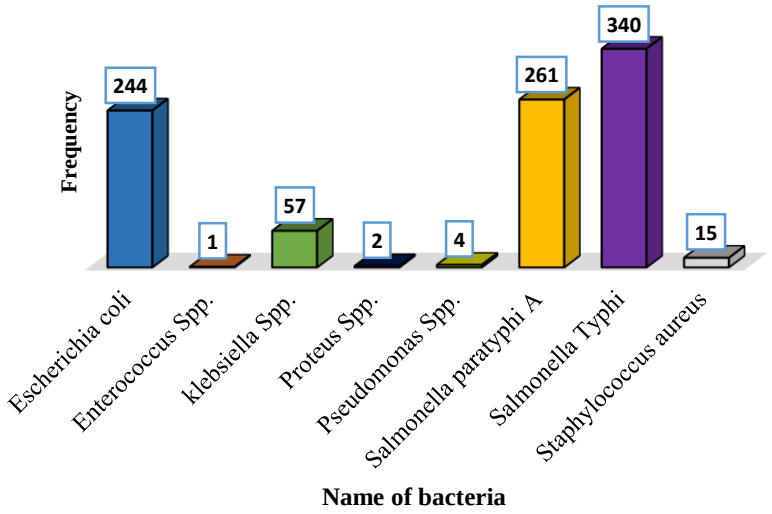

Figure 2. Name of isolated bacteria.

Table 2. Distribution of bacteria among sex of patients.

Name of bacteria	Sex	
	Male	Female
E. coli	40	204
Enterococcus spp.	0	1
Klebsiella Pneumoniae	1	0
Klebsiella spp.	6	51
Proteus spp.	1	1
Pseudomonas spp.	2	2
Salmonella paratyphi A	145	116
Salmonella typhi	184	156
Staphylococcus aureus	6	9
Total	385	540

gentamycin, and amikacin. These bacteria developed resistance to aztreonam, cefepime, and tetracycline. *Proteus* spp. was 100% sensitive tocefepime, imipenem, cotrimoxazole, piperacillin, amikacin, gentamycin, meropenem, amikacin, and amoxiclav. Of the resistance to ceftazidime and cefixime, throughout the study period, only two samples were positive *Proteus* spp. *Enterococcus* spp. was 100% resistance tocefepime, ceftriaxone, imipenem, cotrimoxazole, tetracycline, cefixime, ceftazidime, gentamycin, amikacin and meropenem and resistive for piperacillin, ampicillin, azithromycin. Though, throughout the study period, we have found a single culture positive of *Enterococcus* spp. (Table 3).

DISCUSSION

Bacterial infections are the predominant problem in developing countries like Bangladesh where water, sanitation, and hygiene (WASH) continue to be below international standard. The shortage of reliable microbial and antimicrobial data is also a problem in managing the physicians treating patients with a bacterial infection before the appropriate treatment is applied to get the best outcome (Tjaniadi et al., 2003). The major cause behind antibiotic resistance makes the bacteria to be smart. However, in Bangladesh, prescribers usually diagnose microbial infection based on clinical finding and choose antimicrobial drugs on an experiential basis (Faiz and Rahman, 2004), which critically distresses the sensitivity pattern of microorganisms. Besides, the unwillingness of the policymakers and officials to sanction law to overcome insufficient guidelines and instruction to control antimicrobial prescription and administration leads to the deteriorating of the circumstance.

In the present study, female patients were found to be higher than the males as found in other studies (Derbie et

Table 3. Pattern of antibiotic resistance among isolated bacteria.

Antimicrobials		Bacterial isolates							
		E. coli **(n-244)**	***Salmonella Typhi*** **(n=340)**	***Salmonella paratyphi A*** **(261)**	***Staphylococcus aureus*** **(n=15)**	***Klebsiella*** **spp. (n=57)**	***Pseudomonas*** **spp. (n=4)**	***Proteus*** **spp. (n=2)**	***Enterococcus*** **spp. (n=1)**
Cefepime	S	129(73.30)	285(99.65)	217(83.1)	ND	38 (82.60)	3 (75.0)	1 (100)	0
	R	47(26.70)	1(0.35)	44(16.9)	ND	8 (17.40)	1 (25.0)	0	1 (100)
Ceftriaxone	S	156(63.93)	339(99.71)	259(99.2)	0	38 (82.60)	ND	1 (50)	0
	R	88(36.07)	1(0.29)	2(0.8)	1 (100)	8 (17.40)	ND	1 (50)	1 (100)
Imipenem	S	225(94.94)	331(97.35)	251(96.2)	1(100)	51 (89.47)	3 (100)	2 (100)	0
	R	12(5.06)	9(2.65)	10(3.8)	0	6 (10.53)	0	0	1 (100)
Nitrofurantoin	S	203(88.26)	ND	ND	1 (100)	44(78.57)	ND	2 (100)	1 (100)
	R	27(11.74)	ND	ND	0	12(21.43)	ND	0	0
Tetracycline	S	156(65.27)	332(96.51)	244(93.5)	0	39 (68.42)	ND	1 (50)	0
	R	83(34.73)	13(3.78	17(6.5)	1 (100)	18 (31.58)	ND	1 (50)	1 (100)
Cefixime	S	132(54.32)	338(99.41)	258(98.9)	0	33(57.89)	ND	0	0
	R	111(45.68)	2(0.59)	3(1.1)	1 (100)	24(42.11)	ND	2 (100)	1 (100)
Ceftazidine	S	175(73.53)	338(99.71)	258(98.9)	1 (100)	33(63.46)	3 (75)	0	0
	R	63(26.47)	1(0.29)	2(1.1)	0	19(36.54)	1 (25)	2 (100)	1 (100)
Cephalexin	S	127(53.59)	331(98.51)	250(97.7)	0	32(56.14)	ND	1 (50)	0
	R	110(46.41)	5(1.29)	6(2.3)	1(100)	25(43.86)	ND	1 (50)	1 (100)
Cotrimoxazole	S	149(61.07)	263(77.35)	227(87.3)	9 (90)	42(73.68)	ND	2 (100)	0
	R	95(38.93)	77(22.65)	33(12.7)	1 (10)	15(26.32)	ND	0	1 (100)
Piperacillin	S	117(49.37)	262(78.68)	216(86.4)	1 (100)	33(57.89)	3 (100)	2 (100)	1 (100)
	R	120(50.63)	71(21.32)	34(13.6)	0	24(42.11)	0	0	0
Aztreonam	S	126(54.31)	324(97.30)	251(96.5)	0	35(62.5)	2 (50)	0	0
	R	106(45.69)	9(2.70)	9(3.5)	1 (100)	21(37.5)	2 (50)	2 (100)	1 (100)
Ampicilin	S	97(40.76)	272(80.47)	239(91.9)	1 (100)	10(17.5)	ND	2 (100)	1 (100)
	R	141(59.24)	66(19.53)	21(8.1)	0	47(82.45)	ND	0	0
Cefuroxime	S	135(56.72)	333(98.23)	251(96.2)	1 (100)	37(64.91)	ND	1 (50)	0
	R	103(43.28)	6(1.77)	10(3.8)	0	20(35.8)	ND	1 (50)	1 (100)
Ciprofloxacin	S	132(55.0)	49(14.50)	30(11.5)	1 (7.70)	32(57.14)	3 (100)	1 (50)	0
	R	108(45.0)	289(85.50)	231(88.5)	12 (92.30)	24(42.85)	0	1 (50)	1 (100)
Gentamycin	S	205(85.42)	337(99.12)	260(99.1)	13 (100)	51(91.07)	4 (100)	2 (100)	0
	R	35(14.58)	3(0.88)	1(0.9)	0	5(8.92)	0	0	1 (100)
Meropenem	S	230(96.23)	ND	ND	1 (100)	49(87.5)	ND	2 (100)	0
	R	9(3.77)	ND	ND	0	7(12.5)	ND	0	1 (100)
Amikacin	S	215(92.27)	336(99.41)	257(98.5)	13 (92.85)	46(93.87)	4 (100)	2 (100)	0

Table 3. Contd

Amoxyclav	R	18(7.73)	2(0.59)	4(1.5)	1 (7.15)	3(6.13)	0	0	1 (100)
	S	183(78.54)	325(97.31)	255(98.1)	1 (100)	42 (85.71)	ND	2 (100)	1 (100)
Azithromycin	R	50(21.46)	9(2.69)	5(1.9)	0	7 (14.29)	ND	0	0
	S	1(50.0)	118(34.71)	222(85.5)	4(30.76)	0	ND	ND	1 (100)
Doxycycline	R	1(50.0)	222(65.29)	39(14.9)	9(69.24)	2 (100)	ND	ND	0
	S	ND	ND	ND	12(85.71)	ND	ND	ND	ND
Vancomycin	R	ND	ND	ND	2(14.29)	ND	ND	ND	ND
	S	ND	ND	ND	12(100)	ND	ND	ND	ND
Fusidic acid	R	ND	ND	ND	0	ND	ND	ND	ND
	S	ND	ND	ND	11(84.61)	ND	ND	ND	ND
Cloxacillin	R	ND	ND	ND	2 (15.39)	ND	ND	ND	ND
	S	ND	ND	ND	10(100)	ND	ND	ND	ND
Penicillin	R	ND	ND	ND	0	ND	ND	ND	ND
	S	ND	ND	ND	7(53.84)	ND	ND	ND	ND
	R	ND	ND	ND	6(46.16)	ND	ND	ND	ND

S- Sensitive, R- Resistant , ND - Not Done.

al., 2017; Tahira and Singh, 2017; Kolawole et al., 2009). The females were more infected than males due to their physiological and anatomical differences (Kibret and Abera, 2014). The current study revealed that *Escherichia coli* was highly sensitive (>80%) to imipenem, nitrofurantoin, gentamycin, meropenem, amikacin, and amoxiclav. The susceptibility pattern of *E. coli for* nitrofurantoin was (89.7%), which compares to a study done in London (Bean et al., 2008). *E. coli* is developing resistance (>45%) to some antibiotics such as cefixime, cephalexin, piperacillin, aztreonam, ampicillin, cefuroxime, and ciprofloxacin. Ahmed et al. (2019) showed that *E. coli* was highly resistance to commonly used antibiotics like ampicillin (94.6-100%), amoxiclav (67.1-85.5%), ciprofloxacin (65.2-80.5%) and cotrimoxazole (72-82.2%). Patil and Mule described the isolates of *E. coli* were resistant to ampicillin (96.6%), tetracycline (79%), ceftriaxone (62%) and gentamicin (51.7%) (Patil and Mule, 2019).

Salmonella spp. showed (>80%) sensitive to cefepime, ceftriaxone, imipenem, tetracycline, cefixime, ceftazidime, cephalexin, gentamycin, cotrimoxazole, aztreonam, ampicillin, cefuroxime, amikacin, and amoxiclav. This result is comparable with Ahmed et al. (2019) who demonstrated *Salmonella spp.* was highly sensitive to cefixime and ceftriaxone. *Salmonella typhi* developed resistance to ciprofloxacin and azithromycin. Ebrahim et al. (2016) showed that *Salmonella typhi* has developed resistance from 2003 to 2014 from 0% to 14% in Canada. The previous literature showed Salmonella isolates had 100% sensitivity to ceftriaxone and cefixime (Bhan et al., 2005; Bhetwal et al., 2017; and Mule, 2019).

Klebsiella spp. was above 80% sensitive to cefepime, ceftriaxone, imipenem, gentamycin, meropenem, amikacin, and amoxiclav. These bacteria developed significant resistance (>40%) to some antibiotics like ciprofloxacin, piperacillin, cephalexin, and cefepime. *Klebsiellaspp*.

Sensitive to Meropenem (100%), Nitrofurantoin (83.3%), whereas it is resistant to Penicillin (100%), Ampicillin (93.61%) and intermediate to Gentamicin (18.5%), Augmentin (17.4%) (Ahmed et al., 2019). Antimicrobial sensitivity pattern reveals the development of resistance to common antibiotics, which is comparable with that of previous studies (Akond et al., 2009; Rogers et al., 2011). However, antibiotics like ciprofloxacin, ceftazidime, ampicillin, piperacillin, cotrimoxazole, and ceftriaxone have become ineffective because of the beginning of the resistance. The microbes are still sensitive to imipenem, azithromycin, vancomycin and amikacin, nitrofurantoin, gentamycin, meropenem, and amoxiclav. This outline of sensitivity does not compare with

previous findings (Hasan et al., 2011). Prescribers cited diagnostic insecurity and advent of resistance as pediment reasons for prescribing antimicrobials; nevertheless some of them revealed the possibility of losing patients as one reason (Rahman and Huda, 2014).In this study, we found a very small proportion of *S. aureus* 15(1.7%), *Pseudomonas spp*. 4(0.43%), *Proteus* spp. 2(0.21%), *Enterococcus* spp. 1(0.10%). Therefore, we did not compare it with other studies.

Conclusion

The present study revealed that *E. coli, salmonella typhi,* and *salmonella paratypi* were the most frequently isolated bacterial in the clinical samples. The majority of the isolated bacteria showed certain levels of antimicrobial resistance to commonly recommended drugs like ampicillin, norfloxacin, ciprofloxacin, azithromycin, cephalexin, piperacillin and cotrimoxazole. However, strict policy and appropriate use of antibiotics can assuage the burden of antimicrobial resistance. It is highly suggested to perform antimicrobial susceptibility testing before the administration of antibiotics and ensure the rational use of drugs to reduce antibiotic resistance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Occurrence of multidrug-resistant bacteria in aquaculture farms in Côte d'Ivoire (West Africa)

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Aquaculture provides a significant proportion of the fish consumed around the world. In West Africa, aquaculture is an important economic sector. However, several diseases with high fish mortality are caused by bacterial infections. Due to the lack of surveillance in aquaculture, this study investigated the presence of bacteria in fish farms. The purpose of the study was to isolate bacteria in aquaculture (Ivory Coast). Two hundred and forty fishes and water samples were collected from the pond of two fish farms. Fish was scraped, then, dissected to collect their gills and intestines. Bacterial culture was done for the detection of many species. Isolate identification was done using biochemical tests (API20E) and MALDI-TOF tests. Also, 1696 bacteria strains were isolated, 70.9% of strains were from the fish organs and 29,1% from the water samples. The higher colonization rate was observed in water and on fish's surface. No statistical difference was observed between the two farms. Seven major species were isolated in both farms: *Escherichia coli, Pseudomonas aeruginosa, Enterobacter hormaechei*, *Enterococcus faecalis, Citrobacter freundii, Morganella morganii and Bacillus cereus*. The major isolated strains were *Enterobacter hormachaei*, *Enterococcus faecalis* and *Escherichia coli*. Multi-resistance for 3 classes of antibiotics was observed in some of those strains. This investigation shows microbiological risks for aquatic animals and humans who are in interaction with fish farms.

Key words: Aquaculture, multidrug-resistant strains, fish, West Africa.

INTRODUCTION

Aquaculture belongs to the food industry with an average growth rate of 6.2% in the past years (FAO, 2016). It is an activity that contributes to the production of foods of high nutritional value, generating employment and economic income for the world population (Gabriel et al., 2007). In addition, it strengthens the source of inputs for the food industry and foreign exchange for the country (Kouadio et al., 2019). In Africa, particularly in Côte d'Ivoire, aquaculture has an important place in the food industry and for consumers (Koumi et al., 2017;

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Figure 1. *Oreochromis niloticus* organs collected in two aquaculture farms of Dabou, West Africa, Côte d'Ivoire; **a:** Whole fish (*Oreochromis niloticus);* **b:** Swabs on the surface of fish; **c:** Gills; d: Intestine.

Weichselbaum et al., 2013; Yao et al., 2017). In all aquaculture operations in the country, Tilapia is the major specie reared with frequencies depending on the system. One of the biggest threats to the aquaculture sector is infectious diseases (Abowei and Briyai, 2011). Antibiotics are used in aquatic ecosystems to control the bacteria responsible for infectious diseases (Watts et al., 2017; Ouattara et al., 2014; Salah and Aqel, 2014). Excessive use of antibiotics has led to the resistance of pathogenic bacteria. (Gao et al., 2012; Miller and Harbottle, 2017). Vibriosis, Photobacteriosis and Furunculosis are among other major diseases of marine and estuarine fish in natural and in aquaculture (Toranzo et al., 2005). The agents of Vibriosis are several species of *V. anguillarium, V. vulnificus, V. alginolyticus* and *V. salmonicida*. Photobacteriosis is caused by Photobacterium (Mookerjee et al., 2015). The surveillance of fish disease is under estimated in West Africa and the risk of dissemination is very high, as countries with limited resources do not have veterinary clinics providing clinical and biological diagnostics to monitor the use of antibiotics in animals (Ouedraogo et al., 2017). In West Africa, antibiotic resistance induces the emergence of resistant anterobacteria (Pitout and Laupland, 2008). Several studies have shown that 90% of bacterial strains in marine environment are resistant to more than one antibiotic and 20% are resistant to at least five antibiotics (Kouadio et al., 2017; Benie et al., 2017; Dib et al., 2018). Previous studies have demonstrated the extent of the circulation of bacterial multi resistance. It has been shown in certain animals such as chickens (Koga et al., 2015) in the natural environment and in artificial water systems. (Ouattara et al., 2014). In Côte d'Ivoire, few data are provided on the detection of bacterial multi resistance in aquaculture environments. Previous studies carried out in the DABOU area have focused more on pathogenic bacteria and less on those with antibiotic resistance (Kouadio-Ngbesso et al., 2019). However, surveillance of bacterial multi resistance has become a major concern throughout the world. this study investigated the presence of multi-drug resistant bacteria in two fish farms in Dabou, in the south of Abidjan.

MATERIALS AND METHODS

Study area

Water and fish (*Oreochromis niloticus*) samples were collected from two fish farms in Dabou, located in the South of Ivory Coast (fish farm 1 with GPS data: 5° 19' 34,44'' N, 4° 22' 0.45'' W; fish farm 2: 5°18'41.51'' N, 4° 24'51.66'' W). The study area is characterized by different aquatic ecosystem (lagoon and river) and with natural vegetation. Around these aquaculture zones, some human activities exist. Each fish farm area covers 1 hectare with 12 rectangular ponds (Figure 1).

Sampling in the aquaculture farm

Sampling was done during six months from November 2017 to April 2018 with five ponds per fish farm. Samples were collected once a month. During each visit four water and fish samples were collected

Table 1. Distribution of collected samples.

Type of sample	Farm 1	Farm 2	
	Number of samples (n)	Number of samples (n)	Total
Water	120	120	240
Fishes organs (swabs, gills and intestine)	120	120	240
Total (%)	**240 (50%)**	**240 (50%)**	**480 (100%)**

from different points in each of the five ponds of the fish farms. Water samples were taken using sterile glass vials attached to a rope and fish samples were taken using mowing nets. Afterwards, 100 ml of water sample was taken from four different points in each pond. At each sampling period**,** Fish were sampled by rubbing its surface with swab. Next, the fish were dissected to remove their gills and intestines according to Adingra et al. (2010). A total of 240 were collected (Table 1). The water and fish samples were transported to the laboratory in a cooler containing (4° C) cold accumulator.

Bacteriological examination

Isolation and biochemical identification

Fishes were skinned and gills and intestines were removed. A small portion of the organs and the swabs were transferred into sterile 3 ml of PBS1X (pH= 7.2). The solution was mixed and 100 µl were inoculated into 3 ml of Luria Bertani broth and incubated at 37°C for 24 h. After 24 h cultures were inoculated on different media: trypticase soy agar (TSA) for Enterobacteriaceae, Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS) for Vibrio isolation, Eosin Methyl Blue Agar (EMB) for Gram negative bacteria and selective media for *Pseudomonas* group Cetrimide Agar (biolab, cab. 20500, lot: cab 090419072). After 18-24 h of incubation at 37°C, bacteria colonies were selected to perform Gram staining (Saleh et al., 2017). Bacterial identification was done by API 20E kits (BioMerieux, France).

MALDITOF analysis

Bacteria strains were identified by MALDI-TOF (Biomerieux, VITEK MS) according to the principle described by Carbonnelle and Nassif (2011). The bacterial strains to be analyzed were cultured on microbiological agar and incubated at 37°C for 24 h. the colonies obtained were used for analysis with MALDITOF. The method is based on the ionization of bacterial proteins by a laser beam and the creation of characteristic peaks (spectrum). The preparations were made in "sandwich" form: the sample is deposited on a matrix film before being covered by a final matrix layer. The plate carrying the samples is placed in the spectrometer where they are subjected to the laser beam. From a database of spectra, the software searches for the corresponding species of bacteria according to a reliability index between two spectra. The confirmation of the strains is data based (VITEK MS 3.1).

Antibiotic susceptibility of strains

Fresh bacterial colonies were added to 2 ml of physiological saline. The density of the bacterial suspension was adjusted to 0.5 Mc Farland it was inoculated onto the surface of Mueller Hinton agar (OXOID, CM: 0337). Selected antibiotic discs (Table 3) were placed on the agar and incubated at 37°C for 24 h. The diameter interpretation was based on the EUCAST 2019 (European Committee on Antimicrobial Susceptibility Testing V.1.0) recommendations. The sensitivity or resistance of bacteria to antibiotics is evaluated by comparing the inhibition diameters to the minimum inhibition concentration of the discs. If the measured inhibition diameter on the agar is greater than the minimum inhibition concentration of the disc, the bacterium is sensitive to the antibiotic; otherwise it is resistant (Guessennd et al., 2013).

RESULTS

Bacterial dissemination in fishes and in water samples

Seven bacterial species were isolated from water and in fish organs collected from two aquaculture farms. *Enterobater hormaechei (hor)*, *Enterococcus faecalis (fac), Citrobacter freundii (cit), Bacillus cereus (bac), Pseudomonas aeruginosa (psd), Escherichia coli (col)* and *Morganella morganii (mor)* were isolated and confirmed by biochemical tests and by MALDI-TOF analysis. The strains showed resistance to antibiotics. These isolated strains represent potential sources of pathogenicity for humans. Swab and water samples have similar colonization rates with 29.7 (504/1696) and 29.12% (494/1696), respectively; whereas intestine and gills showed a lower colonization rate by 15% (Table 2). Bacterial species in the aquaculture farms were: *Enterobacter hormachaei, E.coli, B. cereus, Enterococcus faecalis and M. morganii* with presence of 16, 15.9, 14.9, 14.2, and 14% of the samples, respectively (Table 2). The ANOVA 1 test showed no significant difference between the two farms for the presence of bacterial strains.

Antibiotic susceptibility testing

Isolated bacterial strains were tested for sensitivity to different families of antibiotics (Table 3). The bacteria *E. coli*, *Citrobacter freundii M. morganii E. faecalis* and *E. hormachei* are subjected to the same antibiotics. On the other hand, strains of *Pseudomonas aeruginosa* were tested with another group of antibiotics. The results showed that 44.9% (31/69) of the strains were resistant to tested antibiotics and 55.9% were susceptible to those

Table 2. Distribution of bacterial strains isolated in farms according to the different types of samples.

Sample type	Site	Number of isolated bacteria strains (n)							Total
		E. coli	*C. freundii*	*P. aeruginosa*	*M. morganii*	*B.cereus*	*E.faecalis*	*E. hormaechei*	
Swab	F1	45	33	20	37	37	30	45	247
	F2	39	41	33	38	39	26	41	257
Gill	F1	22	17	14	23	26	31	36	169
	F2	27	20	16	17	25	35	28	168
Intestine	F1	23	24	20	31	32	32	29	191
	F2	32	22	20	23	19	32	22	170
Total 1		**188**	**157**	**123**	**169**	**178**	**186**	**201**	**1202**
Water	F1	43	32	25	35	35	30	41	241
	F2	39	40	30	35	40	25	44	253
Total 2		82	72	55	70	75	55	85	494
Total 3		**270**	**229**	**178**	**239**	**253**	**241**	**286**	**1696**
(%)		**15,9%**	**13,5%**	**10,4%**	**14%**	**14,9%**	**14,2%**	**16,8%**	**(100%)**

F1: Farm 1; F2: Farm 2.

Table 3. Distribution of antibiotics susceptibility of isolated bacteria strains.

Family	Antibiotics	Number of tested strains (n)	Number of resistant strains (R)	Number of sensitive strains (S)
Penicillins	Amoxicilline/Acid clavulanic (AMC)	4	4	0
	Ticarcilline/Acid clavulanic (TCC)	1	1	0
Penicillins/Monobactams	Piperacilline/Tazobactam (TZP)	5	5	0
Penicillins	Piperacillin (PIL)	5	5	0
	Ticarcillin (TIC)	5	5	0
Cephalosporins	Ceftazidim (CAZ)	5	1	4
	Cefoxitin (FOX)	5	3	2
Monobactams	Aztreonam (ATM)	5	1	4
Carbapenems	Imipenem (IPM)	6	0	6
Fluoroquinolons	Ciprofloxacin (CIP)	5	0	5
	Acid nalidixic (NAL)	3	1	2
Aminoglucosides	Gentamicin (GMI)	5	0	5
Tetracyclines	Tigecyclin (TCG)	5	1	4
Aminoglucosides	Fosfomycin (FSF)	5	1	4
Sulfonamidess	Cotrimoxazole (SXT)	5	3	2
	Total	**69**	**31 (44.92%)**	**38 (55.07%)**

antibiotics (Table 3). *E. coli, M. morganii* and *P. aeruginosa* strains showed resistance against 3 different antibiotics families (Figure 2A, D, E); while *E. hormachei* and *E. faecalis* for 2 antibiotics (Figure 2B and F) and *C. freundii* strains have multiple resistance against five antibiotics (Figure 2C). Multi-drug resistance for 3 classes of antibiotics is observed in some strains (Figure 2A to F).

DISCUSSION

The bacteriological evaluation of fish and water samples

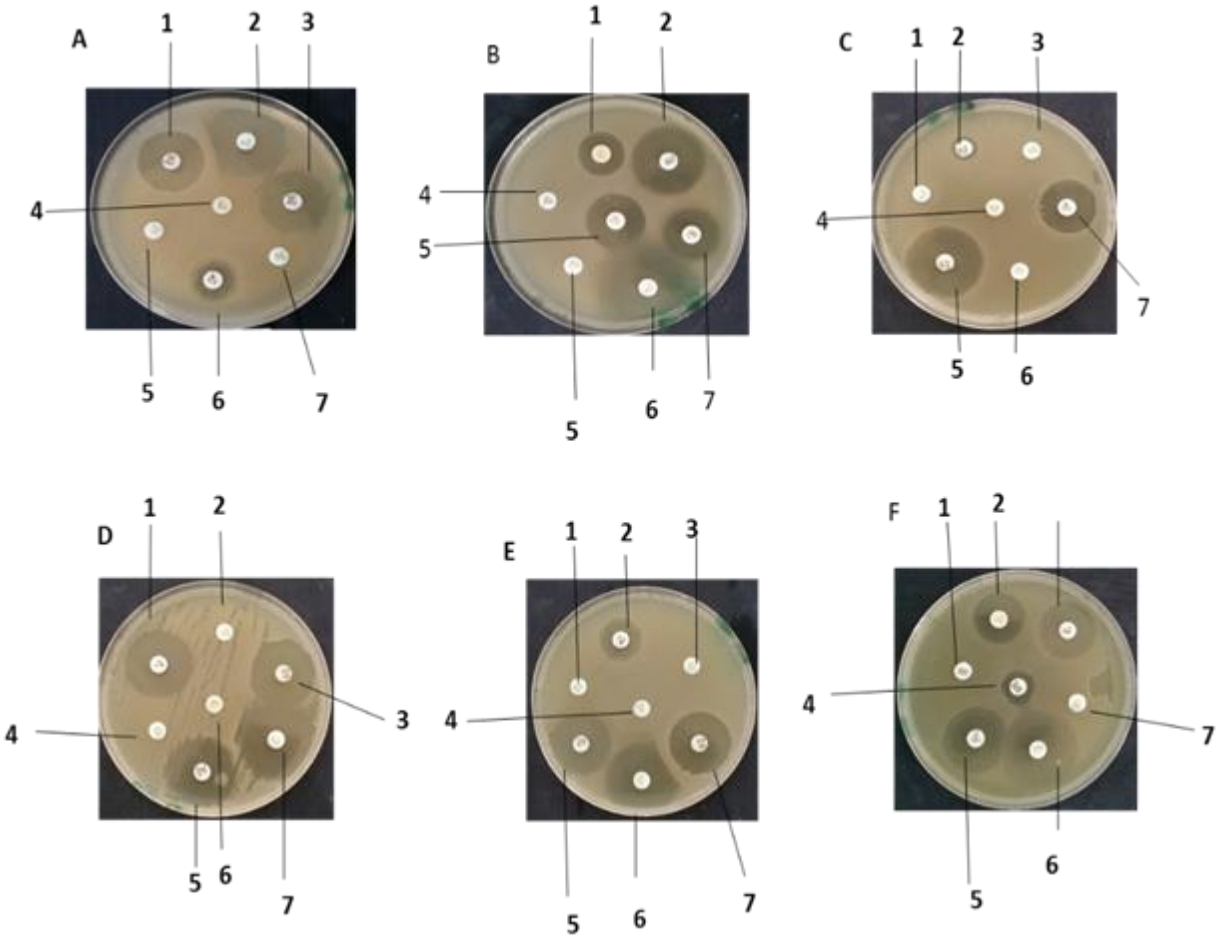

Figure 2. Antibiotic susceptibility for isolated strains in aquaculture farms. **A**: *E. coli* (1:IPM; 2: ATM;3: CAZ;4: AMC;5: PIL;6: FSF;7:TZP); **B**: *E. hormaechei* (1: TGC; 2: SXT; 3: FOX;4: NAL;5: TIC;6: CIP;7: GMI); **C**: *C. freundii* (1: ATM; 2: CAZ; 3: TZP; 4: AMC; 5:IPM; 6:PIL ;7: FSF); **D**: *M. morganii (*1: FSF; 2: PIL;3: IPM; 4: TZP; 5: CAZ; 6: AMC; 7: ATM); **E**: *P. aeruginosa* (1: TZP; 2: FSF; 3: PIL; 4: TCC; 5: CAZ; 6: AMC; 7:IPM); **F**: *E. feacalis* (1: SXT; 2:TGC; 3:GMI; 4: NAL; 5: FOX; 6: CIP; 7:TIC).

collected from two farms revealed different species of bacteria. The bacterial colonization rate showed that *E. coli* was the most isolated bacteria followed by *P. aeruginosa* and *E. faecalis*. The presence of these bacteria has been shown in other studies that also reported the presence of *Bacillus cereus* with multi-resistance in aquaculture tanks (Gao et al., 2012).

This high prevalence of these three bacteria could be linked to contamination of the feeding sources of fish ponds. Several human activities (farming, laundry, fecal matter, market gardening) around aquaculture areas contaminate natural waters such as rivers and lagoons. These waters, which are prone to microbial contamination, are used as a source of water for ponds and are sometimes untreated (Gabriel et al., 2007; Benie et al., 2017). Rasool et al. (2017) reported the presence of *B. cereus* in fishes in India, suggesting that the detection of bacteria in water and farmed fish is not limited to a single bacterial species.

E. faecalis, which showed a high colonization rate in this study, preferentially this bacterium is found in the intestines of warm-blooded animals. Finding these bacteria in fish that is a cold-blooded animal (poikilotherm), could be explained by human contamination. The presence of *E. coli* in the fish indicates fecal contamination of the biotope. Saeidi et al. (2018) have reported that the presence of *E. coli* and other enteric bacteria indicate fecal contamination. *Bacillus cereus* is one of the bacteria responsible for food poisoning. The presence in aquaculture suggests the persistence of this bacterium in the environment. Different genes responsible for enterotoxin production in *B. cereus* have been characterized (Ehling-Schulz et al., 2019). So far, no study has been carried out to relate the presence of virulent genes and enterotoxin production in case of isolates of *B. cereus* from fish.

Environmental factors can influence the bacterial colonization of pond water and fish (Fister et al., 2016). The detection of pathogenic bacteria in fish farms could reflect the list of pathogens in fishes (Toranzo et al., 2005; Soto-Rodriguez et al., 2013) and their geographical distribution of diseases and particularly in sub-Saharan Africa with countries with similar fish farming practices. Some studies have reported identical results for bacterial diseases in fishes (Abowei and Briyai, 2011; Eshetu et al., 2014). The detection of multi-resistant strains in this study correlates with the findings of Ouattara et al. (2016) with the dissemination in the aquaculture environment in Cote d'Ivoire. As a consequence of abusive and uncontrolled use of antibiotics for medical, veterinarian and food production, the increase of MDR is a general treat in West Africa (Gao et al., 2012; Devarajan et al., 2015). The rate of bacterial colonization in fish farms could influence yield (Bentzon et al., 2016) as it would result in a loss through fish mortality. It would be necessary to ensure the quality of the water used in the ponds or their source**.** In fact, there is no boundary between the different environments (industrial, hospital agricultural, animal) and the populations (Rajani et al*.,* 2016; Toule et al., 2017). The results confirm the presence of MDR strains in aquaculture and show the need to investigate the occurrence of enterotoxigenic *B. cereus, E. coli* and other bacteria and to study the relationship between their presence and the presence of diarrheal enterotoxin.

Conclusion

The importance of aquaculture production to provide future fish demands for human consumption is evident. One of the biggest challenges is the surveillance and control of fish production. In Africa, there is lack of surveillance in the food production system. There is a need to correlate human, animal and environment health surveillances. The presence of potential pathogens which are multidrug-resistant in aquaculture farms poses a considerable threat to public hygiene. The distribution of bacteria strains in water and in fish organs correlated in both farms. This study has demonstrated the presence of resistant strains in aquaculture farms and suggests the transmission of bacteria from nearby environments to the fish farms.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Bacillus cereus, Lactobacillus acidophilus and *Succinovibrio dextrinosolvens* promoting the growth of maize and soybean plants

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Plant growth promoting bacteria can be an alternative to increase plant production, and reduce production costs and environmental impacts. Ruminal bacteria have several abilities and some of them are related to plant growth promotion. The aim of this study is to evaluate the increase in maize and soybean plants and in soils promoted by the inoculation of three ruminal bacteria: *Bacillus cereus*, *Lactobacillus acidophilus* and *Succinovibrio dextrinosolvens*. The experiments were conducted in a complete randomized block design with five treatments and six replicates as follows: T1 = control; T2 = *B. cereus*, T3 = *L. acidophilus*, T4 = *S. dextrinosolvens*, T5 = *B. cereus* + *L. acidophilus* + *S. dextrinosolvens*. *In vitro* tests showed that bacteria were able to fix nitrogen, solubilize phosphorus, and synthesize indole acetic acid and amylase. *S. dextrinosolvens* increased the root dry matter of maize plants, *L. acidophylus* increased the phosphorus concentration in maize roots along with the mixture of the three bacteria and increased the shoot dry matter of soybean plants and also the phosphorus and nitrogen concentration in soybean plants. This is the first report showing that *L. acidophilus* and *S. dextrinosolvens* have great potential to be used as plant growth promoting agents.

Key words: Rhizobacteria, indoleacetic acid, nitrogen fixation, *Zea mays*, *Glycine max*.

INTRODUCTION

Maize (*Zea mays* L.) is a crop that originates in Mexico. It is now cultivated in many parts of the world and is of great importance economically or socially (Dowswell, 2019). Brazil ranks third in world's production, second in exports and fourth in consumption. For 2018/2019, the performance of the country will oscillate, reaching 361.4 million tonnes (CONAB, 2019). Soybean (*Glycine max*) probably originated in China, but spread to Europe, North and South America. In 1882, it was brought to Brazil, specifically Bahia State, and taken to the southern region of the country, where it was better adapted (Oliveira and Schneider, 2016). According to CONAB (2019), today, Brazil and the United States are ranked as the largest soybean producers in the world, followed by Argentina

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and China. It is estimated that by the year 2020, Brazil will lead this ranking. The use of chemical inputs in combination with genetic improvement and type of management provide an increase in the yield of these grains (Duncan et al., 2018). There is concern about the excessive use of chemical fertilizers, as they cause soil pollution, soil eutrophication and emission of greenhouse gases (Pavinato et al., 2017).

The major challenge of agriculture is to increase or maintain the productivity of agricultural crops with lower production costs and environmental impact. Plant growth promoting rhizobacteria appear as an alternative, which are a group of microorganisms capable of stimulating plant growth through direct mechanisms (production of plant hormones, enzymes, hydrocyanic acid, phosphate solubilization and nitrogen fixation), and / or indirect mechanisms (biological control, space and nutrient petition, parasitism, resistance induction and cross protection) (Hungria et al., 2010). These rhizobacteria normally inhabit root surfaces, internal plant tissues and rhizosphere. Interestingly, some ruminal probiotic bacteria also have several plant growth promoting characteristics such as rhizospheric bacteria and could be tested for this purpose.

Bacillus cereus is among these bacteria, which is a cylindrical, gram-positive, spore forming, facultative anaerobic and mesophilic bacterium. Its spores facilitate adhesion on surfaces and resistance to high temperatures and sanitization processes, that is, the bacterium can remain in a state of "dormancy" until the environment becomes favorable. *B. cereus* is a producer of phospholipases and food degrading enzymes such as amylases, proteases and lectinase (Granum and Lindbäck, 2013). Bacillus cereus has a positive effect in modulating immunity and intestinal microbiota, which is very important for the exploration of new probiotics (Li et al., 2009).

Lactobacillus acidophilus is a probiotic organism that degrades several enzymes, widely used as a nutritional supplement; it is produced by the food industry, with the function of maintaining the balance of the intestinal microbiota (Flesch et al., 2014). It adheres to specific receptors on the intestinal membrane competing with pathogens, in addition to producing antimicrobial substances, called bactericides (Marco et al., 2006). In addition, *L. acidophilus* is used in food and pharmaceutical application to balance disturbed intestinal microbiota (Sinn et al., 2008).

Succinovibrio dextrinosolvens is an anaerobic, gram-negative amylolytic (degrading starch) bacterium, with optimum pH around 6.0 to 7.0. This bacterium is usually found in the bovine rumen; it helps with other microorganisms to make best use of nutrients (Stewart et al., 1997).

In agriculture, there are many products that have active ingredient species of the genus *Bacillus* such as *Bacillus thuringiensis*, *Bacillus subitilis*, *Bacillus amyloliquefaciens*, among others. Microorganisms promote many gains for plant nutrition and phosphorus solubilization, which is a consequence of the presence of this group of microorganism in the rhizosphere (Canbolat et al., 2006). However, there is no research in literature related to the application of ruminal probiotic bacteria as a growth promoter of maize and soybean plants. There are many benefits to use probiotic bacteria in food and pharmaceutical applications, but they are not used to promote plant growth. As we have demonstrated that these probiotic bacteria have abilities to produce IAA, siderophores, solubilize phosphorus, and fix nitrogen, this study aims to evaluate if *B. cereus*, *L. acidophilus* and *S. dextrinosolvens* would promote maize growth in greenhouse condition.

MATERIAL AND METHODS

Experimental design and statistical analysis

Experiments were conducted in a complete randomized block design with five treatments and six replicates: T1 = control; T2 = *B. cereus*; T3 = *L. acidophilus*; T4 = *S. dextrinosolvens*; T5 = "MIX" (mixture of three microorganisms). Analyses were performed using AgroEstat software (Barbosa and Maldonado, 2010). Data were submitted to analysis of variance (ANOVA) with 5% significance level by the F test and means were compared by the Duncan test at 5% probability. The designs were the same for maize and soybean plants.

Bacterial isolates

Microorganisms used (*B. cereus, L. acidophilose* and *S. dextrinosolvens*) were provided by the Federal University of Viçosa - UFV, and belong to the collection of isolates from one of its laboratories. Bacteria were cultured in 60 mL Erlenmeyer flasks in nutrient broth culture medium at 28°C for 24 h, and suspensions were adjusted in bacterial concentration 10^8 colony forming unit (CFU) mL^{-1}.

In vitro tests of isolates

Production of siderophores

Siderophore production in a liquid medium using the Chrome Azurol Solution (CAS) was performed as previously described (Louden et al., 2011). 5 mL of the PMS7-Ca medium was inoculated and incubated for 72 h. The suspension was then centrifuged at 4000 × g for 10 min and 1 mL of the filter-sterilized supernatant was incubated 1:1 with the CAS. The OD630 was then measured and a 10% difference between the sample and un-inoculated PMS7-Ca with CAS was considered as positive (Machuca and Milagres, 2003).

Starch agar

The following reagents were used for the preparation of the starch production medium: K_2HPO_4 0.3 g/L; $MgSO_4.7H_2O$ 1.0 g/L; NaCl 0.5 g/L; $NaNO_3$ 1.0 g/L; Starch 10 g/L; pH = 6.9.

Cellulolytic activity

Cellulolytic activity was assayed by monitoring the oxidation of L-

Table 1. Soil chemical analysis prior to the assembly of the experiment.

pH $CaCl_2$	MO g dm^3	P resin $mgdm^3$	S $mgdm^3$	K	Ca	Mg	H+Al	SB	CTC	V%
				$mmol_c dm^3$						
6.5	11	20	12	0.7	19	5	17	24.4	41.3	59

3,4-dihydroxyphenylalanine (L-DOPA; Sigma) in the presence of hydrogen peroxide (28). A final volume of 1.0 ml of reaction mixture contained 4.0 mM hydrogen peroxide, 0.1 M potassium phosphate buffer (pH 7.0), and 1.0 mM L-DOPA. A concentrated crude enzyme preparation (100 to 200 ul) was used in the assay. The reaction was initiated by the addition of hydrogen peroxide, and the increase in the A470 was monitored for 5 min at 37°C. Reactions containing all reagents except the crude enzyme extract served as controls. One unit of enzyme was expressed as the amount of enzyme. The methodology of culture medium described by Ramachandra, Crawford and Pometto with no alterations was used (Ramachandra et al., 1987).

Production of indoleacetic acid

The bacteria evaluated were screened for IAA production (15). Briefly, the bacterial culture was inoculated in the respective medium (Jensen's/nutrient broth) with tryptophan (1, 2, and 5 mg/ml) or without tryptophan incubated at 28 ± 2°C for 15 days. Cultures were centrifuged at 3000 rpm for 30 min. Two milliliters of the supernatant was mixed with 2 drops of orthophosphoric acid and 4 ml of Solawaski's reagent (50 ml, 35% perchloric acid; 1 ml 0.5 $FeCl_3$). Development of a pink colour indicates IAA production. O.D. was read at 530 nm using Spectronic 20D+. The level of IAA produced was estimated by a standard IAA graph.

P quantification in test tubes

For phosphate solubilization quantification, the modified methodology of Malavolta et al. (1997) was used. In a 120 ml Erlenmeyer flask containing 50 ml of Nahas medium (1994), 200 µl of inoculum from each isolate was added. Erlenmeyer flasks were incubated for 48 h at ± 28°C with stirring at 180 rpm, and after incubation, 5 ml of each sample was transferred to tubes and centrifuged at 9000 rpm for 15 min. Then, 1 ml of supernatant from each isolate, 4 ml of distilled water and 1 ml of ammonium molybdate-vanadate reagent formed by mixing equal volumes were added to a new tube for further reading (after 5 min) in spectrophotometer at 470 nm.

Nitrogen quantification in test tubes

The method of Kuss et al. (2007) was used in the nitrogen quantification analyses by isolates. In the determination of nitrogen in foodstuffs (1), a digestion mixture of 40 g of sodium sulfate and 1.6 g of copper sulfate per 100 ml of acid is recommended, with a digestion time of 6 h. For the micro determination of protein in 50% glycerol (51), bromine is used as an oxidizing agent, supplemented by 30% hydrogen peroxide.

Planting

Experiments were conducted in greenhouse belonging to the Laboratory of Agricultural Microbiology of UNESP-FCAV (coordinates - Latitude: 21° 14 '05 "S Longitude: 48° 17' 09" W). For studies with maize, seeds of variety 2B587PW Dow Agro-Science-transgenic were used; in experiments with soybean, seeds of variety 95R95IPRO Piornner were used. In both cases, seeds were pre-inoculated with *B. cereus, L. acidophiluse* and *S. dextrinosolvens*, deposited in pots (5 L), filled with red eutrophic latosol type soil, sieved and fertilized. Fertilization was performed according to soil chemical analysis and recommended for crops (Table 1).

Inoculations

Four inoculations were performed, the first through seeds, which were immersed in 125 ml Erlenmeyer flasks containing nutrient broth in bacterial concentration of 10^8 CFU ml^{-1} for 15 min in 120 rpm orbital shaking and then sown. The second, third and fourth inoculations were performed every week, seven days of sowing, adding 20 ml of each inoculum at the same concentration as above.

Evaluations in corn and soybean plants

Dry mass

Roots were collected from both cultures, washed in running water to remove excess soil and dried on absorbent paper. Shoots were separated from roots and both were dried in oven with forced air circulation at 65°C for approximately 72 h until reaching constant weight. The last step was the weighing of all the material, using analytical scale to determine the mass (g) of root dry matter (RDM) and shoot dry matter (SDM).

Nitrogen concentration in shoots and roots

In order to determine the nitrogen concentration (N), the plant material was ground in Willey mill (mesh 20) and submitted to N-leaf analysis using the method proposed by Bremmer and Mulvaney (1982) and modified by Bezerra and Barreto (2011).

Shoot and root phosphorus concentration

Phosphorus concentrations (P) were determined by nitroperchloric digestion, followed by the molybdo-vanadate colorimetric method according to methodology proposed by Haag et al. (1975) with modifications by Bezerra and Barreto (2011).

Soil assessments

Simple soil samples were collected from the maize and soybean rhizosphere; they were collected at random points from pots and then divided into two parts: the first was kept in plastic bags at 4°C until the moment of use for total bacteria counting and the second air was dried and stored at room temperature (28°C) to determine the amount of soluble phosphorus, total nitrogen and carbon of the bacterial biomass (Nahas and Assis, 1992).

Table 2. *In vitro* tests of isolates.

Isolate	Siderophores	Amylolytic activity	Cellulolytic activity	IAA µg. mL-^{1}	P solubilization mg P	N fixation mg N
B. cereus	-	+	-	9.08	14.93	0.7
S. dextrinosolvens	+	+	-	10.25	41.38	0.42
L.acidóphilus	+	-	-	7.25	5.58	0.5

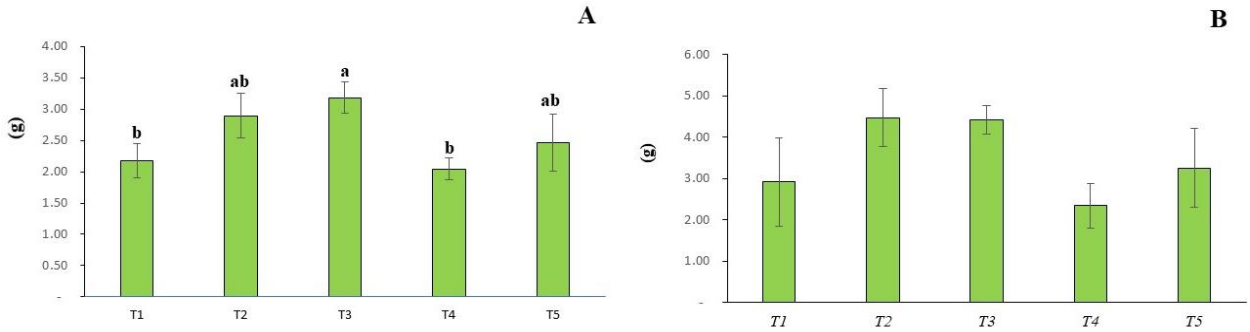

Figure 1. RDM (*VC = 25.54) (A) and SDM (*VC = 47.85) (B) of maize plants. Control (T1), *B. cereus* (T2), *L. acidophilus* (T3), *S. dextrinosolvens* (T4), MIX (T5). Bars represent the standard error of the mean. Means with different letters indicate significant differences among treatments. Statistical analysis was performed according to the Duncan test (p≤0.05). * Variation coefficient.

Total nitrogen

Total nitrogen content was determined by Berigari (1975). For nitrogen determination, the plant material was ground in Willey-type mill (20 mesh) and submitted to leaf nitrogen analysis according to Bremmer and Mulvaney (1982) and modified by Bezerra and Barreto (2011); 0.1 g of plant sample was weighed, placed in a digester and digested using 7 ml of sulfuric acid. The material was digested, and then, 10 ml of distilled water was added. Distillation was performed by using the Kjeldahl method with the aid of 25 ml of NaOH (50%). The distilled material was recollected in 10 ml of boric acid as an indicator solution, resulting in 20 ml of distilled material. Ammonium titration was performed using 0.05 N H_2SO_4 as the standard.

Soluble phosphorus

Soluble phosphorus was measured according to Watanabe and Olsen (1965), where 0.6 g of dry soil was sampled and transferred to Erlenmeyer flasks containing 12 ml of extractor sodium bicarbonate solution and Whatman filter paper. For determination, 2.0 ml of sodium bicarbonate was pipetted, and 0.2 ml of sulfur solution (5 M) and 0.8 ml of B reagent were filtered. Then, the material was incubated at 45°C for 20 min. Next, a reading was taken using a spectrophotometer at 820 nm.

RESULTS

The results of *in vitro* laboratory analyses to evaluate the ability of isolates to produce siderophores, amylolytic and cellulolytic activity, and the quantitative values of IAA production, nitrogen fixation and phosphorus solubilization in tubes are shown in Table 2. For root dry matter (RDM) (Figure 1A), *L. acidophilus* (T3) bacterium promoted a 3.2 g increase (p> 0.05) compared to control treatment. For shoot dry matter (SDM) (Figure 1B), treatments did not differ from each other, although numerically, *B. cereus* isolate was almost twice as large as control (without application of bacteria).

The root nitrogen content ranged from 25.36 to 30.73 g N / kg^{-1}, and there was no significant difference (p <0.05) among treatments (Figure 2A). The shoot nitrogen content ranged from 34.5 to 43.5 g N / kg^{-1} and there was no significant difference among treatments (Figure 2B).

The bacterial MIX promoted an increase in the phosphorus concentration of 1.4 g of P / kg of plant compared to control, followed by *L. acidophilus* bacterium (Figure 3A). Interestingly, the bacterial MIX decreased shoot P concentration of 1.7 g of P per kg of maize plant compared to control of 2.3 g of P per kg of plant (Figure 3B). Nitrogen concentrations ranged from 0.06 to 0.9 g N per kg of dry soil while phosphorus concentrations ranged from 5 to 35 g P / kg $^{-1}$ of dry soil. However, there was no significant difference among treatments (Figure 4). *L. acidophilus* showed the highest root dry matter compared to control treatment and the other treatments (Figure 5A), while, there was no significant difference (p <0.05) in the shoot dry matter (Figure 5B).

L. acidophilus and *S. dextrinosolvens* bacteria promoted an increase in the nitrogen concentration in soybean roots (p > 0.05), 23 and 25 g of N kg^{-1}, respectively,

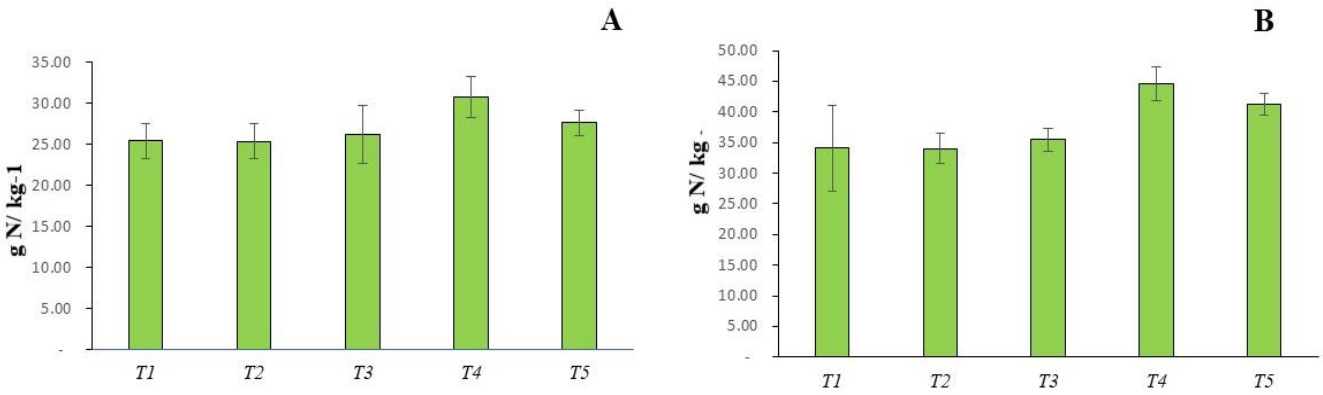

Figure 2. Root nitrogen concentration (*VC = 23.27) (A) and shoot nitrogen concentration (*VC = 25.57) (B) of maize plants. Control (T1), *B. cereus* (T2), *L. acidophilus* (T3), *S. dextrinosolvens* (T4), MIX (T5). Bars represent the standard error of the mean. Means with different letters indicate significant differences among treatments. Statistical analysis was performed according to the Duncan test (p≤0.05). *Variation coefficient.

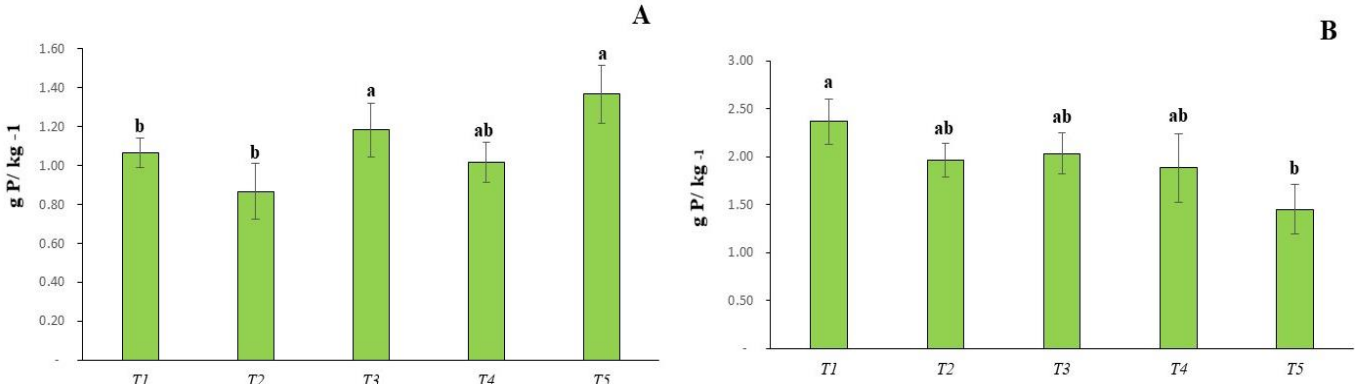

Figure 3. Root phosphorus concentration (*VC = 25.00) (A) and shoot phosphorus concentration (*VC = 29.04) (B) of maize plants. Control (T1), *B. cereus* (T2), *L. acidophilus* (T3), *S. dextrinosolvens* (T4), MIX (T5). Bars represent the standard error of the mean. Means with different letters indicate significant differences among treatments. Statistical analysis was performed according to the Duncan test (p≤0.05). * Variation coefficient.

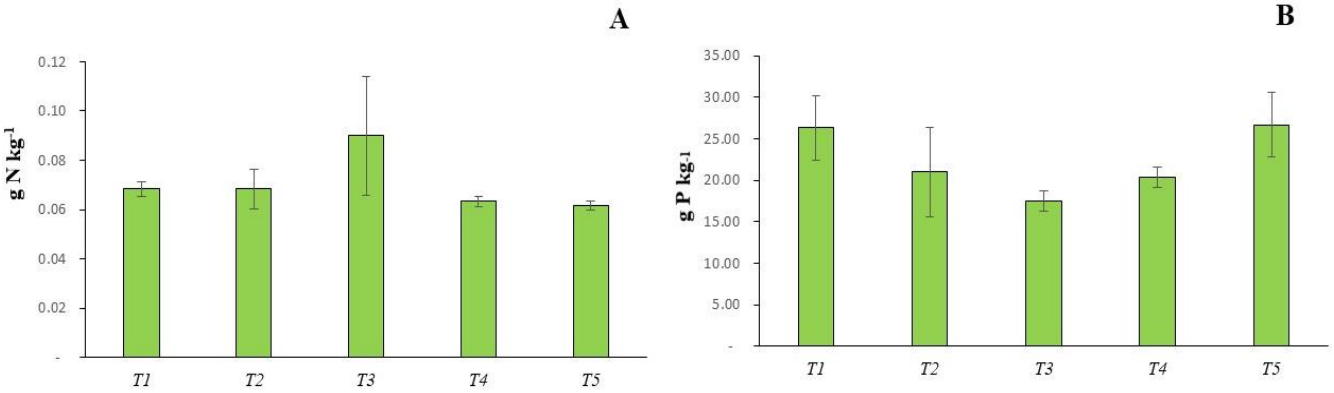

Figure 4. Soil nitrogen concentration (*VC = 40.96) (A) and soil phosphorus concentration (*VC = 40.25) (B) of maize plants. Control (T1), *B. cereus* (T2), *L. acidophilus* (T3), *S. dextrinosolvens* (T4), MIX (T5). Bars represent the standard error of the mean. Means with different letters indicate significant differences among treatments. Statistical analysis was performed according to the Duncan test (p≤0.05). * Variation coefficient.

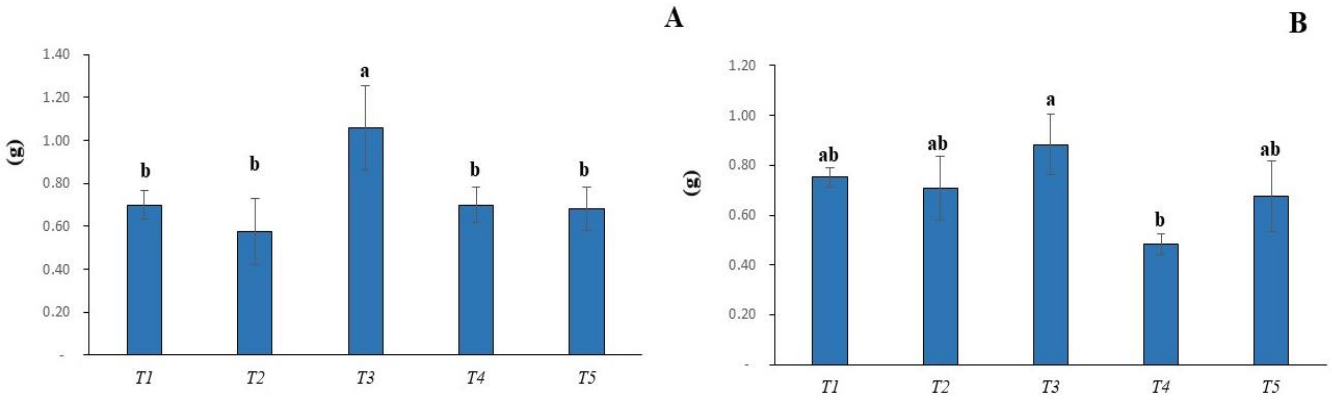

Figure 5. RDM (*VC = 38.89) (A) and SDM (*VC = 35.24) (B) of soybean plants (T1). Control (T1), *B. cereus* (T2), *L. acidophilus* (T3), *S. dextrinosolvens* (T4), MIX (T5). Bars represent the standard error of the mean. Means with different letters indicate significant differences among treatments. Statistical analysis was performed according to the Duncan test (p≤0.05). * Variation coefficient.

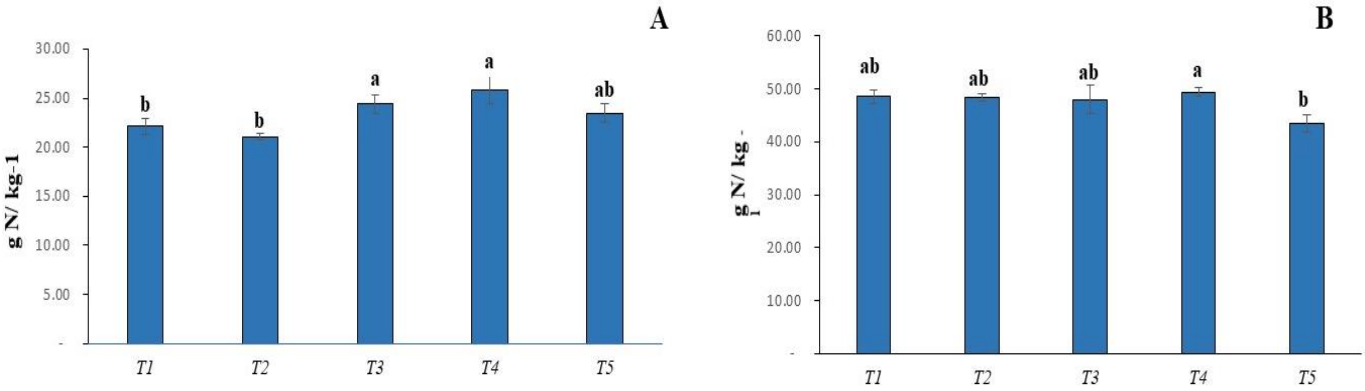

Figure 6. Root nitrogen concentration (*VC = 9.56) (A) and shoot nitrogen concentration (*VC = 8.22) (B) of soybean plants. Control (T1), *B. cereus* (T2), *L. acidophilus* (T3), *S. dextrinosolvens* (T4), MIX (T5). Bars represent the standard error of the mean. Means with different letters indicate significant differences among treatments. Statistical analysis was performed according to the Duncan test (p≤0.05). * Variation coefficient.

compared to the control treatment, 22 g of N kg^{-1} plant (Figure 6). *L. acidophilus* bacterium (T3) promoted the highest root phosphorus concentration, 1.1 g of P kg^{-1}, while *S. dextrinosolvens* promoted the highest shoot phosphorus concentration, 2.8 g of P kg^{-1}, compared to control treatments (p> 0.05). However, no significant difference (p <0.05) in relation to root and shoot concentrations for the other treatments was observed (Figure 7A-B). In relation to soil nitrogen, *S. dextrinosolvens* bacterium (T3) promoted the highest concentration, 0.065 g of N kg^{-1}, compared to control treatment (p> 0.05). On the other hand, *L. acidophilus* decreased the soil nitrogen concentration, 0.05 g of N kg^{-1}, compared to control treatment (Figure 8A). In relation to soil phosphorus concentrations, no significant difference between control and the other treatments was observed (Figure 8B).

DISCUSSION

Ruminal probiotic bacteria presented important characteristics related to plant growth promotion such as synthesis of siderophores, indole acetic acid, nitrogen fixation and phosphorus solubilization. Therefore, these bacteria were evaluated in maize and soybean plants to verify the potential of each for their plant growth promoting effect. Probiotic bacteria are bacteria whose inadequate amounts promote any benefit to the host (Martin and Langella, 2019). These benefits may be the consequence of nutrient supply and / or the reduction of pathogens that impair host development (Kleerebezem et al., 2019).

Plant growth promoting bacteria are generally isolated from the rhizosphere or from within plant tissues and have plant growth promoting abilities (Calvo et al., 2019).

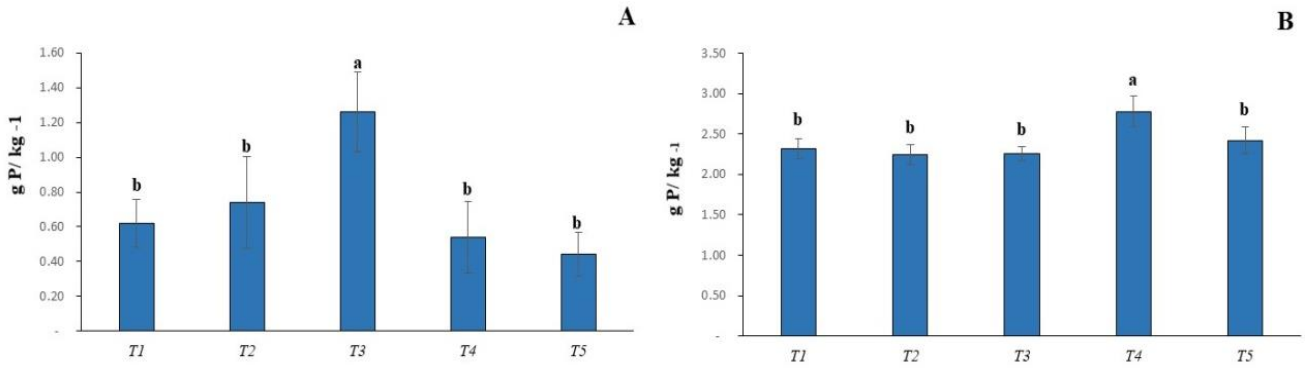

Figure 7. Root phosphorus concentration (*VC = 76.73) (A) and shoot phosphorus concentration (*VC = 13.74) (B) of soybean plants. Control (T1), *B. cereus* (T2), *L. acidophilus* (T3), *S. dextrinosolvens* (T4), MIX (T5). Bars represent the standard error of the mean. Means with different letters indicate significant differences among treatments. Statistical analysis was performed according to the Duncan test (p≤0.05). * Variation coefficient.

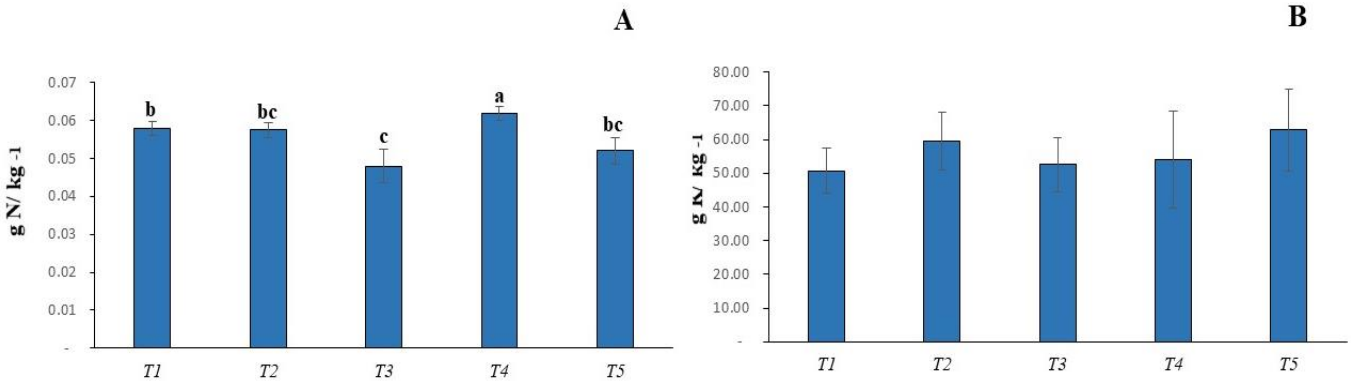

Figure 8. Soil nitrogen concentration (*VC = 11.24) (A) and soil phosphorus concentration (*VC = 41.78) (B) of soybean plants. Control (T1), *B. cereus* (T2), *L. acidophilus* (T3), *S. dextrinosolvens* (T4), MIX (T5). Bars represent the standard error of the mean. Means with different letters indicate significant differences among treatments. Statistical analysis was performed according to the Duncan test (p≤0.05). * Variation coefficient.

In the same way that probiotic bacteria promote host development, plant growth promoting bacteria also provide nutrients and reduce the harmful effects of plant pathogens. In a way, it was found that the mode of action of probiotic bacteria and plant growth promoting bacteria is very similar. As a consequence of these similarities, *B. cereus*, *L. acidophilus* and *S. dextrinosolvens* were inoculated in maize and soybean plants and some plant growth parameters were evaluated in comparison with control treatment.

Interestingly, *L. acidophilus* bacterium increased root dry matter and the phosphorus concentration in the roots of maize plants. *L. acidophilus* also increased root dry matter and the nitrogen and phosphorus concentration in the roots of soybean plants. Probably, these effects promoted by *L. acidophilus* were due to its ability to synthesize phytohormones that in certain amounts can stimulate or inhibit the root development of plants and as a consequence, increase the concentration of certain nutrients in the plant (Barnawal et al., 2019).

The increase of phosphorus concentration in maize roots and phosphorus and nitrogen concentration in soybean roots is a very interesting aspect promoted by the plant / microorganisms interaction, in which the nutritional efficiency of plants is increased. Nutrients such as phosphorus and nitrogen are essential for plant growth and development (Stewart et al., 2019; Klamer et al., 2019) and when the association with a microorganism allows their absorption more efficiently, this microorganism has great potential to be used in a more sustainable agricultural production system (Syed and Tollamadugu, 2019), allowing reductions in production costs and environmental impact (Baron et al., 2018).

S. dextrinosolvens increased the nitrogen concentration in roots and soil as well as phosphorus concentration. These results are very interesting from the point of view

of plant nutrition and show that ruminal probiotic bacteria have potential to be used as plant growth promoting bacteria. There is a positive correlation between plant nutritional status, microbioma and productivity (Pii et al., 2016). In this sense, the action of bacteria such as *S. dextrinosolvens* and *L. acidophilus* can be very positive for plant production. The bacteria / plant interaction depends on several factors such as plant species, soil type, climatic conditions and characteristics that are intrinsic to microorganisms used (Bulgarelli et al., 2013). *L. acidophilus* and *S. dextrinosolvens* bacteria showed a certain affinity with the plant species tested, promoting increases in plant and nutritional development and soil fertility. In this sense, more studies are needed to verify the best conditions of use of these bacteria such as dose, mode of application and plant species in order to optimize the increases promoted by the microorganisms.

Conclusion

This is the first report on the use of ruminal probiotic bacteria as plant growth promoting bacteria. It shows great potential for their use, since *L. acidophilus* increased dry matter in soybean and corn plants and *S. dextrinosolvens* promoted increases in the nutritional status of soybean and soil plants. In the future these ruminal probiotic bacteria could be used in agricultural production as inoculates, allowing significant reduction of mineral fertilizer levels and contributing to more sustainable production.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Distribution of β-Lactam resistant Gram-negative bacteria isolated from clinical and environmental sources in two tertiary hospitals in Makurdi, Benue State, Nigeria

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For appropriate control of infections, it is necessary to possess updated awareness about occurrence of the causative agents. Gram-negative bacteria are considered important microorganisms that cause hospital infections. Clinical multidrug resistant Gram-negative bacteria were obtained from clinical samples including urine, high vaginal swab (HVS), wound swab (WS), stools, ear swab (ES), endocervical swab (ECS), sputum and blood, from Federal Medical Centre (FMC) and Benue State University Teaching Hospital (BSUTH) located at Makurdi. Sewer wastewater and sediment samples were also collected from both hospitals using standard sampling techniques and bacteria were isolated using pour plate technique. Identification was done using API 20E kit. Out of the 403 clinical bacteria obtained, 271 were from FMC and 132 from BSUTH; of these, 299 were confirmed Gram-negative (218 from FMC and 81 BSUTH, respectively). Thirty-nine Gram-negative bacteria were also isolated from the sewer samples, that is, from the environmental samples. Pooled frequencies of Gram negative bacteria isolated from clinical samples in both hospitals were: Urine (56.9%), HVS (11.7%), WS (11.4%), stools (7.7%), ES (6.0%), ECS (3.3%), sputum (2.3%) and blood (0.7%). The identified bacteria from the clinical samples from FMC and BSUTH were *Escherichia coli* (92; 55), *Pseudomonas* sp. (104; 17), *Klebsiella* sp. (19; 5) and *Proteus* sp. (3; 4) respectively.

Key words: Antibiotic resistance, Gram-negative bacteria, β-lactams.

INTRODUCTION

The use of antibiotics for the treatment of bacterial infections is very important; however, increase in the rate at which bacteria develop resistance to these agents all over the world is a public health challenge because the antimicrobial agents become less effective (Neu, 1992; Witte, 1998; Alhaj et al., 2007). Antibiotic resistance in

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pathogenic bacteria has been an increasing medical problem for decades (Mazel and Davies, 1999; Kastner et al., 2005). The development of antibiotic resistance by bacterial pathogens may be due to selection or acquiring resistance determinants even when the organisms were not directly exposed to the antibiotics. In addition, potential and obligate pathogens acquire resistance determinants which are spread among species and genera (Kastner et al., 2005). The intestine of humans and animals are especially favored in settings that allow association of densely packed microorganisms (Salyers et al., 2004; Kastner et al., 2005).

The beta–lactamases target the peptidases of bacterial cell-wall in biosynthetic processes (Ittoo et al., 2010). The beta-lactam antibiotics are the largest and most commonly used group of antimicrobial agents all over the world which are distinguished by a chemical structure known as the beta-lactam ring. Based on this, they can be divided into five groups, depending on the ring structure fused to the beta-lactam ring (Penicillins, cephalosporins, carbapenems, monobactam and beta-lactamase inhibitors) (Walsh, 2003). The beta-lactam antibiotics work by blocking the peptidoglycan of the cell wall component, through transpeptidation inhibition of penicillin binding proteins (Walsh, 2003; Andes and Craig, 2005; Chambers, 2005). Toxicity to the beta-lactams is very low in animals since penicillin binding proteins are not found in their cells, but allergy against penicillins and other beta-lactams can be very serious (Weiss and Adkinson, 2005). The spectrum of action can be narrow or broad and targets both Gram-positive and Gram-negative bacteria. Resistance against beta-lactams is primarily mediated by a structural change of the penicillin binding proteins (leading to lower affinity of the drug) or by bacterial production of enzymes cleaving to the beta-lactam ring. Other mechanisms include decreased permeability or active transportation via efflux pumps (Chambers, 2005).

The β-lactamases is the collective name of enzymes that open the β-lactam ring by adding a water molecule to the common β-lactam bond, and this inactivates the β-lactam antibiotic from penicillin to carbapenems. This hydrolyzation was first observed in 1940 by Abraham and Chain as penicillinase in a strain of *Escherichia coli* (Abraham and Chain, 1940). Although, the clinical effect of such hydrolyzation was not noted until the beginning of the 1950s, when the first β -lactam-resistant *Staphylococcus aureus* isolates appeared in hospitals (Kirby, 1944).

Gram-negative bacteria are bacteria that do not retain the crystal violet stain used in the gram-staining method of bacterial differentiation (Baron et al., 1996). They are characterized by their cell envelopes, which are composed of a thin peptidoglycan cell wall pack in between an inner cytoplasmic cell membrane and a bacterial outer membrane. The gram-negative bacteria include *E. coli*, as well as many pathogenic bacteria, such as *Pseudomonas aeruginosa, Neisseria gonorrhoeae, Chlamydia trachomatis,* and *Yersinia pestis.*

Based upon a number of different observations including that the gram-positive bacteria are the major reactors to antibiotics and that gram-negative bacteria are, in general, resistant to them, it has been proposed that the outer cell membrane in gram-negative bacteria (diderms) evolved as a protective mechanism against antibiotic selection pressure (Gupta, 2011). The diderm bacteria can also be further differentiated between simple diderms lacking lipopolysaccharide (LPS); the archetypical diderm bacteria, in which the outer cell membrane contains lipopolysaccharide; and the diderm bacteria, in which the outer cell membrane is made up of mycolic acid (for example, Mycobacterium) (Desvaux et al., 2009).

The proteobacteria are a major phylum of gram-negative bacteria, including *E. coli, Salmonella, Shigella,* and other *Enterobacteriaceae, Pseudomonas, Moraxella, Helicobacter, Stenotrophomonas, Bdellovibrio*, acetic acid bacteria, and *Legionella*. Other notable groups of gram-negative bacteria include the cyanobacteria, spirochaetes, green sulfur, and green non-sulfur bacteria. Medically relevant gram-negative bacilli include a multitude of species. Some of them cause primarily respiratory problems (*Klebsiella pneumoniae, Legionella pneumophila, P. aeruginosa*), primarily urinary problems (*E. coli, Proteus mirabilis, Enterobacter cloacae, Serratia marcescens*), and primarily gastrointestinal problems (*Helicobacter pylori, Salmonella enteritidis, Salmonella* Typhi). Gram-negative bacteria associated with hospital-acquired infections include *Acinetobacter baumannii*, which cause bacteremia, secondary meningitis, and ventilator-associated pneumonia in hospital intensive-care units. The aim of this work is to illustrate the distribution of β-Lactam resistant Gram-negative bacteria from clinical and environmental sources in two healthcare facilities.

MATERIALS AND METHODS

Sample site/collection

Approval was obtained from the two hospitals before the commencement of the study. Ethical approval was obtained from the government of Benue State of Nigeria Ministry of Health and Human Services with reference number MOH/STA/204/VOL.1/31. Clinical isolates (Stock culture) were obtained from the laboratory benches of the Medical Microbiology Department by 10th March to 3rd June, 2016. Also, samples of wastewater and wastewater sediments were collected in the month of September, 2016 from sewers (gutters) from the two tertiary hospitals. Sites selected for the study were drains from various wards which includes; The Theatre, Female Surgical Ward, Pediatric Ward, Male and Female Medical Wards, Amenity Ward (ward block), Resident doctors hostel and cafeteria, Laboratory (Chemical Pathology, Microbiology, Hematology and Histopathology), Administrative Block, and Accident and Emergency Ward (A and E) BSUTH, whereas FMC samples sites include Laboratory, A Ward (Male Ward 18 years and above), Gynecology Ward, Theatre and Female Ward. Samples were routinely subcultured onto slants prepared from nutrient agar.

Wastewater and sediment samples

Wastewater and sediment samples were collected in the month of September, 2016. The water samples were collected into sterile bottles from the various units aseptically by using disposable micro pipette at each collection unit. Samples were safely transported by road to the laboratory, and immediately analysed.

Sediments were collected by wearing gloves and using clean hand trowel from different wastewater sampling sites to scoop sediments from the bottom of the sewers and introduced into sterile Bijou bottles. The trowel was properly cleaned using alcohol (70% ethanol) before using it in another site to avoid contamination.

Clinical bacterial isolates

Clinical bacterial isolates (Gram-negative multidrug resistant stock culture) were collected from stocks from the laboratory benches of the Department of Medical Microbiology of the hospitals listed above. Collection of clinical isolates was done between 10 March and 3 June, 2016. Isolates were confirmed using different laboratory synthetic media and biochemical tests were done using API 20E. The clinical samples were collected from samples of body fluids (urine and blood samples), swab (high vaginal, endocervical, wound, ear and sputum samples) and stool samples.

Isolation of β-lactam resistant Gram-negative bacteria from environmental sources

Beta lactam resistant Gram-negative bacteria were isolated from wastewater and wastewater sediments. This was done by supplementing peptone water with ampicillin antimicrobial susceptibility test disc 10 µ/g (Oxoid). Stock solution of peptone water was prepared according to manufacturer instructions. 5 ml each was dispensed into an incubating bottle sterilized at 121°C for 15 min and allowed to cool. The sterile ampicillin discs 10 µ/g were aseptically introduced into the sterile peptone water at 50°C to a final concentration of 60 µg/ml.

Water: For the wastewater samples, 1 ml each was introduced into the sterile incubating bottles containing sterile peptone water supplemented with ampicillin discs (60 µ/ml) and incubated for 18-24 h at 37°C.

Sediments: Serial dilutions were carried out with the sediment samples and 1 ml of 10^{-1} diluent was introduced into the sterile peptone water supplemented with ampicillin discs (60 µ/ml) and also incubated for 18-24 h at 37°C.

The 18-24 h incubated water and sediments samples above were subsequently streaked on MacConkey agar with the aid of sterile wire loop and incubated at 37°C for 18-24 h. This was done for all the wastewater and sediments samples.

Statistical analysis

Data obtained were subjected to frequencies and Chi-square analysis, using IBM Statistical Package and Service Solution (SPSS) version 20. The level of significance was defined as $P \leq 0.05$.

RESULTS

A total of 403 clinical isolates reportedly, multidrug Gram-negative bacteria were collected from two (2) tertiary hospitals; Federal Medical Centre (FMC), and Benue State Teaching Hospital (BSUTH). Of these, two hundred and seventy-one (271) were from FMC, and 132 from BSUTH. The 403 clinical isolates were Gram stained; out of which two hundred and ninety-nine (299) bacteria were actually confirmed Gram-negative bacteria while 104 of the total of 403 isolates collected were eventually proven to be Gram-positive. Of these two hundred and ninety-nine (299) confirmed Gram-negative bacteria, 218 were from FMC while 81 were from BSUTH.

Of a total of 218 Gram-negative bacteria from FMC, urine had 127(58.26%), high vagina 33(15.14%), wound 26(11.93%), ear 11(5.05%), endocervical 10(4.59%), stool 8(3.67%) and sputum 3(1.38%). Out of a total 81 isolates from BSUTH, urine had 43(53.09%), stool 15(18.52%), wound 8(9.88%), ear 7(8.64%), sputum 4(4.94%), high vaginal swab and blood had 2(2.47%) each (Table 1).

The number/percentage distribution of probable identity of the isolates are shown in Table 2, *Pseudomonas* sp. had 102(46.79%) and 17(20.99%)*; E. coli* 94(43.12%) and 55(67.90%); *Klebsiella* sp. 19(8.72%), and 5(6.17%), while *Proteus* sp*.* had 3(1.38%) and 4(4.94%) from FMC and BSUTH, respectively. From FMC, *Pseudomonas* sp*.* was more abundant while from BSUTH, *E. coli* was more abundant in the clinical sample collected. *Proteus* sp*.* is the least dominant from the two hospitals.

The identified Gram-negative bacteria in the clinical samples from both hospitals were *E. coli*, *Pseudomonas* sp., *Klebsiella* sp., and *Proteus* sp. with a prevalence of 147(49.16%), 121(40.47%), 24(8.03%) and 7(2.34), respectively (Table 3).

Klebsiella sp*.* has higher percentage distribution in FMC compared to that in BSUTH with a distribution of 95(79.20%) and 25(20.80%) respectively while *Proteus* sp*.* is higher in BSUTH than FMC with a distribution of 20(57.10%) and 15(42.90%), respectively. The distribution is statistically significant (Table 4).

In the distribution of environmental bacteria isolated from wastewater and sediments from FMC, the highest bacterial distribution is *Proteus stuartii,* with a distribution of 15(100%), while the least is *Proteus vulgaris* with a distribution of 10(20%). From BSUTH, the highest bacterial distribution is *Citrobacter freudii* with a distribution of 100% while the least is *Proteus mirabilis* with a 25% distribution. The bacteria distribution from the two hospitals was statistically significant (Table 5).

Eleven (11) different bacterial species belonging to Six (6) genera were isolated from environmental sources (water and sediment) of the two hospitals which include; *E. coli*, *Shigella sonnei*, *Citrobacter diversus*, *C. freudii, Citrobacter koseri*, *Erwinia chrysanthemi*, *K. pneumonia*, *P. mirabilis*, *P. vulgaris*, *Providencia stuartii* and *Serratia liquifaciens*. Among the 11 bacterial isolated, *S. sonnei* has the highest frequency of isolates from water while the least bacterial isolate is *C. freundii* also from water; *C. koseri* is isolated from both water and sediment, *E.*

Table 1. Percentage distribution of specimen types and their sources in clinical Gram-negative bacteria from FMC and BSUTH.

S/N	Specimen type	BSUTH {No. (%)}	FMC {No. (%)}	Total {No. (%)}
1	Urine	43 (53.09)	127 (58.26)	170 (56.86)
2	High vagina swab	2 (2.47)	33 (15.14)	35 (11.71)
3	Wound swab	8 (9.90)	26 (11.93)	34 (11.37)
4	Stool	15 (18.52)	8 (3.67)	23 (7.69)
5	Ear swab	7 (8.64)	11 (5.05)	18 (6.02)
6	Endocervical swab	0 (0.00)	10 (4.59)	10 (3.34)
7	Sputum	4 (4.94)	3 (1.38)	7 (2.34)
8	Blood	2 (2.47)	0 (0.00)	2 (0.67)
	Total	81 (100.00)	218 (100.00)	299 (100.00)

BSUTH- Benue State University Teaching Hospital; FMC- Federal Medical Centre.

Table 2. Distribution of Gram- negative bacteria from FMC and BSUTH.

S/N	Gram-negative bacteria	BSUTH {No. (%)}	FMC {No. (%)}	Total {No. (%)}
1	*E. coli.*	55 (67.90)	94 (43.13)	147 (49.16)
2	*Pseudomonas* sp.	17 (20.99)	102 (46.79)	121 (40.47)
3	*Klebsiella* sp.	5 (6.17)	19 (8.72)	24 (8.03)
4	*Proteus* sp.	4 (4.94)	3 (1.38)	7 (2.34)
	Total	81 (27.09)	218 (72.91)	299 (100.00)

BSUTH- Benue State University Teaching Hospital; FMC- Federal Medical Centre.

Table 3. Distribution of Gram-negative bacterial in clinical samples from both FMC and BSUTH.

Sample	*Pseudomonas* sp. {No. (%)}	*E. coli* {No. (%)}	*Klebsiella* sp. {No. (%)}	*Proteus* sp. {No. (%)}	Total {No. (%)}
Urine	63 (21.07)	86 (28.76)	17 (5.69)	4 (1.34)	170 (56.86)
HVS	16 (5.35)	19 (6.35)	0 (0.00)	0 (0.00)	35 (11.71)
Stool	3 (1.00)	19 (6.35)	0 (0.00)	1 (0.33)	23 (7.69)
W/S	18 (6.20)	11 (3.68)	3 (1.00)	2 (0.67)	34 (11.37)
E/S	13 (4.35)	5 (1.67)	0 (0.00)	0 (0.00)	18 (6.20)
ECS	7 (2.34)	3 (1.00)	0 (0.00)	0 (0.00)	10 (3.34)
Sputum	1 (0.33)	2 (0.67)	4 (1.34)	0 (0.00)	7 (2.34)
Blood	0 (0.00)	2 (0.67)	0 (0.00)	0 (0.00)	2 (0.67)
Total	121 (40.47)	147 (49.16)	24 (8.03)	7 (2.34)	299 (100.00)

HVS- High Vagina Swab; W/S- Wound Swab; E/S- Ear Swab; ECS- Endocervical Swab.

Table 4. Percentage distribution of *Klebsiella*, *Proteus* and *Pseudomonas species* in clinical from BSUTH and FMC.

Bacterial species	Hospital		Total {No. (%)}
	BSUTH {No. (%)}	FMC {No. (%)}	
Klebsiella sp.	25 (20.8)	95 (79.20)	120 (100.00)
Proteus sp.	20 (57.10)	15 (42.90)	35 (100.00)
Pseudomonas sp.	85 (100.00)	0 (0.00)	85 (100.00)
Total	130 (54.2)	110 (45.80)	240 (100.00)

X^2 = 125.754, df = 2, p = 0.00; BSUTH- Benue State University Teaching Hospital; FMC- Federal Medical Centre.

Table 5. Distribution of environmental Gram-negative bacterial from BSUTH and FMC.

Gram-negative bacterial species	Hospital		Total No. (%)
	BSUTH {No. (%)}	FMC {No. (%)}	
Citrobacter diversus	25 (55.60)	20 (44.40)	45 (100.00)
Citrobacter freundii	5 (100.00)	0 (0.00)	5 (100.00)
Citrobacter koseri	5 (50.00)	5 (50.00)	10 (100.00)
Erwinia chrysanthemi	0 (0.00)	5 (100.00)	5 (100.00)
E. coli	5 (33.30)	10 (66.70)	15 (100.00)
Klesiella pneumoniae	5 (33.30)	10 (66.70)	15 (100.00)
Proteus mirabilis	5 (25.00)	15 (75.00)	20 (100.00)
Proteus stuartii	0 (0.00)	15 (100.00)	15 (100.00)
Proteus vulgaris	40 (80.00)	10 (20.00)	50 (100.00)
Shigella liquefaciens	0 (0.00)	5 (100.00)	5 (100.00)
Shigella sonnei	5 (50.00)	5 (50.00)	10 (100.00)
Total	95 (48.70)	100 (51.3)	195 (100.00)

X^2 = 56.798, df = 10, p = 0.00; BSUTH- Benue State University Teaching Hospital; FMC- Federal Medical Centre.

Table 6. Frequency of Gram-negative bacterial isolated from Environmental samples in BSUTH and FMC.

S/N	Gram-negative bacterial	Sources	BSUTH	FMC	Total
1	*Citrobacter diversus*	Water	2	2	4
		Sediment	3	2	5
2	*Citrobacter freundii*	Sediment	1	0	1
3	*Citrobacter koseri*	Water	1	0	1
		Sediment	0	1	1
4	*E. coli*	Water	0	1	1
		Sediment	1	1	2
5	*Erwinia chrysanthemi*	Water	0	1	1
6	*Klebsiella pneumonia*	Water	1	1	2
		Sediment	0	1	1
7	*Providencia stuartii*	Water	0	1	1
		Sediment	0	2	2
8	*Proteus mirabilis*	Water	1	1	2
		Sediment	0	2	2
9	*Proteus vulgaris*	Water	4	1	5
		Sediment	4	1	5
10	*Serratia liquefaciens*	Water	0	1	1
		Sediment	1	0	1
11	*Shigella sonnei*	Water	0	1	1
		Sediment	1	0	1
	Total		19	20	39

BSUTH- Benue State University Teaching Hospital; FMC- Federal Medical Centre.

chrysanthemi from sediment and *S. liquefaciens* from water of equal distribution each (Table 6).

DISCUSSION

The most prevalent clinical bacterial isolate from the 299 confirmed Gram-negative bacteria from the two hospitals were *E. coli* 147(49.16%), followed by *Pseudomonas* sp. 121(40.47%), *Klebsiella* sp. 24(8.03%) and *Proteus* sp. 7(2.34%) as shown in Table 2. This is similar to a study conducted by Okesola and Ige (2012) who recorded *P. mirabilis* as the least dominant bacterial etiology of community acquired pneumonia. This study also correlates

with Rugira et al. (2016) who reported predominance of *E. coli* (51.2%) and *P. mirabilis* among the least occurrence of 2.3% in a tertiary hospital. El-Mahalawy et al. (2005) stated that it is important to recognize the importance of organisms like *E. coli, P. aeruginosa and Klebsiella* species as they cause higher mortality rates compared to Gram positive organisms.

These results indicate that the prevalence of urinary tract infection (UTI) is high in the two hospitals as urine samples (Tables 1 and 3) had the highest prevalence of bacterial isolates. A similar result was reported by Alauvdeen et al. (2017) who also isolated high prevalence of bacteria from urine. This may likely have been related to contamination of colonic bacteria (Ruston, 1997). The most prevalent bacterial isolate from the urine samples are *E. coli* from both hospitals. This result is similar with the report of Devanand and Ramchandra (2013) who reported high *E. coli* isolates from urine samples. Gram-negative bacteria, related to Enterobacteriaceae, in causing UTI have many factors which are responsible for their attachment to the uroepithelium. Their ability to colonize the urogenital mucosa with adhesins, pili, and fimbriae was established (Das et al., 2006).

Eleven (11) different bacterial species (Table 6) belonging to 6 genera were isolated which includes; *E. coli*, *S. sonnei*, *P. stuartii*, *Citrobacter diversus*, *K. pneumonia*, *C. freundii*, *C. koseri*, *E. chrysanthemi*, *P. mirabilis*, *P. vulgaris*, and *S. liquifaciens*. This is similar to the study of Mandal et al. (2011) who also isolated some of these organisms from the environment.

Conclusion

The study shows high level of distribution of *E*. *coli* and *pseudomonas* species in the clinical samples from the two hospitals while the wastewater and sediment of the two hospitals are contaminated with β-lactam resistant bacteria and can contribute to the spread of these bacteria.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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