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*Full Length Research Paper*

# Improving ethanol production by co-culturing of *Saccharomyces cerevisiae* with *Candida tropicalis* from rice husk hydrolysate media

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**The use of agricultural by-product as feed stock and co-culture fermentation is a good strategy for improving the efficiency of fermentation and ethanol production. Most rice husks have low protein and nitrogen content and need to be supplemented with nitrogen for fermentation process. This research sought to determine the optimal supplementation of rice husk stream–based fermentation medium with nitrogen and molasses sources, initial pH and incubation time for maximizing ethanol production by co-culturing *Saccharomyces cerevisiae* with *Candida tropicalis*. Urea, sodium nitrate and ammonium nitrate were used as nitrogen sources and molasses was used as carbon sources. Co-cultures of *S. cerevisiae* and *C. tropicalis* can use different nitrogen sources and molasses for growth and ethanol production. Molasses supplemented with rice husk hydrolysate medium, initial pH and incubation period significantly influenced ethanol yield and content of nitrogen and carbon in distillers grains (DDG). Maximum ethanol yield (20.32 ± 0.42%) with nitrogen (4.40 ± 0.11%) and carbon (9.20 ± 1.01%) content of DDG were obtained in the rice husk hydrolysate medium containing 16.0 g/l urea, 12.0 g/l $NaNO_3$, 12.0 g/l $NH_4NO_3$, 1.0 g/l $KH_2PO_4$, 0.7 g/l $MgSO_4 \cdot 7H_2O$, 20 ml/l molasses, 1.0 g/l $KH_2PO_4$ and 0.7 g/l $MgSO_4 \cdot 7H_2O$ with initial pH 5.5 and 6 days incubation period at 28 to 29°C, 50% relative humidity in the dark for 5 d in a rotary incubator at 60 rpm.**

**Key words:** Rice husk, *Saccharomyces cerevisiae, Candida tropicalis,* co-culture, ethanol yield, nitrogen and molasses.

## INTRODUCTION

The use of agricultural by-product as feed stock and co-culture fermentation is a good strategy for improving the efficiency of fermentation and ethanol production. Lignocellulosic biomass including agricultural by-product has been considered as possible raw material for ethanol production due to its renewability, large quantities,

relatively low prices compared to grain or sugar, potential environmental benefits and competactiveness with food (Cardona and Sa´nchez, 2007; Kumar et al., 2008; Lee and Huang, 2000; Mielenz, 2001; Service, 2007; Zaldivar et al., 2001; Ishola and Taherzadeeh, 2014).

The lack of a microorganism able to ferment efficiently all sugars released by hydrolysis from lignocellulosic materials has been one of the main factors preventing utilization of lignocellulose (Zaldivar et al., 2001). In a previous study, the simple sugar content in rice husk hydrolysate consists of 35.97% glucose, 8.87% xylose and 1.21% arabinose (Sopandi and Wardah, 2015). *Saccharomyces cerevisiae*, which is by far the dominant yeast used for ethanol production, naturally converts glucose to ethanol but does not metabolize xylose (Jeffries and Jin, 2004; Lin and Tanakan, 2006). In addition, other problem associated with efficient conversion of cellulose and hemicellulose sugars to ethanol is that during dilution of acid hydrolysis, a broad range of compounds which inhibit the fermenting microorganism are liberated or formed along with the sugars (Larsson et al., 2001). The ethanol yield and productivity obtained during fermentation of lignocellulosic hydrolysates decreases due to the presence of inhibiting compounds, such as weak acids, furans and phenolic compounds formed or released during thermo-chemical pre-treatment step such as acid and steam explosion (Parawira and Tekere, 2011).

Although it varies, most rice husks have low protein and nitrogen content and need to be supplemented with nitrogen for fermentation process. In one study, crude protein and nitrogen of rice husks were 4.38 and 0.7%, respectively, with C/N ratio of 57.93 (Ofoefule et al., 2011). In another study, crude protein, crude fiber and gross energy of rice husks were 1.92%, 37.33% and 302.33 kcal/kg, respectively (Telew et al., 2013). Nitrogen sources such as ammonium (Jones et al., 1994; Srichuwong et al., 2009) and urea (Jones and Ingledew, 1994; Yue et al., 2010) are widely used to increase yeast growth, and rate of sugar utilization and to reduce fermentation time (Chniti et al., 2015). Urea not only promoted the specific growth rate and ethanol tolerance, but also increased the ethanol yield and reduced the formation of side products (Yue et al., 2010). However, several investigators have reported the negative effects of using ammonium and urea as nitrogen supplements in ethanol fermentation (Laopaiboon et al., 2009; Wang et al., 2003; Beltran et al., 2005; Chniti et al., 2015).

Carbon and nitrogen are both required in yeast metabolism. The type and concentration of carbon and nitrogen sources as well as the C/N ratio of the medium, *S. cerevisiae* cultivation influence cellular growth and metabolites biosynthesis (Thomas et al., 1996). Molasses is a waste product of the sugar industry which can be used as a substrate for ethanol production by *S. cerevisiae* (Fern´andez-L´opez et al., 2012; Sadik and Halema, 2014). Molasses contains readily utilizable carbohydrates available in the form of fermentable sugars and can be used by the alcohol producing yeasts without any pretreatment (Murtagh, 1999).

Co-culture is a potential bioprocess whereby, there are no cross-interactions among microorganisms and each microorganism metabolizing its substrate is unaffected by the presence of other microorganism (Park et al., 2012). Co-culture of *S. cerevisiae* and other microorganism increases ethanol productivity which might be due to enhanced substrate utilization (Tesfaw and Assefa, 2014). Co-culture of *S. cerevisiae* with other microbes reduces inhibitory compounds in lignocellulosic hydrolysates (Tom´as et al., 2013; Taherzadeh and Karimi, 2011; Wan et al., 2012) which increases ethanol yield and production rate (Singh et al., 2014; Wan, 2012), shortens fermentation time, and reduces process cost (Hickert et al., 2013; Tesfaw and Assefa, 2014).

*C. tropicalis* have been demonstrated to produce ethanol from a mixed-sugar stream (Oberoi et al., 2010) and acid hydrolysate olive pruning (Mateo et al., 2015). It is able to degrade acetate, furfural, and 5-hydromethylfurfural and metabolite xylose to ethanol under anaerobic simultaneous saccharification and fermentation (Cheng et al., 2014). In a previous study, ethanol production from rice husks hydrolysate medium by co-culturing of *S. cerevisiae* and *C. tropicalis* higher than mono cultures of *S. cerevisiae* or *C. tropicalis* and other mono and co-cultures fermentation was more efficient in metabolizing and converting fermentable sugars than other selected microorganisms (Sopandi and Wardah, 2015). The present study explored the supplementation of inorganic nitrogen sources and molasses used to improve ethanol production by co-culturing of *S. cerevisiae* with *C. tropicalis* from rice husk hydrolisate.

## MATERIALS AND METHODS

### Culture microorganism

*S. cerevisiae* Food and Nutrition Culture Collection (FNCC) 3012

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and *C. tropicalis* FNCC 3033 were obtained from Microbiology Laboratories, PPAU Gadjah Mada University, Yogyakarta, Indonesia. Sabouraud agar (Oxoid) was used to maintain the strains. Working stock cultures were prepared from stock in 7 days at 28°C SA plate cultures subcultures from the master stock. Colonies were aseptically sampled by scraping the top with an inoculating loop and transferred to 10 ml sterile water. Inoculum stock suspensions were prepared from working stock and diluted to $1.7 \times 10^6$ cell/ml, as enumerated using a haemocytometer.

**Rice husk hydrolysis**

Locally farm-sourced rice husk from Sidoarjo, Indonesia was air-dried and then ground to approximately 2-mm diameter particles using a grinder mill. The milled rice husks (900 g) were steamed at 130°C for 3 h, cooled to room temperature, mixed with 15 l, 2.5% $H_2SO_4$ and autoclaved for 15 min at 121°C. Hydrolysate was cooled and stored at 1 to 5°C in the dark until it was used.

**Inorganic nitrogen supplementation**

The effect of inorganic nitrogen supplemented with rice husk hydrolysate medium on ethanol yield, N and C content of DDG was conducted using completely randomized design with 4 treatments of N sources where each treatment was replicated 5 times. The basal medium containing 1500 ml rice husk hydrolysate, 1.0 g/l $KH_2PO_4$, and 0.7 g/l $MgSO_4.7H_2O$ was dispensed into three Erlenmeyer flasks. Each 1000 ml urea, sodium nitrate ($NaNO_3$) and ammonium nitrate ($NH_4NO_3$) was added to final individual concentrations of 9.0 g/l, respectively. Media were mixed thoroughly, adjusted to pH 5.5 with an addition of NaOH, which is autoclaved for 15 min at 121°C and cooled to room temperature.

One hundred millilitres was aseptically dispensed into individual Erlenmeyer flasks (250 ml), inoculated with 1.0 ml of *S. cerevisiae* FNCC 3012 and 1.0 ml of *C. tropicalis* FNCC 3033 inoculum stock suspension. All flasks were incubated at 28 to 29°C with 50% relative humidity in the dark, for 5 d in a rotary incubator at 60 rpm.

**Molasses supplementation**

The effect of molasses supplemented with rice husk hydrolysate medium on ethanol yield, N and C content of DDG was conducted using completely randomized design with 5 treatments of molasses proportion in a medium and each treatment was replicated 5 times. Molasses was obtained from locally sugar industry, Mojekerto, Indonesia. Rice husk hydrolysate basal medium (2500 ml) containing 3.0 g/l urea, 3.0 g/l $NaNO_3$, 3.0 g/l $NH_4NO$, 1.0 g/l $KH_2PO_4$ and 0.7 g/l $MgSO_4 \cdot 7H_2O$ was dispensed into five 1000 ml Erlenmeyer flasks. Molasses was added to final concentrations of 0.0, 5.0, 10.0, 15.0 and 20.0 ml/l.

Media were mixed thoroughly, adjusted to pH 5.5 with an addition of NaOH or HCl 1 N, autoclaved for 15 min at 121°C and cooled to room temperature. One hundred millilitres was then aseptically dispensed into individual Erlenmeyer flasks (250 ml) with one ml of *S. cerevisiae* FNCC 3012 and *C.tropicalis* FNCC 3033 inoculum stock suspension and all flasks were incubated as described above.

**Formulation of rice husk hydrolysate**

The effect of formulation rice husk hydrolysate on ethanol yield, N and C content of DDG was conducted using completely randomized design with 4 treatments and each treatment was replicated five times. Four formulations of rice husk hydolysate media were examined to improve ethanol production by co-culturing *S. cerevisiae* FNCC 3012 with *C. tropicalis* FNCC 3033. Rice husk hydrolysate basal medium (2000 ml) containing 1.0 g/l $KH_2PO_4$ and 0.7 g/l $MgSO_4 \cdot 7H_2O$ was dispensed into four 1000 ml Erlenmeyer flasks. Individually were added 4.0 g/l urea, 3.0 g/l $NaNO_3$, 3.0 g/l $NH_4NO_3$, and 20 ml/l molasses ($F_1$), 8.0 g/l urea, 6.0 g/l $NaNO_3$, 6.0 g/l $NH_4NO_3$, and 20 ml/l molasses ($F_2$), 12.0 g/l urea, 9.0 g/l $NaNO_3$, 9.0 g/l $NH_4NO_3$, and 20 ml/l molasses ($F_3$), and 16.0 g/l urea, 12.0 g/l $NaNO_3$, 12.0 g/l $NH_4NO_3$ and 20 ml/l molasses ($F_4$), respectively.

Media were mixed thoroughly, adjusted to pH 5.5 with an addition of NaOH or HCl 1 N, autoclaved for 15 min at 121°C and cooled to room temperature. One hundred millilitres was then aseptically dispensed into individual Erlenmeyer flasks (250 ml) with one ml of *S. cerevisiae* FNCC 3012 and *C.tropicalis* FNCC 3033 inoculum stock suspension and all flasks were incubated as described above.

**Initial medium pH**

The effect of initial medium pH on ethanol yield, N and C content of DDG was conducted using completely randomized design with 8 treatments of initial pH medium (3.5 to 7.0) and each of the treatment was replicated 5 times.

To examine the effect of initial medium pH, 100 ml rice husk hydrolysate basal medium containing 1.0 g/l $KH_2PO_4$, 0.7 g/l $MgSO_4 \cdot 7H_2O$, 16.0 g/l urea, 12.0 g/l $NaNO_3$, 12.0 g/l $NH_4NO_3$ and 20 ml/l molasses was aliquoted into 8. 250-ml Erlenmeyer flasks and the pH of each was adjusted to 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0 prior to autoclaving for 15 min at 121°C with NaOH or HCl 0.1 N added. After cooling to room temperature, flasks were inoculated with 1-ml *S. cerevisiae* FNCC 3012 and 1- ml *C.tropicalis* FNCC 3033 inoculum stock suspension and incubated as described above.

**Incubation period**

The effect of incubation period on ethanol yield, N and C content of DDG was conducted using completely randomized design with 9 treatments of incubation period (1 to 9 d) and each of the treatment was replicated 5 times. The effect of incubation period on ethanol yield, nitrogen and carbon content distillate residue was examined using a rice husk hydrolysate basal medium containing 1.0 g/l $KH_2PO_4$, 0.7 g/l $MgSO_4 \cdot 7H_2O$, 16.0 g/l urea, 12.0 g/l $NaNO_3$, 12.0 g/l $NH_4NO_3$ and 20 ml/l molasses and adjusted to pH 5.5 by adding NaOH and autoclaved for 15 min at 121°C.

Erlenmeyer flasks (250 ml) containing 100-ml sterile medium were inoculated with 1-ml *S. cerevisiae* FNCC 3012 and 1- ml *C.tropicalis* FNCC 3033 inoculum stock suspension and incubated as described above. Ethanol yield, nitrogen and carbon content distillate residue analyses were carried out every day up to 9-days incubation. All data presented are means of four simultaneously incubated fermentation culture replicates.

**Determination of ethanol yield**

Whole flask cultures were distillated at 78°C for 60 min and ethanol in distillated were measured using a gas chromatograph Carbomax

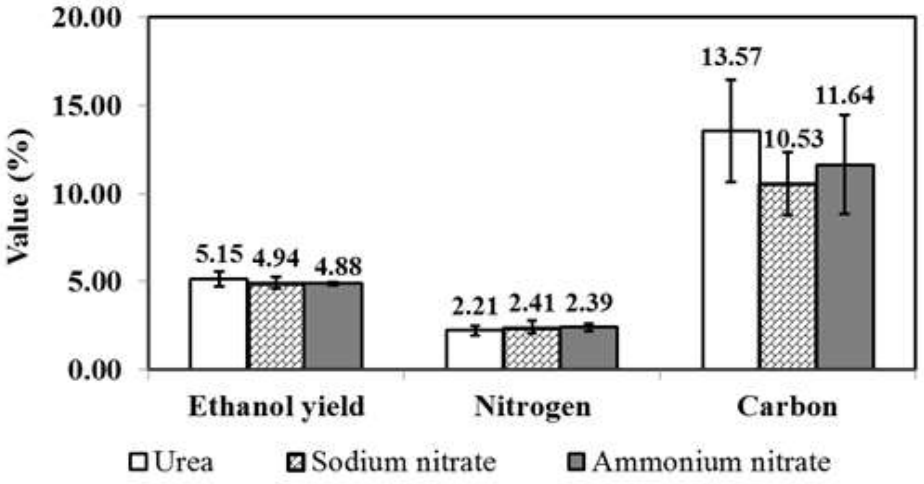


**Figure 1.** Ethanol yield, nitrogen and carbon content in DDG from rice husk hydrolysate medium supplemented inorganic nitrogen different that fermented by co-culture *S. cerevisiae* with *C. tropicalis*. Values and error bars represent means ± SD (n=5).

t70-10-0 column, FID t220 detector, helium as carrier gas with flow rate of 40.3 mL/min, tin column Porapack Q, detector temperature at 160°C and column temperature at 180°C with injection volume 1.0 µL. Fermented media were filtered through Whatmann No.1 paper prior to analysis.

$$\text{Ethanol yield (\%)} = \frac{\text{Concentration of ethanol from GC analysis (ml)}}{\text{Volume of medium (ml)}} \times 100$$

#### Determination of distillers dried grains

To analyze distillers' dried grains, whole flask cultures were distillated at 78°C for 60 min and residue was poured through predried (100°C) and preweighed Whatman No.1 filter paper. Retained material was washed with distilled water and ethanol until it became colourless and dried at 100°C to constant weight (48 h).

#### Determination of organic carbon

Levels of total organic carbon (TOC) were determined using the wet oxidation method of Walkey and Black (1965). One hundred millilitres (100 ml) of liquid culture was evaporated at 100°C for approximately 2 h to obtain a dried powder, 0.5 g of which was used for TOC determination.

#### Nitrogen determination

Nitrogen ($NH_4$-N) concentration was determined using the method of the American Society of Agronomy and Soil Science Society of America (1982). Ten-millilitre culture medium was evaporated at 100°C for approximately 2 h to obtain a dried powder. Samples (50 mg) were added to digestion tubes. 1-g selenium mixture (mashed 1.55 g $CuSO_4$, 96.9 g $Na_2SO_4$ and 1.55 g selenium) and 3-ml 97% $H_2SO_4$ were added, mixed and digested at 350°C for 4 h to obtain a colourless extract, cooled to room temperature, diluted to 50 ml with distilled water, shaken vigorously and left to stand overnight. Two-millilitre of extract was placed and transferred to a new borosilicate glass test tube. 4 ml potassium sodium tartrate buffer (50 g NaOH and 50 g $KNaC_4H_4O_6$ in 1000 ml distillated water) and sodium phenate solution (100 g NaOH and 125 g phenol in 1000 ml distillated water) were successively added, mixed and allowed to stand for 10 min. Four-millilitre of 5% NaOCl was also added, shaken and allowed to stand for 10 min with an absorbance measurement at 636 nm. $(NH_4)_2SO_4$ which was used to prepare N standards.

#### Statistical analysis

Tukey's honestly significant difference multiple comparison tests were used to segregate significantly different treatments using SPSS 16 software. Analysis of variance (ANOVA) was performed to determine significant differences between experiments ($P < 0.05$).

## RESULTS

### Effect of inorganic nitrogen supplementation

No significant ($P>0.05$) differences in ethanol yield or nitrogen and carbon content of distillers' dried grains (DDG) was observed between types of nitrogen source (Figure 1). Also, no significant ($P > 0.05$) differences were

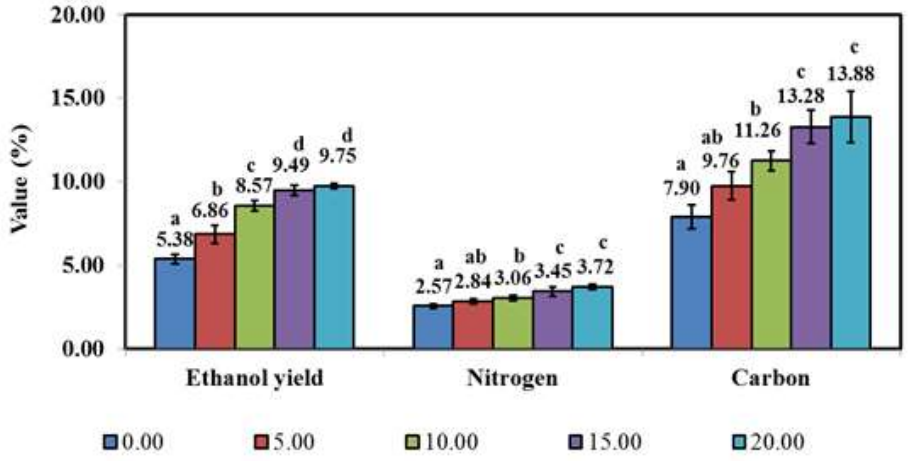


**Figure 2.** Effect of molasses addition on ethanol yield, nitrogen and carbon content of DDG rice husk hydrolysate medium that fermented by co-culture by co-culture *S. cerevisiae* with *C. tropicalis*. Values and error bars represent means ± SD (n=5) in same variable (ethanol yield, nitrogen and carbon) with different subscript shown ANOVA Tukey's test. a, b, c, d P<0.05 within respective groups.

observed between ethanol yields from rice husk hydrolysate basal media supplemented with urea. A similar lack of effect was observed for nitrogen content in DDG. Addition of inorganic nitrogen to the rice husk hydrolysate basal medium significantly affected (P > 0.05) carbon content of DDG.

**Effect of molasses supplementation**

Addition of 5 to 20 ml/l molasses to the rice husk hydrolysate basal medium significantly (P<0.05) increased ethanol yield, nitrogen and carbon content of DDG (Figure 2). Increasing amounts of molasses (5, 10, 15 and 20 ml/l) in the medium progressively increased ethanol yield. Ethanol yield in the basal medium alone is significantly (P<0.05) lower than that in the basal medium plus 5, 10, 15, and 20 ml/l molasses, but no significant (P>0.05) difference between 15 and 20 ml/l molasses. Nitrogen content of DDG from the rice husk hydrolysate basal medium was also significantly (P < 0.05) lower than that in the rice husk hydrolysate basal medium plus molasses (10, 15, and 20 ml/l).

Nitrogen content of DDG in the basal medium was not significantly different (P>0.05) from the basal medium plus of 5 ml/l molasses, but significantly (P<0.05) lower than that in basal medium plus 10, 15 and 20 ml/l molasses. However, there is no significant (P>0.05) difference between 15 ml/l and 20 ml/l molasses basal medium plus. This indicates molasses-concentration stimulates growth of yeast and ethanol production. While the mean carbon content of DDG in the basal medium was not significantly different (P>0.05) from that in the basal medium plus 5 ml/l molasses, it was significantly (P<0.05) lower than that in the basal medium plus 10, 15 and 20 ml/l molasses. However, no significant (P>0.05) difference was seen between 15 ml/l and 20 ml/l molasses.

**Formulation of rice husk hydrolysate media**

Formulation of rice husk hydrolysate media supplemented with inorganic nitrogen and molasses significantly (P<0.05) influenced ethanol yield, nitrogen and carbon content of DDG (Figure 3). The addition of nitrogen source and molasses to the rice husks hydrolysate fermentation media increased ethanol yield and nitrogen levels but lowered the carbon content of DDG.

Values and error bars represent means ± SD (n=5) in same variable (ethanol yield, nitrogen and carbon) with different subscripts shown in ANOVA Tukey's test. a, ab, b, bc, c P<0.05 within respective groups. F1; 1000 ml rice husk hydrolysate, 4.0 g/l urea, 3.0 g/l $NaNO_3$, 3.0 g/l $NH_4NO_3$, 1.0 g/l $KH_2PO_4$, 0.7 g/l $MgSO_4 \cdot 7H_2O$, 20 ml/l molasses, F2; $7H_2O$, 20 ml/l molasses, F3; 1000 ml rice husk hydrolysate 12.0 g/l urea, 9.0 g/l $NaNO_3$, 9.0 g/l

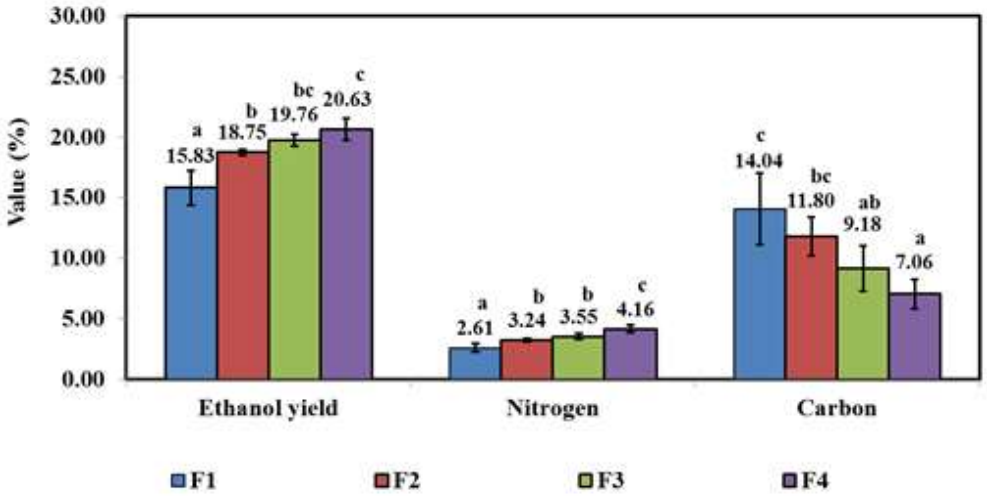


**Figure 3.** Effect of different formulation of rice husk hydrolysate culture medium on ethanol yield, nitrogen and carbon content of DDG were fermented by co-culture *S. cerevisiae* with *C. tropicalis*.

$NH_4NO_3$, 1.0 g/l $KH_2PO_4$, 0.7 g/l $MgSO_4 \cdot 7H_2O$, 20 ml/l molasses and F4; 1000 ml rice husk hydrolysate, 16.0 g/l urea, 12.0 g/l $NaNO_3$, 12.0 g/l $NH_4NO_3$, 1.0 g/l $KH_2PO_4$, 0.7 g/l $MgSO_4 \cdot 7H_2O$, 20 ml/l molasses.

Maximum ethanol yield, nitrogen and carbon content of DDG were obtained in the rice husk hydrolysate medium F4. Ethanol yield in F1 medium is significantly ($P<0.05$) lower than F2, F3 and F4. While mean nitrogen content of DDG in the F1 medium was significantly ($P<0.05$) lower than that in the F2, F3 and F4 medium, but no significant ($P > 0.05$) difference was observed between F2 and F3 medium. Carbon content of DDG in the F1 medium was also significantly ($P < 0.05$) higher than that in the F3 and F4 medium, but no significant ($P>0.05$) differences between F1 and F2 and between F3 and F4 also were observed in the medium.

## Effect of initial medium pH

Initial medium pH significantly ($P<0.05$) affected ethanol yield, nitrogen and carbon content of DDG (Figure 4). This study showed that *S. cerevisiae* and *C. tropicalis* grew and produced ethanol in co-culture, over a broad pH range (3.0-7.0).

An initial medium pH outside 5.5 to 6.5, decreased ethanol yield, nitrogen and carbon content of DDG. Ethanol yield at pH 5.5 and 6.0 was significantly ($P<0.05$) higher than that at pH 3.0, 3.5, 4.0, 4.5, 5.0, 6.5 or 7.0, with no significant ($P > 0.05$) difference observed between pH 5.5 and 6.0 and 6.0 and 6.5. Nitrogen contents of DDG pH 5.5, 6.0 and 6.5 were significantly ($P<0.05$) higher than those at pH 3.5, 4.0, 4.5, 5.0, or 7.0; no significant ($P > 0.05$) difference was observed between pH 5.0, 5.5, 6.0 and 6.5. There was significant diference in the carbon content mean of DDG at pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, or 7.0.

## Effect of incubation period

Incubation period significantly ($P<0.05$) affected ethanol yield (Figure 5A), nitrogen and carbon content of DDG (Figure 5B). Ethanol yield increased between 3 and 5 d, then was stagnant from 6 to 9 d total incubation. Ethanol yields at 2 and 3 d were significantly ($P<0.05$) lower than that at 4 d; 4 d ethanol yield was significantly ($P < 0.05$) lower than that at 5, 6, 7, 8, and 9 d. Differences in ethanol yield between 5 and 6 d incubation were not significant ($P > 0.05$). Ethanol yield at 5 d was also significantly ($P < 0.05$) lower than 7, 8 or 9 d, but there were no significant ($P < 0.05$) differences between 6, 7, 8 and 9 d ethanol yields.

Nitrogen content of DDG increased between 1 and 7d, then relatively stagnant from 7 to 9 days of total incubation. Nitrogen contents of DDG 1, 2, 3, 4, 5, and 6 d were significantly ($P<0.05$) lower than those at 7, 8 and 9 d. Differences in nitrogen content of DDG between 1, 2, 3, 4, 5 and 6 d incubation were relatively small ($P < 0.05$), but no significant ($P > 0.05$) differences were observed

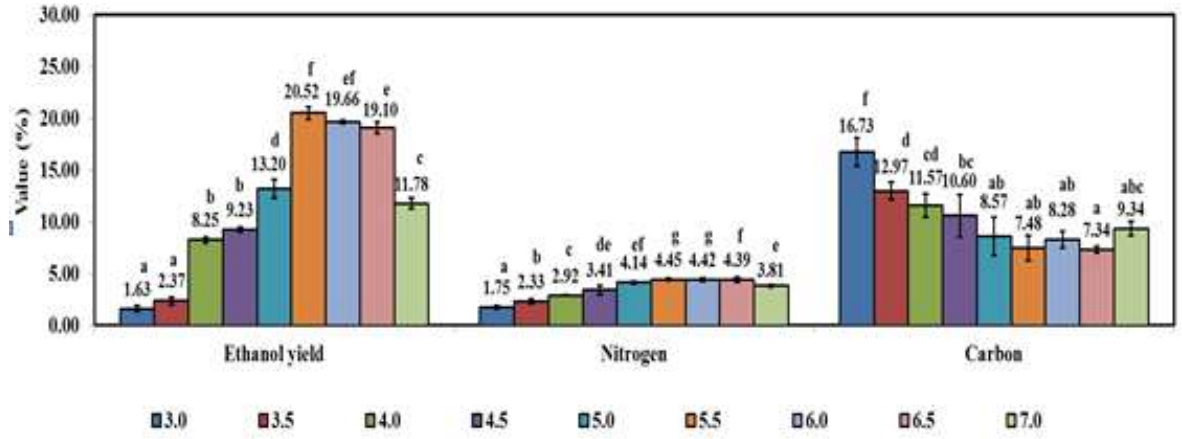


**Figure 4.** Effect of initial pH medium on ethanol yield, nitrogen and carbon content of DDG from rice husk hydrolysate culture medium with supplemented and fermented by co-culture *S. cerevisiae* and *C. tropicalis.* Values and error bars represent means ± SD (n=5) in same variable (ethanol yield, nitrogen and carbon) with different subscript shown ANOVA Tukey's test. a, b, c, d, e, g P<0.05 within respective groups.

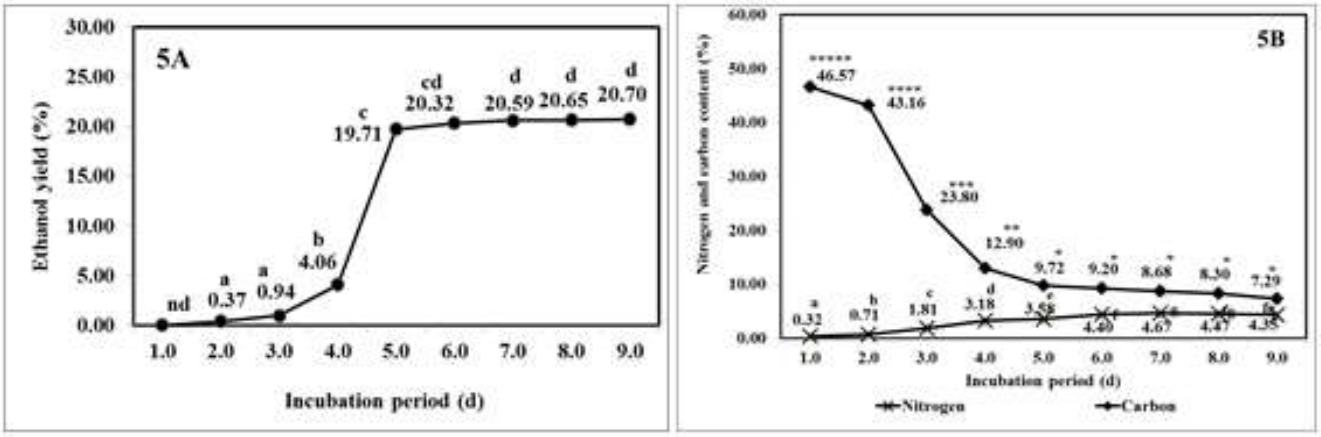


**Figure 5.** Effect of incubation period on ethanol yield (5A) and nitrogen and carbon content (5B) of distillate residue fermented rice husk hydrolysate medium by co-culture *S. cerevisiae* and *C. tropicalis.* Values and error bars represent means ± SD (n=5) in same variable (ethanol yield, nitrogen and carbon) with different subscript shown ANOVA Tukey's test. a, b, c, d, e, f, fg, g and *, **, ***, ****, **** P<0.05 within respective groups.

between 7, 8 and 9 d incubation. The mean nitrogen content of DDG decreased between 1 and 4 days, but was relatively stagnant from 5 to 9 days total incubation. Carbon contents of DDG 1, 2, 3, and 4 d were significantly (P<0.05) higher than those at 5, 6, 7, 8 and 9 d. Differences in nitrogen content of DDG between 1, 2, 3 and 4 days incubation were relatively small (P < 0.05), but no significant (P > 0.05) differences were observed between 5, 6, 7, 8 and 9 d incubation.

## DISCUSSION

Studies in other fermentation systems have revealed that N deficiency in the fermentation medium leads to slow and stuck fermentation rate (Vilanova et al., 2007). N sources are very crucial and strongly influence the yeast growth and metabolism during fermentation (Beltran et al., 2005). The present study shows no significant differences in ethanol yield or N and C content of DDG at

exogenous N sources supplemented ($NH_4NO_3$, urea and $NaNO_3$) with rice husk fermentation media. This indicates that the co-culture of *S. cerevisiae* and *C. tropicalis* can be utilized on the various sources of N for growth and stimulation of ethanol production.

Some investigators have reported varying effects of exogenous N source supplemented with lignocellulosic fermentation media on ethanol production by yeast. The results of this work are similar to several studies which reported that supplementation of the various sources of N with fermentation media does not significantly affect the production of ethanol. Fern´andez-L´opez et al. (2012) reported the addition of yeast extract, ammonium sulfate, urea, and their combination to medium of sugar rich molasses which was obtained during the second step of crystallization but did not improve ethanol productivity significantly. Wang et al. (2012) reported that, for the integrated ethanol-methane fermentation system, ammonium and other component in the effluent promoted yeast growth and fermentation rate but did not increase the yield of ethanol. However, the results of this work differ from several studies which reported that the supplementation of various N sources to fermentation media affected ethanol production. Mongkolchaiarunya et al. (2016) reported that ammonium nitrate is better than ammonium chloride, ammonium sulfate, urea and peptone as N sources for ethanol production from cattail. Li et al. (2016) reported that the combination of urea and ammonium sulfate as nitrogen sources synergistically enhanced ethanol production by *S. cerevisiae* in a very high gravity fermentation of corn starch.

Initial sugar concentrations before fermentation in the growth media can influence the specific rate of yeast growth and ethanol production (Tesfaw and Assefa, 2014). There are varieties of yeast, which are used to convert molasses into ethanol and $CO_2$, such as *S. cerevisiae and Klyureomyces marxianus* (Parkash, 2015). The present study shows that supplementation of molasses in the growth media significantly ($P<0.05$) increased ethanol yield and the N and C content in the distillers grains. Production of ethanol from molasses-based media by co-culture fermentation has been reported. Eiadpum et al. (2012) reported that immobilized co-culture of *K. marxianus* and *S. cerevisiae* can improve ethanol production from both sugarcane juice and blackstrap molasses when the operating temperature ranged between 33°C and 45°C and generate maximal ethanol concentrations of 81.4 and 77.3 g/l, respectively.

Carbohydrates and nitrogenous compounds are two major components affecting yeast performance in fermentation. A high level of N sources significantly increased the efficiency of fermentation and yeast yield (Tyagi and Ghose, 1980). Increasing the N concentration in the fermentation medium can increase the rate of fermentation, decrease the duration and lack of nitrogen triggers sluggish fermentations **(**Alexandre and Charpentier, 1998; Fleet and Heard, 1992; Varela et al., 2004). The ratio of N sources to carbon sources in the medium can influence yeast growth and metabolism of *S. cerevisiae* (Larsson et al., 1993). N deficiency with a high sugar transporter turnover rate results in a loss of sugar uptake capacity in the cells (Salmon, 1989; Bisson, 1999). In the present study, 4 formulations of rice husk hydrolysate media with different supplemented inorganic nitrogen and molasses significantly ($P<0.05$) influenced ethanol yield, nitrogen and carbon content of DDG.

The specific rate of yeast growth and ethanol production were influenced by pH fermentation medium (Tesfaw and Assefa, 2014). In the present work, initial pH of the medium affected ethanol yield and the content of N and C at DDG. A wide range of optimum pH (4.0 to 8.0) was reported for *S. cerevisiae* JZ1C isolated from rhizosphere of Jerusalem artichoke using inulin and Jerusalem artichoke tuber as substrate at 35°C (Hu et al., 2012). Optimum pH for *S. cerevisiae* BY4742 was in the range of 4.0 to 5.0. When the pH was lower than 4.0, the incubation period was prolonged though the ethanol concentration was not reduced significantly and when the pH was above 5.0, the concentration of ethanol diminished substantially (Lin et al., 2012). Some investigators have reported the effect of incubation period on ethanol production from lignocellulosic medium by co-culture fermentation. Wright (1988) reported the maximum ethanol production of 4% (w/v) from wheat straw medium after 48 h of incubation, employing process of simultaneous saccharification and fermentation using *T. reesei* cellulase and *Kluyveromyces fragilis*. Sharma (2000) reported maximum ethanol yield and fermentation efficiency of 0.397 g/g and 77.84%, respectively after 36 h of incubation at 30°C using mixed culture of *S. cerevisiae* and *P. tannophilus*. Verma *et al*. (2000) reported maximum ethanol concentration of 24.8 g/l at 48 h of incubation from starch medium in a single step process by co-culturing of amylolytic yeasts and *S.cerevisiae.*

In the present study, the maximum ethanol yield (20.32%) lower than the theoretical maximum ethanol yield of broth hexoses and pentoses is 0.511 kg/kg sugar, but higher than the ethanol yield from rice husk which has been reported by some investigators. Reddy and Pushpa (2012) reported the maximum ethanol yield (1.60%) obtained from rice husks, treated with 5% sodium hydroxide and fermented by *S. cereveceae* type 181 at pH 5.0 for 7 d. Sopandi and Wardah (2015) reported the maximum ethanol yield (2.13 %) gained from rice husk hydrolysate medium with supplement of 4 g/l urea, 3 g/l

$NaNO_3$, 3 g/l $NH_4NO_3$, 1 g/l $KH_3PO_4$ and 0.7 g/l $MgSO_4 \cdot 7H_2O$ fermented by co-culturing of *S. cerevisiae* and *C. tropicalis* for 3 d at 30°C, 60 to 70% relative humidity, under dark condition, and 150 rpm agitation) incubation. Gaffa and Krakwowiak (1997) reported the maximum ethanol yield (10.5%) by *S. cerevisiae* continuous fermentation process from molasses diluted tap water (1:2) for 14 d at 27°C.

## Conclusion

Inorganic nitrogen and molasses supplementation can increase the production of ethanol from rice husk hydrolysate medium by co-culturing of *S. cerevisiae* and *C. tropicalis*. Initial pH medium and incubation period demonstrated can influence ethanol production by co-culturing of *S. cerevisiae* and *C. tropicalis* from the rice husk medium supplemented with molasses. The best formulation medium to obtain maximum production of ethanol with pH 5.5 and incubation period of 6 days comprised of 16.0 g/l urea, 12.0 g/l $NaNO_3$, 12.0 g/l $NH_4NO_3$, 1.0 g/l $KH_2PO_4$, 0.7 g/l $MgSO_4 \cdot 7H_2O$, and 20 ml/l molasses in 1000 ml rice husks hydrolysate.

## Conflict of Interests

The authors have not declared any conflict of interest.

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## REFERENCES

Alexandre H, Charpentier C (1998). Biochemical aspects of stuck and sluggish fermentation in grape must. J. Ind. Microbiol Biotechnol. 20:20-27.

American Society of Agronomy and Soil Science Society of America (1982). Methods of Soil Analysis. 2nd edition, Part 2. *In* Page et al (*Eds*.). Chemical and Microbiological Properties. Madison, Wisconsin, USA.

Beltran G, Esteve-Zarzoso B, Rozes N, Mas A, Guillamon JM (2005).Influence of the timing of nitrogen additions during synthetic grape must fermentations on fermentation kinetics and nitrogen consumption. J. Agric. Food. Chem. 53(4):996-1002.

Bisson L (1999). Stuck and sluggish fermentations. Am. J. Enol. Vitic. 50:107-119.

Cardona CA, Sa´nchez OJ (2007). Fuel ethanol production: process design trends and integration opportunities. Bioresour. Technol. 98:2415-2457.

Cheng KK, Wu J, Lin ZN, Zhang JZA (2014). Aerobic and sequential anaerobic fermentation to produce xylitol and ethanol using non-detoxified acid pretreated corncob. Biotechnol. Biofuels. 7:166.

Chniti S, Jemni M, Rejeb ZB, Chaabane H, Hassouna M, Amrane A, Djelal H (2015). Effect of the Nitrogen Source on Bioethanol Production from Syrup Dates by *Saccharomyces cerevisiae.* Inter. Agric. Innov. Res. 4(3):530-535.

Eiadpum A, Limtong S, Phisalaphong M (2012). High-temperature ethanol fermentation by immobilized coculture of *Kluyveromyces marxianus* and *Saccharomyces cerevisiae*. 114(3):325-329.

Fernández-López CL, Torrestiana-Sánchez B, Salgado-Cervantes MA, García PG, Aguilar-Uscanga MG (2012). Use of sugarcane molasses "B" as an alternative for ethanol production with wild-type yeast *Saccharomyces cerevisiae* ITV-01 at high sugar concentrations. Bioproc. Biosys. Eng. 35(4): 605-614.

Fleet G, Heard G (1992). Yeasts—growth during fermentation, p. 27-54. *In* G. H. Fleet (ed.), Wine microbiology and biotechnology. Harwood Academic Publishers, Camberwell, Australia.

Gaffa T, Krakwowkiak A (1997). Production of ethyl alcohol from molasses using continuous process. Nig. J. Biotech. 8(1):35-39.

Hickert LR, Da Cunha-Pereira F, De Souza-Cruz PB, Rosa CA, Ayub MAZ (2013). Ethanogenic fermentation of cocultures of *Candida shehatae* HM 52.2 and *Saccharomyces cerevisiae* ICVD254 in synthetic mediumand rice hull hydrolysate. Biores. Technol. 131:508-514.

Hu N, Yuan B, Sun J, Wang SA, Li FL (2012). Thermotolerant *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* strains representing potentials for bioethanol production from Jerusalem artichoke by consolidated bioprocessing. Appl. Microbiol. Biotechnol. 95(5):1359-1368.

Ishola MM, Taherzadeh MJ (2014). Effect of fungal and phosphoric acid pretreatment on ethanol production from oil palm empty fruit bunches (OPEFB). Biores Technol. 165:9-12.

Jeffries TW, Jin YS (2004). Metabolic engineering for improved fermentation of pentoses by yeasts. Appl. Microbiol. Biotechnol. 63(5):495-509.

Jones AM, Thomas KC, Ingledew WM (1994). Ethanolic fermentation of blackstrap molasses and sugarcane juice using very high gravity technology. J. Agric. Food. Chem. 42(5):1242-1246.

Jones AM, Ingledew W (1994). Fuel alcohol production: appraisal of nitrogenous yeast foods for very high gravity wheat mash fermentation. Process Biochem.29:483-488.

Kumar R, Singh S, Singh OV (2008). Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. J. Ind. Microbiol. Biotechnol. 35:377-391.

Laopaiboon L, Nuanpang S, Srinophakun P, Klanrit P, Laopaiboon P (2009). Ethanol production fromsweet sorghumjuice using very high gravity technology: Effects of carbon and nitrogen supplementations. Bioresour. Technol. 100(18):4176-4182.

Larsson C, von Stockar U, Marison I, Gustafsson L (1993). Growth and metabolism of *Saccharomyces cerevisiae* in chemostat cultures under carbon-, nitrogen-, or carbon- and nitrogen-limiting conditions. J. Bacteriol. 175:4809-4816.

Larsson C, von Stockar U, Marison I, Gustafsson L (1993). Growth and metabolism of Saccharomyces cerevisiae in chemostat cultures under carbon-, nitrogen-, or carbon- and nitrogen-limiting conditions. J. Bacteriol.175:4809-4816.

Larsson S, Cassland P, Jonsson LJ (2001). Development of a *Saccharomyces cerevisiae* Strain with Enhanced Resistance to Phenolic Fermentation Inhibitors in Lignocellulose Hydrolysates by Heterologous Expression of Laccase. Appl. Environ. Microbiol. 67(3): 1163-1170.

Lee WC, Huang CT (2000). Modeling of ethanol fermentation using *Zymomonas mobilis* ATCC 10988 grown on the media containing glucose and fructose. Biochem. Eng. J. 4 (3):217-227.

Li Z, Wang D, Shi YC (2016). Effects of nitrogen source on ethanol production in very high gravity fermentation of corn starch. J. Taiwan. Inst. Chem. Eng. 70:229-235.
Lin Y, Tanaka S (2006). Ethanol fermentation from biomass resources: current state and prospects. Appl. Environ. Microbiol. 69(6):627–642.
Lin Y, Zhang W, Li C, Sakakibara K, Tanaka S, Kong H (2012). Factors affecting ethanol fermentation using *Saccharomyces cerevisiae* BY4742. Biomass and Bioenergy. 47:395-401.
Mateo S, Puentes JG, Moya AJ, Sánchez S (2015). Ethanol and xylitol production by fermentation of acid hydrolysate from olive pruning with Candida tropicalis NBRC 0618. Bioresour. Technol.190:1-6.
Mielenz JR (2001). Ethanol production from biomass: technology and commercialization status. Curr. Opin. Microbiol. 4:324-329.
Mongkolchaiarunya S, Vaithanomsat P, Chuntranuluck S (2016). Effect of Nitrogen Source on Ethanol Production from Weeds by a Simultaneous Saccharification and Fermentation Process. KKU Res.j. 22(1):210-213.
Murtagh TE (1999). Molasses as a feedstock for alcohol production. In: Jacques KA, Lyons TP, Kelsall DR, editors. The Alcohol Textbook. 2nd edition. London, UK: Nottingham University Press.
Oberoi HS, Vadlani PV, Brijwani K, Bhargav VK, Patil RT (2010). Enhanced ethanol production via fermentation of rice straw with hydrolysate-adapted *Candida tropicalis* ATCC 13803. Process Biochem. 45(8):1299-1306.
Ofoefule AU, Onyeoziri MC, Uzodinma EO (2011). Comparative study of biogas production from chemically-treated powdered and un-powdered rice husks. Envir. Chem. Ecotoxicol. 3(4):75-79.
Parawira W, Tekere M (2011). Biotechnological strategies to overcome inhibitors in lignocellulose hydrolysates for ethanol production: review. Crit. Rev. Biotechnol. 1(1):20-31.
Parkash A (2015). Modeling of Ethanol Production from Molasses: A Review. Ind. Chem. 1:2.
Park EY, Naruse K, Kato T (2012). One-pot bioethanol production from cellulose by co-culture of Acremonium cellulolyticus and Saccharomyces cerevisiae. Biotechnol. Biofuels. 5(64):2-11.
Reddy TKS, Pushpa A (2012). Studies on characterizations of agriculture waste (rice husk) for the production of ethanol. J. Environ. Res. Develop. 7(2):1076-1084.
Sadik MW, Halema AA (2014). Production of Ethanol from Molasses and Whey Permeate Using Yeasts and Bacterial Strains. Int. J. Curr. Microbiol. App. Sci. 3(3): 804-818.
Salmon J (1989). Effect of sugar transport inactivation in *Saccharomyces cerevisiae* on sluggish and stuck enological fermentations. Appl. Environ. Microbiol. 55:953-958.
Service RF (2007). Cellulosic ethanol: biofuel researchers prepare to reap a new harvest. Science 315:1488-1491.
Sharma SK (2000). Saccharification and bioethanol production from sunflower stalks and hulls. Ph.D. Thesis, Punjab Agricultural University, Ludhiana, India.
Singh A, Bajar S, Bishnoi NR (2014). Enzymatic hydrolysis of microwave alkali pretreated rice husk for ethanol production by *Saccharomyces cerevisiae*, *Scheffersomyces stipitis* and their coculture. Fuel. 116:699-702.
Sopandi T, Wardah A (2015). Sugar consumption in mono and co-culture *Saccharomyces cerevisiae* and others selected microorganism for bioethanol production from stream rice husk medium. Asian. J. Microbiol. Biotechnol. Environ. Sci. 17(3):89-98.
Srichuwong S, Fujiwara M, Wang X, Sevama T, Shiroma R (2009). Simultaneous saccharification and fermentation (SSF) of very high gravity (VHG) potato mash for the production of ethanol. Biomass Bioenergy. 33(5):890-898.
Taherzadeh MJ, Karimi K (2011). Fermentation inhibitors in ethanol processes and different strategies to reduce their effects. Biofuels. 287-311.
Telew C, Kereh VG, Untu IM, Rembet (2013). The improvement of the rice husk nutritional value with a biotechnology Effective microorganisms (EM4) as an organic feed ingredient. Zootek J. 32(5):1-8.
Tesfaw A, Assefa F (2014). Current Trends in Bioethanol Production by *Saccharomyces cerevisiae*: Substrate, Inhibitor Reduction, Growth Variables, Coculture, and Immobilization. Rev. Art. Inter. Scho. Res Not. pp. 1-11.
Thomas KC, Hynes SH, Ingledew WM (1996). Effect of nitrogen limitation on synthesis of enzymes in *Saccharomyces cerevisiae* during fermentation of high concentration of carbohydrates. Biotechnol. Lett. 18:1165-1168.
Tom´as AF, Karag¨oz P, Karakashev D, Angelidaki I (2013). Extreme thermophilic ethanol production from rapeseed straw: using the newly isolated *Thermoanaerobacter pentosaceus* and combining it with *Saccharomyces cerevisiae* in a two-step process. Biotechnol. Bioeng. 110(6):1574-1582.
Tyagi RD, Ghose TK (1980). Batch and multistage continuous ethanol fermentation of cellulose hydrolysate and optimum design of fermentor by graphical analysis. Biotechnol. Bioeng. 22(9):1907-1928.
Varela C, Pizarro F, Agosin E (2004). Biomass Content Governs Fermentation Rate in Nitrogen-Deficient Wine Musts. Appl. Environ. Microbiol. 70(6):3392-3400.
Verma G, Nigam P, Singh D, Chaudhary K (2000). Bioconversion of starch to ethanol in a single step process by co-culture of amylolytic yeasts and *Saccharomyces cerevisiae.* Bioresour. 72:261-266.
Vilanova M, Ugliano M, Varela C, Siebert T, Pretorius IS, Henschke PA (2007). Assimilable nitrogen utilisation and production of volatile and non-volatile compounds in chemically defined medium by Saccharomyces cerevisiae wine yeasts. Appl. Microbiol. Biotechnol. 77(1):145-157.
Walkey C, Black CA. (1965). *S*oil organic matter in methods of soil analysis. C.A. Black, Ed. Agronomy No 9, Part 2, American Society of Agronomy, Madison, WI.
Wan P, Zhai D, Wang Z, Yang X, Tian S (2012). Ethanol Production from Nondetoxified Dilute-Acid Lignocellulosic Hydrolysate by Cocultures of *Saccharomyces cerevisiae* Y5 and *Pichia stipitis* CBS6054. Biotechnol, Res. Inter. pp. 6-11.
Wang XD, Bohlscheid JC, Edwards CG (2003). Fermentative activity and production of volatile compounds by Saccharomyces grown in synthetic grape juice media deficient in assimilable nitrogen and/or panthotenic acid. J. Appl. Microbiol. 94:349-359.
Wang M, Han J, Dunn JB, Cai H, Elgowainy A (2012). Well-to-wheels energy use and greenhouse gas emissions of ethanol from corn, sugarcane and cellulosic biomass for US use. Environ. Res. Lett. 7(4):1-13.
Wright JD (1988). Ethanol from biomass by enzymatic hydrolysis. Chem. Eng. Prog. 84:62-74.
Yue G, Yu J, Zhang X, Tan T (2010). The influence of nitrogen sources on ethanol production by yeast from concentrated sweet sorghum juice. Biomass. Bioenerg. 39:48-52.
Zaldivar J, Nielsen J, Olsson L (2001). Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. Appl. Microbiol. Biotechnol. 56:17-34.

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# Engineered microbial consortium for the efficient conversion of biomass to biofuels: A preliminary study

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**This work showed ethanol production by a microbial consortium of *Clostridium cellulolyticum* and a recombinant *Zymomonas mobilis* (ZM4 pAA1). The ZM4 pAA1 and wild type ZM4 (ZM4 WT) were first tested on RM medium (ATCC 1341) containing 2% cellobiose as the carbon source. Ethanol production from *Z*M4 pAA1 was three times higher than that observed from the *Z*M4 WT. Concomitant with ethanol production was the reduction in OD from 2.00 to 1.580. The ZM4 pAA1 was then co-cultured with *C. cellulolyticum* using cellobiose and microcrystalline cellulose, respectively, as carbon sources. Results indicate that the ZM4 pAA1 with *C. cellulolyticum* utilized 2.0 g/L cellobiose, producing as much as 0.40 mM of ethanol, whereas only 0.20 mM ethanol was detected for the ZM4 WT co-cultured with *C. cellulolyticum* under similar conditions. A consortium of the ZM4 pAA1 and *C. cellulolyticum* using 7.5 g/L microcrystalline cellulose gave a far lower ethanol yield than when using cellobiose. In the latter case, ethanol production was detected within 5 days, whereas it took about 10 days for ethanol to be detectable for the ZM4 WT and *C. cellulolyticum*. Future efforts will concentrate on identifying suitable partners for the ZM4 pAA1, the correct concentration of feedstocks at which synergy will be observed, as well as optimize medium formulations and inoculation techniques.**

**Key words:** Biofuel, ethanol, cellulosome, consortium, *Zymomonas mobilis, Clostridium cellulolyticum.*

## INTRODUCTION

*Zymomonas mobilis* is a facultative anaerobic Gram-negative bacterium belonging to the alpha subdivision of the phylum Proteobacteria, class Alpha-Proteobacteria, order Sphingomonadales and family Sphingomonadaceae. It is rod shaped with dimensions 1.0-2.0 × 4.0-5.0 µm, motile, does not sporulate, does not produce capsules, intracellular lipids or glycogen, optimal pH range for growth is 6 to 7.0, optimal temperature range is 25 to 31°C, the G + C content of the cellular DNA is about 47.5 to 49.5% with an average *T*m of 89.3 to 89.5°C (Gunasekeran et al., 1990). *Z. mobilis* uses the Entner-Doudoroff (ED) pathway which is found in microrganisms that are strictly aerobic, conducts fermentation with 50% less ATP produced relative to the Embden-Meyerhof-Parnas (EMP) pathway, which leads to improved ethanol yield (Yang et al., 2016).

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*Clostridium cellulolyticum* ATCC 35319 formerly identified as strain $H_{10}$ was isolated in the fall of 1982 from decayed grass compost packed for 3 to 4 months at the Université de Nancy, France. it is a Gram-positive, straight to slightly curved rods that are 3 to 6 µm long by 0.6 to 1.0 µm wide, with a mean G + C content of 41% and forms spores in cultures of cellulose media 3 or more days old (Petitdemange et al., 1984). *C. cellulolyticum* produces several cellulases, which are re-grouped into an extracellular enzymatic complex called cellulosomes and cellulolytic activities allow the release of soluble cellodextrins from cellulose, which in return permits microbial growth (Desvaux, 2005).

The wild-type *Z. mobilis* was primarily isolated from alcoholic liquids in natural environments containing fermentable sugars such as plant saps, and can only utilize a limited carbon source, including glucose, fructose and sucrose (Weir, 2016). This drawback is a major reason why it has not been used extensively for bioethanol production on an industrial scale. *C. cellulolyticum* is excellent at cellulose utilization due to the presence of cellulosomes, a complex mass of enzymes comprised of an array of cellulases and hemicellulases, but unlike *Z. mobilis* has a low yield of ethanol production due to its inability to utilize higher concentrations of pure cellulosic substrates. It has been suggested that there is also an overflow of pyruvate higher than the rate of procession of pyruvate ferridoxin oxidoreductase (PFO) and lactate dehydrogenase (LDH) (Senthikumar and Gunasekaran, 2005) in *C. cellulolyticum*.

Engineered microbial consortia and co-culturing of wild type bacteria have become pivotal tools in biotechnology and have been utilized in the production of a wide range of products from biofuels to pharmaceuticals. For examples, Abate et al. (1996) described ethanol production by a co-culture of *Z. mobilis* and *Saccharomyces* sp. with higher yields and production rates than with either microorganism in pure culture. Shin et al. (2010) genetically engineered two *E. coli* strains for xylan utilization, with one strain expressing two hemicellulases to hydrolyze xylan into xylooligosaccharides and another one importing the xylooligosaccharides to produce ethanol, with a 55% theoretical yield. Similarly, Shou et al. (2007) demonstrated a slightly more ideal cooperation using two engineered *Saccharomyces cerevisiae* strains. One strain required adenine and overproduced lysine while the other strain required lysine and overproduced adenine. Singh et al. (2014) co-cultured *Pichia stipitis* and *Z. mobilis* for bioethanol production from kans grass biomass with significant yields and Zhang et al. (2016a) employed *C. cellulolyticum* and hydrogen fermentation bacteria for enhanced biohydrogen production from corn stover with significant differences seen in the metabolites of the co-culture system over the mono-cultures. Other reports of successful bio-catalysis based on microbial consortia have equally been reported (Fu et al., 2009; He et al., 2011; Ho et al., 2011; Li et al., 2011; Quinn et al., 2016; Reddy and Basappa, 1996; Yaun et al., 2016; Zhang et al., 2016b; Zhong et al., 2016).

In order to improve its industrial appeal in substrate utilization and sugar transport, *Z. mobilis* has been genetically modified extensively with significant improvements over the wild type, for example Luo and Bao (2015), expressed a heterologous β-D-glucosidase from *Bacillus polymyxa* in *Z. mobilis,* where the signal peptide ZMO 1086 facilitated its secretion. Other reported efforts include the works of Deanda et al. (1996), Dunn and Rao (2014), Yanase et al. (2005) and Yanase et al. (2012). To the best of the author's knowledge, there has not been any reported microbial consortium involving *Z. mobilis and C. cellulolyticum* whether as wild types or engineered clones for the production of bioethanol. Given the immense potentials as previously outlined of these two bacteria, it became necessary to establish a testbed from which further research would be conducted in order to improve the process of bioethanol production. The present study aimed to study the effects of using a consortium of a recombinant *Z. mobilis* and *C. cellulolyticum* on the conversion of biomass to bioethanol and using this study as a Launchpad for further experimental studies and process improvement.

## MATERIALS AND METHODS

### Construction of ZM4 pAA1

#### *Amplification of pBBR1 MCS-3*

The vector backbone, pBBR1 MCS-3 (Kovach et al., 1995) was linearized with the restriction enzyme *Kpn*I, the linearized vector verified for size correctness on electrophoresis gel to give a band size of 5.2 kb. The fragment was PCR amplified using Phusion DNA polymerase (NEB), with the PCR conditions set at 98°C for 1 min, 98°C for 30 seconds, 56°C for 30 s for annealing and 72°C for 90 s for extension. The cycle was repeated 35 times from the second to the fourth step, a final extension for 5 min at 72°C and a hold at 4°C.

#### *Amplification of celZ and celY genes from Erwinia chrysenthemi*

The *celY* endoglucanase gene and *celZ* endoglucanase gene with the ZM4 promoter from pLOI 2352 (kindly provided by Professor L. Ingram, University of Florida) were individually amplified using Phusion DNA polymerase (NEB). For the *celY* gene, the PCR condition was set at 98°C for 1 min, 98°C for 30 s, 72°C for 45 s (to include annealing and extension). The cycle was repeated 35 times from the second to the merged annealing and extension steps, a final extension for 5 min at 72°C and a hold at 4°C. Similar conditions were used for the *celZ* gene with the ZM4 promoter but with the annealing temperature set at 54°C for 30 s and extension time for 45 s. The amplicons were verified on the gel for size correctness and further sequenced for correctness.

**Table 1.** List of primers.

| Primer name | Sequences |
|---|---|
| pBBR1mcs-3 (forward) | AGGGATAAGGTACCGGGCCCCCC |
| pBBR1mcs-3 (reverse) | GGTTGATCCAGCTTTTGTTCCCTTT |
| *celZ* with ZM4 promoter (forward) | AAAAGCTGGATCAACCGGCAATTT |
| *celZ* with ZM4 promoter (reverse) | CTCCTTCTTCAATTAGTTACAGCTACCAA |
| *celY* (forward) | CTAATTGAAGAAGGAGAATGAATGGGAAAGCC |
| *celY* (reverse) | CTCCTTCTTTATTTACCGCGCGCCAACATCAC |
| *gfor-betaglc* fusion (forward) | GTAAATAAAGAAGGAGTAAGAATGACGAACAA |
| *gfor-betaglc* fusion (reverse) | CCGGTACCTTATCCCTCTAACATGGAATTCAG |

***Amplification of glucose-fructose oxidoreductase (GFOR) leader sequence of Z. mobilis and B-glucosidase gene of Ruminococcus albus in Z. mobilis.***

The *β-glucosidase* gene from *R. albus* was cloned and translationally fused to the glucose-fructose oxidoreductase (gfor) leader sequence of *Z. mobilis* for export; resulting in 61% secretion and 0.49 g ethanol yield per g cellobiose (Yanase et al., 2005). To amplify the 159 bp leader sequence of the glucose-fructose oxidoreductase gene, the reverse primer was designed to include 10 bp forward primer of the *β-glucosidase* gene. Similarly, the forward primer of the *β-glucosidase* gene was designed to include 10 bp of the complimentary sequence of the gfor leader reverse primer sequence. The *β-glucosidase* gene from the genomic DNA of *R. albus* (kindly provided by Professor P. Weimer, University of Wisconsin) and the leader sequence of the gfor gene of *Z. mobilis*, which was fused to the *β-glucosidase* gene, were amplified using synthetic oligonucleotide primers (Invitrogen). The PCR condition used was the same as previously described; however, the annealing temperature was 65°C. The amplicons were verified by DNA gel electrophoresis and sequenced for correctness (Table 1).

***Cloning and assembly of plasmid pAA1***

The cloning and expression of these three genes was to expand the substrate utilization range of *Z. mobilis* to include larger oligodextrins. These three fragments, ZM4 promoter with *celZ*, *celY* and *gfor-betaglucosidase* fusion were cloned into the *Kpn*I site of the broad host range vector pBBR1MCS-3 ($tc^r$). The cloning was performed using the Life Technologies Gene Art Seamless Cloning and Assembly kit (Life technologies). This kit was optimized to clone up to 4 DNA fragments with a total vector and insert size of 13 kb.

*Escherichia coli* NEB-10 Beta competent cells were transformed with the cloned vector pAA1 as described in New England Biolabs (NEB) manual. The transformants were plated out on lysogeny medium (LB) containing 40 µL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) for blue/white screening and with 15 µg/ml of tetracycline. To select for the right clone, the plasmid pAA1 and the vector backbone pBBR1MCS-3 were extracted from their respective host cells and subsequently digested using *Kpn*I and *Not*I HF restriction enzymes (NEB) which cut at different positions within the vector backbone. Furthermore, the vectors pAA1 and pBBR1MCS-3 were digested with the restriction enzyme *Nde*I (NEB). *Nde*I cut site CA^TATG exists within the insert that produced pAA1 but not on the vector backbone pBBRIMCS-3.

The cloned pAA1 was completely sequenced by Eurofins MWG operon using the Sanger sequencing method and verified for correctness and thereafter used for the transformation of ZM4 WT using the Gene Pulser (Bio-Rad) as described by Liang and Lee (1998) to create ZM4 pAA1. The *Z. mobilis* cultures were grown in a stationary flask at 30°C to an absorbance (600 nm) of 0.3 to 0.4. The cells were harvested by centrifugation at 13000 g for 10 minutes at 4°C. The cells from an original 100-ml culture were suspended in 10 ml of sterile 10% glycerol (supplemented with 0.85% NaCl), centrifuged, and finally re-suspended in 2 to 3 ml of 10% glycerol. The plasmid pAA1 was extracted from *E. coli* NEB-10 Beta, suspended in water and concentrated to 3000 ng/µl DNA before electroporation.

The Gene Pulser (Bio-Rad) for generating exponential decay pulses were set at a peak voltage of 1.5 kV and 25 µF capacitance. A 200 µL aliquot of the *Z. mobilis* cultures were mixed with 10 µL of pAA1 in a chilled electroporation chamber with an electrode gap of 0.2 cm and held on ice for 5 min. Thereafter, the mixture of cells and DNA was pulsed and immediately after pulsing, the cells were mixed with 1 ml of RM medium for outgrowing at 30°C for 4 h. At the end of this outgrowth period, the cells were diluted with RM medium and plated on RM agar containing 15 µg/ml of tetracycline. The *Z. mobilis* cultures were also transformed with the original vector backbone pBBR1MCS-3 to create ZM4 pBBR1MCS-3 and plated on RM agar (15 µg/ml of tetracycline) to verify the expression of the plasmid with the antibiotic marker in a new host but without the inserts as seen in pAA1. ZM4 pAA1 and ZM4 pBBR1MCS-3 were inoculated into RM broth containing 15 µg/ml of tetracycline and 30 µg/ml of gentamicin and incubated at 30°C for 48 h. Gentamicin was added because *Z. mobilis* is naturally resistant to it up to 50 µg/ml, therefore most potential contaminants were not expected to survive under that condition. Thereafter, OD was determined at 600 nm using the spectrophotometer (Spectronic 20D+).

To further verify that the recombinants ZM4 pAA1 and ZM4 pBBR1MCS-3 were indeed *Z. mobilis*, the cells were once again grown in RM medium with 2% glucose as the carbon source and supplemented with 15 µg/ml of tetracycline. The ZM4 WT was also grown in RM medium with 2 percent glucose as the carbon source and supplemented with 30 µg/ml of gentamicin. Cells were harvested after 48 h and genomic DNA extraction was performed using the FastDNA spin kit (MP Biomedicals). The leader sequence of the gfor is unique to *Z. mobilis* and so this gene fragment was individually amplified from the genomic DNA extracted from ZM4 pAA1, ZM4 pBBR1MCS-3 and ZM4 WT. Band sizes of approximately 200 bp was observed for the 3 amplicons on the electrophoresis gel.

The recombinant ZM4 pAA1 (tetracycline resistant) was grown in RM medium containing 2% glucose supplemented with 15 µg/ml of tetracycline. The pAA1 was then extracted from ZM4 pAA1 using the 5 Prime fast plasmid extraction kit (5 Prime) and the inserts of

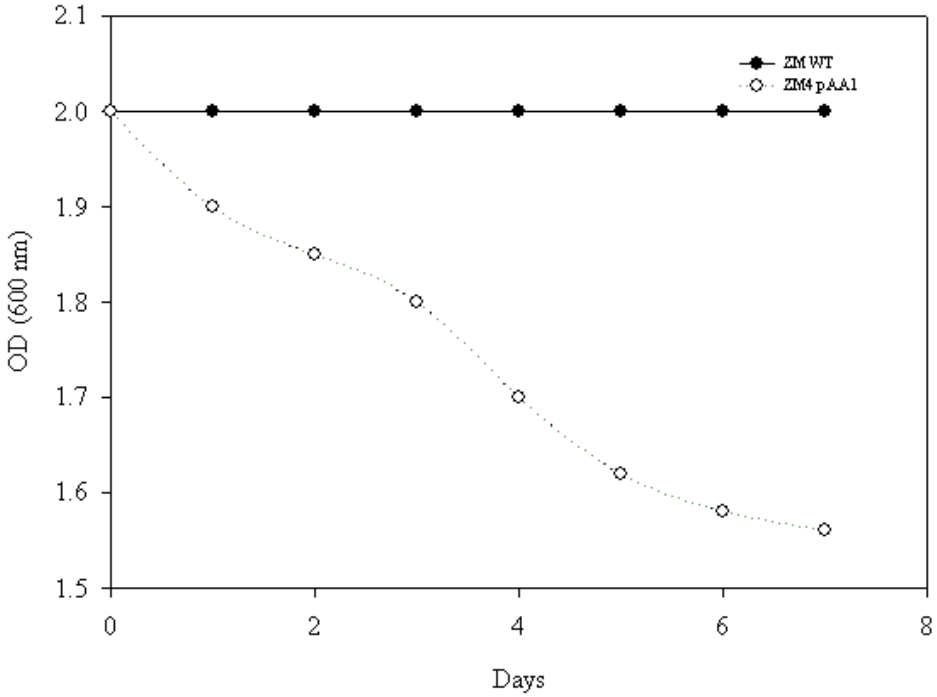


**Figure 1.** Optical density of the ZM4 pAA1 and ZM WT. Starting OD was 2.0 and decreased as insoluble cellobiose was consumed and ethanol production increased for the ZM4 pAA1. OD for the ZM4 WT remained the same, indicating it failed to utilize the substrate.

ZM4*celZ*, *celY* and *gfor-beta-glucosidase* individually amplified from the plasmid pAA1 using the Q5 high fidelity DNA polymerase (New England Biolabs), verified for size correctness on gel electrophoresis and subsequently sequenced for correctness.

### Ethanol production test

#### *Ethanol production from cellobiose using recombinant ZM4 pAA1 and ZM4 WT*

Cultures of ZM WT were grown in RM medium containing 2% glucose and supplemented with gentamicin (30 µg/ml). The recombinant ZM4 pAA1 were similarly grown in RM medium but were supplemented with 15 µg/ml of tetracycline in order to maintain the vector (pAA1) in addition to 30 µg/ml of gentamicin. Cells were harvested after 24 h, washed thoroughly in phosphate buffered saline (PBS, pH 7.2), re-suspended in RM medium containing 2% cellobiose to give an optical density (OD) of 2.0 and the appropriate antibiotics added in each medium as previously described. The cultures were incubated at 30°C in a shaking incubator (New Brunswick) at 150 rpm. The ODs were determined every twenty-four hours for 7 days and samples taken from the cultures for ethanol quantification starting from 48 h after original inoculation and every 24 h thereafter for three days.

#### *Ethanol production from cellobiose and microcrystalline cellulose using ZM4 pAA1, ZM4 WT and Clostridium cellulolyticum*

ZM4 pAA1 and ZM4 WT cultures were grown to OD of 0.4 to 0.6 in RM medium containing glucose as carbon source. The cultures were washed in PBS (pH 7.2), re-suspended, and then kept on ice until further use (maximum time <20 min). *C. cellulolyticum* was grown in Clostridium medium (ATCC 1368) containing 7.5 g/L of microcrystalline cellulose as the carbon source to an OD of 0.4 to 0.6. The cultures were subsequently used to inoculate Clostridium medium containing cellobiose and microcrystalline cellulose as carbon sources, respectively. For the mono cultures of ZM4 pAA1, ZM4 WT and *C. cellulolyticum,* the medium was inoculated with 5% inoculum size (v/v) and for the consortium, the medium was inoculated with 2.5% each of the inoculum size. For the Clostridium medium containing cellobiose, 2 g/L of the oligosaccharide cellobiose was used, whereas for the regular Clostridium medium, 7.5 g/L of cellulose was used.

## RESULTS AND DISCUSSION

From Figures 1 and 2, it can be seen that the ZM4 pAA1 utilized the cellobiose and produced three times as much ethanol after 5 days, respectively, than did the ZM4 WT. The ethanol produced and analyzed using the gas chromatography technique (Shimadzu) by the ZM4 pAA1 was significantly different from that produced by the ZM4 WT ($p = 0.03761$, SigmaPlot). The ethanol detected in the wild type could have come from the residual glucose from after wash transferred into the medium at inoculation. As ethanol was being produced, the optical density (OD) of the recombinant culture declined gradually

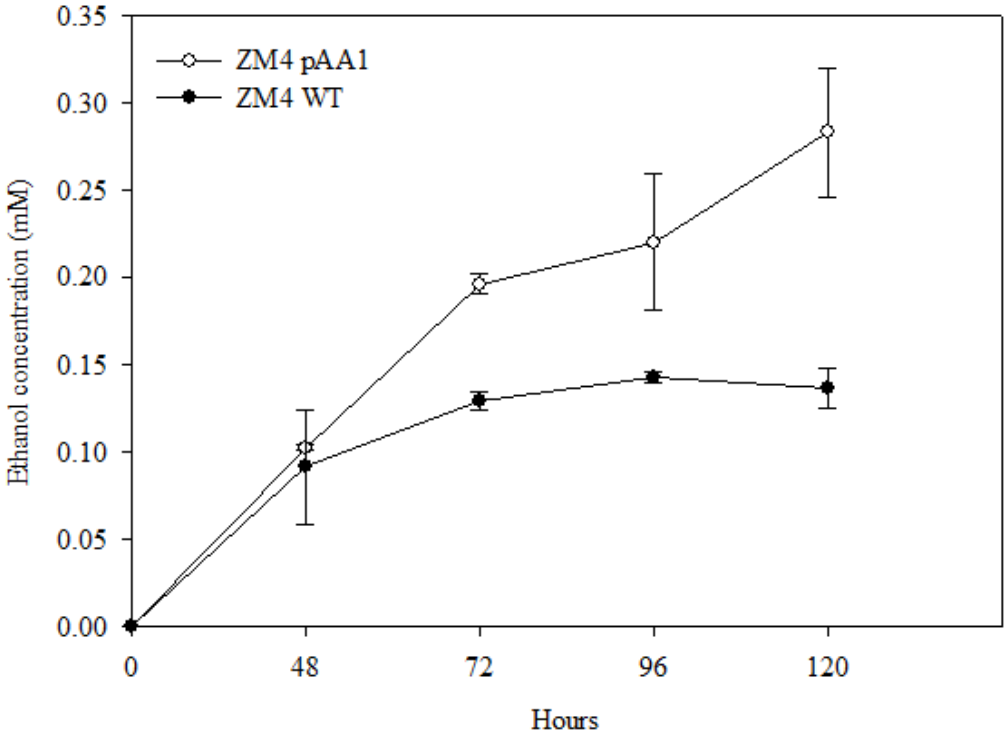


**Figure 2.** Ethanol production from RM medium containing 2% cellobiose as the carbon source. Error bars represent standard deviations among three replicates.

to 1.58 after 7 days. Further decline in OD was observed but with no further change after 1.44. No change was detected for the ZM4 WT for the entire duration.

As seen in Figure 3, ZM4 pAA1 produced the most ethanol and was consistent across the three batches whereas ethanol production was not observed at all for ZM4 WT across the three batches. *C. cellulolyticum* produced ethanol once as can be seen from batch 1 (Figure 3) but none in subsequent batches.

In Figure 4, ethanol production can be observed from the two conditions, with ZM4 pAA1 and *C. cellulolyticum* producing more ethanol than ZM4 WT and *C. cellulolyticum*. In a study by Payot et al. (1998) detailing the metabolism of cellobiose by *C. cellulolyticum* growing in continuous culture, it was reported that *C. cellulolyticum* was able to metabolize only a small quantity of soluble carbohydrates (3 g/L), with the molar growth yield reduced when the initial cellobiose concentration exceeded (2 g/L). In this work, the concentration of cellobiose utilized was originally set at 5 g/L and the results obtained (not shown) clearly indicated that such concentration negatively impacted growth of the *C. cellulolyticum* but the recombinant ZM4 pAA1 could have aided in rescuing the situation, with subsequent tests using 2 g/L cellobiose showing an improvement. The ZM4 WT however, was unable to do same, possibly due to the lack of the *β-glucosidase* gene and also by the use of a sugar source that could not adequately support its growth. Furthermore, as described by Payot et al. (1998), *C. cellulolyticum* growth was limited due to low rate of NADH re-oxidation leading to an intracellular accumulation of the reduced nucleotide and as described by Giallo et al. (1983), acetate was the main product for the continuous cultures of *Clostridium*. The acetate formation was found to increase with increasing carbon flow, leading to a high ATP production and to an insufficient rate of NADH regeneration (Giallo et al., 1983). They further described the ability of *C. acetobutylicum* to induce metabolic shifts to produce solvents such as ethanol, butanol and acetone and this shift was associated with high intracellular ATP and NAD(P)H. It does not appear that *C. cellulolyticum* is able to induce such metabolic shifts to produce reduced compounds such as ethanol. The result obtained from the consortium of recombinant ZM4 with *C. cellulolyticum* and ZM4 WT with *C. cellulolyticum* is consistent with previously observed findings, therefore the ethanol produced in the medium is likely as a result of the secretion of the fused *β-glucosidase* into the medium by ZM4 pAA1 and the ability of ZM4 WT to convert glucose released from cellobiose to ethanol, respectively. Figure 5 shows ethanol production from microcrystalline cellulose by *C. cellulolyticum,* ZM4 pAA1 and ZM4 WT in five days.

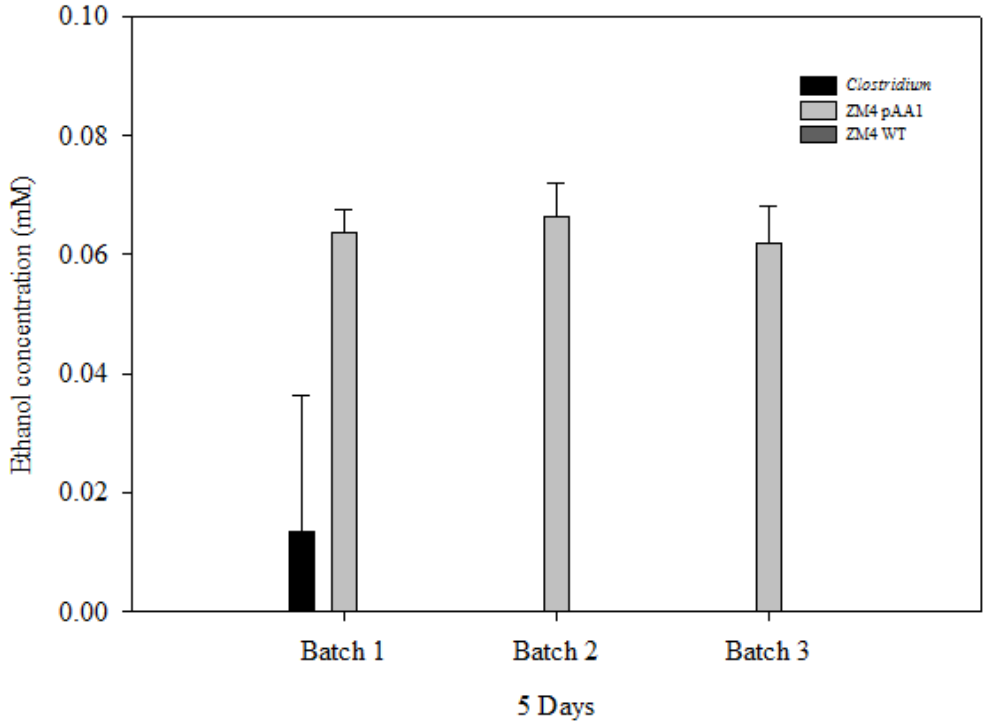


**Figure 3.** Ethanol production from cellobiose. Error bars represent standard deviations among three replicates.

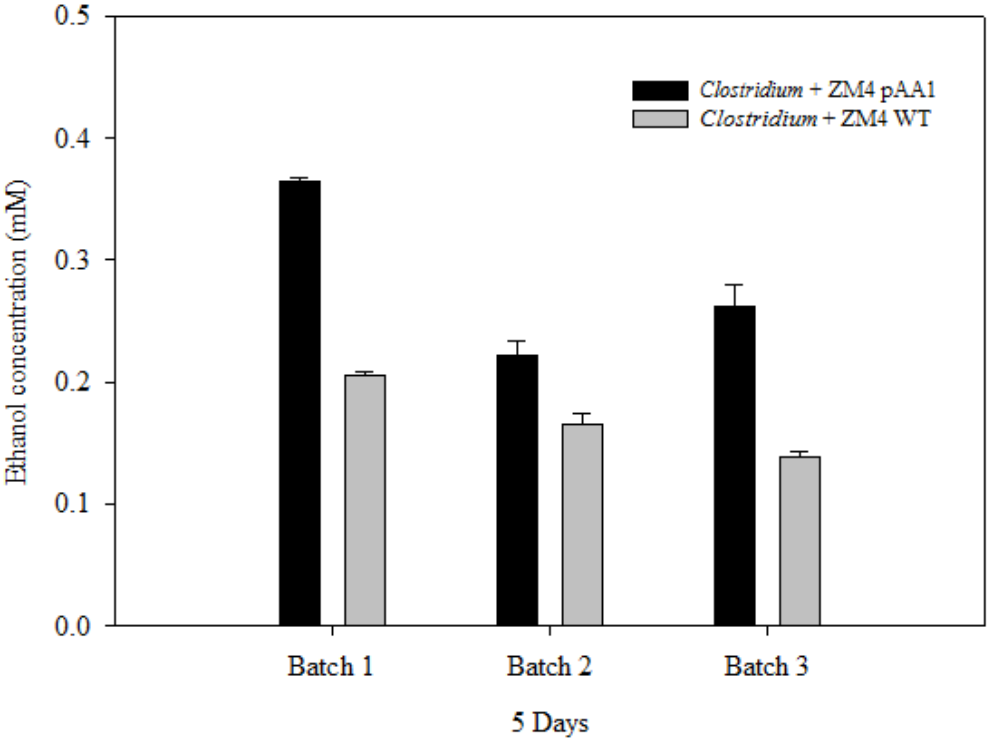


**Figure 4.** Ethanol production from cellobioise. Error bars represent standard deviations among triplicate samples.

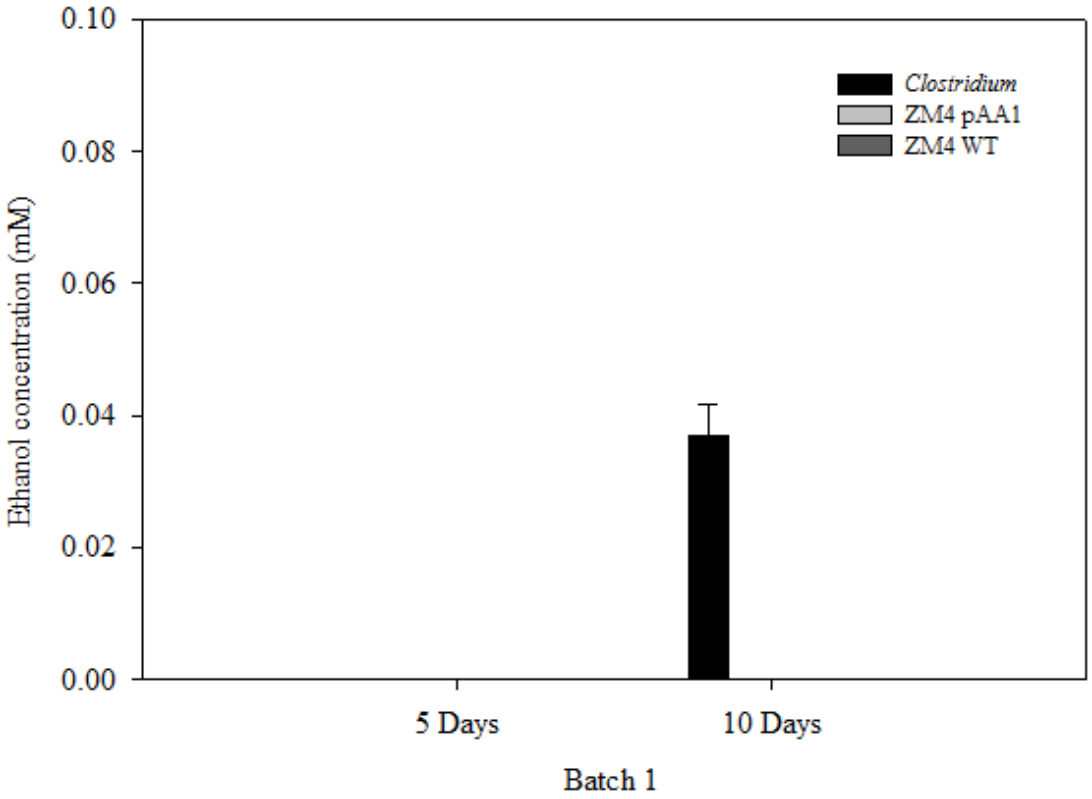


**Figure 5.** Ethanol production from microcrystalline cellulose. Error bars represent standard deviations among three replicates.

*C. cellulolyticum* showed ethanol production for only one batch but no ethanol production was detected from ZM4 pAA1 and ZM4 WT respectively. No ethanol production was detected in the first 5 days but was seen within 10 days for *C. cellulolyticum.* The amount of ethanol produced by *C. cellulolyticum* only was far lower than seen when *C. cellulolyticum* was co-cultured with ZM4 pAA1 using cellobiose as the carbon source.

In the consortium, Figures 6a and b, ethanol production was detected for *C. cellulolyticum* with ZM4 pAA1 for the three batches after 5 days and increased after 10 days while *C. cellulolyticum* with ZM4 WT produced ethanol in batches 1 and 2 but not batch 3 and only detected after 10 days. This observation suggested that the consortium involving recombinant ZM4 was more efficient than that involving the wild type. This pattern could be seen from both the time it took for ethanol to be detected and the quantity of ethanol detected. There appear to be only slight increases in ethanol production from the consortium involving the recombinant ZM4 pAA1 from the 10-day culture as compared to that produced after 5 days, suggesting that maximum ethanol yield could be possible in slightly over 5 days. The consortium involving the ZM4 WT produced less ethanol and that was detected only after 10 days, suggesting less efficiency in synergy.

## Conclusion

The recombinant *Z. mobilis* bearing the plasmid vector pAA1 supported the findings of Yanase et al. (2005) in which ZM4 re-engineered with *β-glucosidase* gene from *Ruminococcus albus* was able to secrete 61% through the cytoplasmic membrane which resulted in the production of 0.49 g ethanol per gram of cellobiose. From this work, the highest ethanol concentration determined for recombinant ZM4 pAA1 was approximately 0.06 mM, whereas the wild type showed no evidence of ethanol production after 5 days on *C. cellulolyticum* medium containing cellobiose. Despite the tagging of the *β-glucosidase* gene to gfor, only about 36% of the total activity was reported to be located on the cell surface fraction, with 20% of the activity on the periplasmic fraction (Yanase et al., 2005). With the introduction of *celZ* and *celY* genes, the recombinant ZM4 with *C. cellulolyticum* was able to indicate ethanol production from microcrystalline cellulose within 5 days whereas the consortium of the ZM4 WT took twice that amount of time and produced significantly less ethanol. The recombinant ZM4 and ZM4 WT did not produce ethanol using cellulose as the carbon source as expected but *C. cellulolyticum* alone did after 10 days, twice the time it

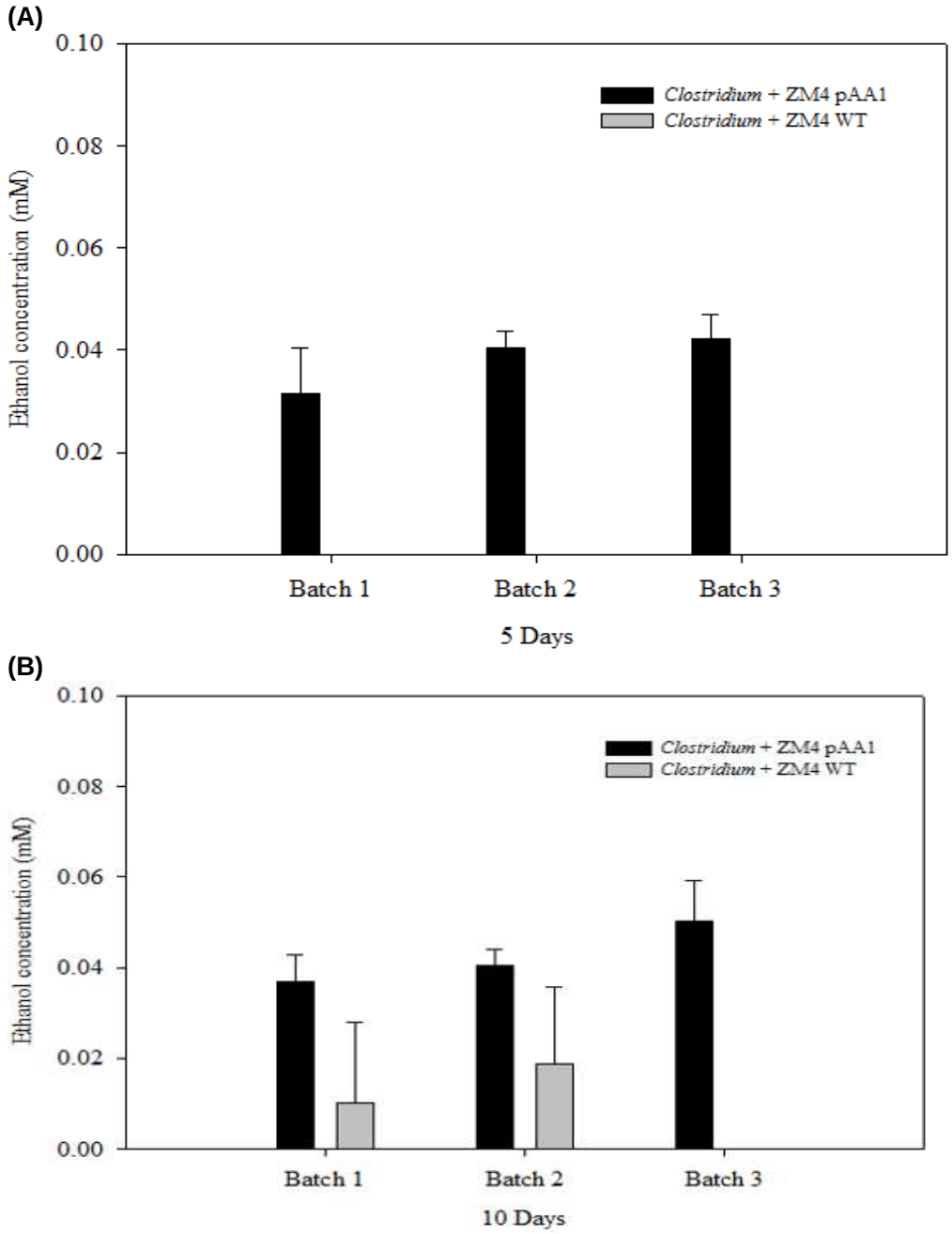


**Figure 6.** Ethanol production after 5 (A) and 10 (B) days using microcrystalline cellulose as a carbon source. Error bars represent standard deviations among three replicates.

took to make ethanol for the consortium.

This preliminary study was initiated to study the effect of the microbial consortium on ethanol production. It is far from exhaustive, did not establish a convincing case for the consortium using the two microorganisms and more investigation is required in order to optimize the desired synergy between the ZM4 pAA1 and *C. cellulolyticum.* The concentration of microcrystalline cellulose used was 7.5 g/L, but further investigation is needed to determine the best concentration at which a synergy can be clearly observed. Furthermore, the culture growth conditions and incubation times chosen could also be a major factor in contributing to the lack of a clear evidence of synergy; therefore, different culture conditions and time points

would be tested to better investigate the consortium.

Based on the wide differences in the genetics and physiology of the two bacteria used, the low yields could simply have been as a result of metabolic bottlenecks, which were not investigated in this preliminary study. Batch culture conditions were investigated and the need to investigate same conditions using the continuous culture conditions cannot be overemphasized. Using pure cellobiose and pure microcrystalline cellulose only, were insufficient, other carbon sources to reflect natural conditions would be investigated

Finally, the recombinant ZM4 pAA1 would be partnered with other known cellulose degraders other than the one used here to further understand its efficiency in a consortium.

## Conflict of interest

There is no conflict of interest in preparation of the manuscript.

## REFERENCES


Abate C, Callieri D, Rodriguez E, Garro O (1996). Ethanol production by a mixed culture of flocculent strains of *Zymomonas mobilis* and *Saccharomyces* sp. Appl. Microbiol. Biol. 45(5):580-583.

Bayer TS, Widmaier DM, Temme K, Mirsky EA, Santi DV, Voigt CA (2009). Synthesis of Methyl Halides from Biomass Using Engineered Microbes. J. Am. Chem. Soc. 131(18):6508-6515.

Deanda K, Zhang M, Eddy C, Picataggio S (1996). Development of an arabinose-fermenting *Zymomonas mobilis* strain by metabolic pathway engineering. Appl. Environ. Microbiol. 62(12):4465-4470.

Desvaux M (2005). The cellulosome of *Clostridium cellulolyticum.* Enzym. Microb. Technol. 37(4):373-385.

Dunn KL, Rao CV (2014). Expression of a xylose-specific transporter improves ethanol production by metabolically engineered *Zymomonas mobilis.* Appl. Microbiol. Biotechnol. 98(15):6897-6905.

Fu N, Peiris P, Markham J, Bavor J (2009). A novel co-culture process with *Zymomonas mobilis* and *Pichia stipitis* for efficient ethanol production on glucose/xylose mixtures. Enzym. Microbiol. Technol. 45(3):210-217.

Giallo J, Gaudin C, Belaich JP, Petitdemange H, Caillet-Mangin F (1983). Metabolism of glucose and cellobiose by cellulolytic mesophilic *Clostridium* sp. strain H10. Appl. Environ. Microbiol. 45(3):843-849.

Guedon E, Dexvaux M, Payot S, Petitdemange H (1999). Growth inhibition of *Clostridium cellulolyticum* by an inefficiently regulated carbon flow. Microbiology 145(8):1831-1838.

Gunasekeran P, Karunakaran T, Nellaiah H, Kamini N.R, Mukundan A.G (1990). Current status and prospective of an ethanol producer, *Zymomonas mobilis.* Indian J. Microbiol 30: 107-133.

He Q, Hemme C.L, Jiang H, He Z, Zhou J (2011). Mechanisms of enhanced cellulosic bioethanol fermentation by co-cultivation of *Clostridium* and *Thermoanaerobacter* spp. Bioresour. Technol. 102(20):9586-9592.

Ho Q, Chang JJ, Lin JJ, Chin TY, Matthew GM, Huang CC (2011). Establishment of functional rumen bacterial consortia (FRBC) for simultaneous biohydrogen and bioethanol production from lignocellulose. Int. J. Hydrogen Energ. 36(19):12168-12176.

Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Loop RM, Peterson KM (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166(1):175-176.

Li Y, Park J-Y, Shiroma R, Tokuyasu K (2011). Bioethanol production from rice straw by a sequential use of *Saccharomyces cerevisiae* and *Pichia stipitis* with heat inactivation of *Saccharomyces cerevisiae* cells prior to xylose fermentation. J. Biosci. Bioeng. 111(6): 682-686.

Liang CC, Lee WC (1998). Characteristics and transformation of *Zymomonas mobilis* with plasmid pKT230 by electroporation. Bioprocess Eng. 19(2):81-85.

Luo Z, Bao J (2015). Secretive expression of heterologous β-glucosidase in *Zymomonas mobilis.* Bioresour. Bioprocess. 2:1-6.

Payot S, Guedon E, Cailliez C, Gelhaye E, Petitdemange H (1998). Metabolism of cellobiose by cellulolytic mesophilic *Clostridium* sp. strain H10. Appl. Environ. Microbiol. 45(3):843-849.

Petitdemange E, Caillet F, Giallo J, Gaudin C (1984). *Clostridium cellulolyticum* sp. nov, a Cellulolytic, Mesophilic Species from Decayed Grass. Int. J. Syst. Bacteriol. 34:155-159.

Quinn BM, Apolinario EA, Gross A, Sowers KR (2016). Characterization of a microbial consortium that converts mariculture fish waste to biomethane. Aquaculture 453:154-162.

Reddy OV, Basappa SC (1996). Direct fermentation of cassava starch to ethanol by mixed cultures of *Endomycopsis fibuligera* and *Zymomonas mobilis;* Synergism and limitation. Biotechnol. Lett. 18(11):1315-1318.

Senthikumar V, Gunasekaran P (2005). Bioethanol production from cellulosic substrates: Engineered bacteria and process integration challenges. J. Sci. Ind. Res. India 64(11):845-853.

Shin HD, McClendon S, Vo T, Chen R.R (2010). *Escherichia coli* Binary Cultured Engineered for Direct Fermentation of Hemicellulose to a Biofuel. Appl. Environ. Microbiol. 76(24):8150-8159.

Shou W, Ram S, Vilar J.M.G (2007). Synthetic cooperation in engineered yeast populations. Proc. Natl. Acad. Sci. U.S.A 104(6): 1877-1882.

Singh LK, Majumder CB, Ghosh S (2014). Development of sequential co-culture system (*Pichia stipitis* and *Zymomonas mobilis*) for ethanol production from Kans grass biomass. Biochem. Eng. J. 82: 150-157.

Weir PM (2016). The ecology of *Zymomonas:* a review. Folia Microbiol (Praha) 61:385-392.

Yanase H, Nozaki K, Okamoto K (2005). Ethanol production from cellulosic materials by genetically engineered *Zymomonas mobilis.* Biotechnol. Lett. 27(4):259-263.

Yanase H, Miyawaki H, Sakurai M, Kawakami A, Matsumoto M, Haga K, Kojima M, Okamoto K (2012). Ethanol production from wood hydrolysate using genetically engineered *Zymomonas mobilis.* Appl. Microbiol. Biotechnol. 94(6):1667-1678.

Yang S, Fei Q, Zhang Y, Contreras L.M, Utturkar S.D, Himmel M.E, Zhang M (2016). *Zymomonas mobilis* as a model system for production of biofuels and biochemical. Microbiol. Biotechnol. 9(6):699-717.

Yaun X, Ma L, Wen B, Zhou D, Kuang M, Yang W, Cui Z (2016). Enhancing anaerobic digestion of cotton stalk by pretreatment with a microbial consortium (MC1). Bioresour. Technol. 207:293-301.

Zhang SC, Lai QH, Lu Y, Liu ZD, Wang TM, Zhang C, Xing XH (2016a). Enhanced biohydrogen production from corn stover by the combination of *Clostridium cellulolyticum* and hydrogen fermentation bacteria. J. Biosci. Bioeng. 122:482-487.

Zhang H, Zhang P, Ye J, Wu Y, Fang W, Gou X, Zeng G (2016b). Improvement of methane production from rice straw with rumen fluid pretreatment. A feasibility study. Int. J. Biodeterior. Biodegr. 113:9-16.

Zhong C, Wang C, Wang F, Jia H, Wei P, Zhao W (2016). Enhanced biogas production from wheat straw with the application of synergistic microbial consortium pretreatment. RSC Adv. 6:60187-60195.

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*Full Length Research Paper*

# Antibiotic resistance and molecular characterization of *Staphylococcus* species from mastitic milk

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**Species within the *Staphylococcus* genus are important mastitis pathogens. Studies to describe virulence and antibiotic resistance as well as rapid techniques that permit analyses strains are needed. The aims were to identify and characterize *Staphylococcus* spp. isolated from mastitic milk, and to optimize multiplex polymerase chain reactions (PCR). Staphylococci previously isolated from milk of dairy cows with subclinical mastitis were analyzed. PCR was completed to amplify *nuc, sodA, spa, agr* locus, virulence factors, and antibiotic resistance genes. DNA sequencing of *sod*A and *spa* genes was performed and antibiograms were carried out on all isolates. In a group of 49 staphylococci, *S. aureus* was the most prevalent, followed by *S. hyicus*, *S. xylosus*, *S. chromogenes*. Following optimization of multiplex PCR, virulence factor genes were identified in the majority of isolates. The enterotoxin genes, *seh* and *sel*x were highlighted. All hemolysin genes were detected in 28.6% of isolates. Antibiotic resistance was evaluated and the majority of isolates (69.4%) were resistant to penicillin. Among the genes encoding antibiotic resistance, *mec*A was identified, while two methicillin-resistant *S. aureus* were typed as *spa* type 605, *agr* type II, and one identified as SCC*mec* type IVa. The types t605 and *agr* II were detected in the majority of *S. aureus* assessed. The findings emphasized the importance of preventing *Staphylococcus* infection in dairy cows. Effective dairy herd management and information on milk quality are essential to prevent mastitis pathogens.**

**Key-words:** Antibiotic, staphylococci, toxins, virulence, genes.

## INTRODUCTION

Bovine mastitis affects the dairy industry worldwide, and is associated with reduced milk quality and production (Silva et al., 2013). Coagulase-positive staphylococci (CPS) are widely studied as a common cause of clinical

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and subclinical mastitis (Ote et al., 2011; Rajic-Savic et al., 2015), and the most important causative agent in this bacterial group is *Staphylococcus aureus* (Ote et al., 2011). In addition, the relevance of coagulase-negative staphylococci (CNS) as a cause of mastitis in dairy cows has also been shown (Silva et al., 2014). CNS research has predominantly focused on humans, and the enterotoxigenic potential of CNS has not been extensively explored, although it has been suggested that CNS from bovine intrammamary infection (IMI) could be a potential source of staphylococcal superantigens (SAgs) (Park et al., 2011).

SAgs, e.g. staphylococcal enterotoxin (SE) and toxic shock syndrome toxin-1 (TSST-1), were first identified in *S. aureus* (Park et al., 2011), and have been well characterized. The emetic activity of SEs has been demonstrated (Hu and Nakane, 2014); thus, there is a potential to cause foodborne disease (Jorgensen et al., 2005). *Staphylococcus aureus* may carry genes for production of other toxins such as Panton-Valentine leukocidin, toxic shock syndrome toxin and exfoliative toxins (Jarraud et al., 2002).

The importance of virulence factors in *Staphylococcus* genus and the highly clonal structure within the *S. aureus* population have been highlighted in medicine, and could potentially help in treatments (Ote et al., 2011). However, antibiotic resistance is a concern since studies have demonstrated the emergence of resistant isolates from bovine mastitis (Moon et al., 2007; Silva et al., 2014). Thus, the aims of the present study were to identify, and characterize *Staphylococcus* spp. isolated from mastitic milk, and to optimize several multiplex polymerase chain reaction (PCR) in order to simultaneously identify the presence of different virulence factor genes.

## MATERIALS AND METHODS

### Origin and collection of isolates

The collection of bacterial isolates belonging to Hygiene and Dairy Laboratory, University of São Paulo, was used. From this collection, isolates from mastitic milk previously identified as *Staphylococcus* spp. were selected. Forty-nine isolates were selected from three different dairy farms located in São Paulo State, region of Piracicaba city, Brazil.

The isolates were obtained in a previous study performed by Hygiene and Dairy Laboratory's group, in which dairy cows were diagnosed with subclinical mastitis after screening using California Mastitis Test. Mastitic milk samples collected from September to October of 2013 were used. For the bacterial culturing, standard microbiological methods included colony morphology on Baird Parker Agar (BPA, Difco BD®, Nova Jersey, EUA) with egg yolk tellurite supplement (Laborclin®, Pinhais, Brazil), Gram staining, catalase, and coagulase test were completed to identify staphylococci, and all isolates were stored at -20°C.

### DNA extraction, polymerase chain reaction and molecular typing

Each isolate was inoculated into Brain Heart Infusion (BHI, Oxoid™, Hampshire, UK) broth and incubated at 37°C for 24 h. Aliquots of each culture were centrifuged and the supernatant was discarded. The pellet was used to extract DNA using "AxyPrep™ Blood Genomic DNA Miniprep kit" (Axygen Scientific Inc., Union City, USA), according to manufacturer's instructions. Agarose gel electrophoresis was completed to verify the extraction, and the genomic DNA was stored at -20°C.

Coagulase-positive and coagulase-negative staphylococci previously identified by coagulase test were confirmed by detecting the *coa* gene through PCR. The PCR amplification of *coa* gene described by Aarestrup et al. (1995) was modified by using 0.75 mM of $MgCl_2$ in each reaction, and the PCR cycles used were as follows: 95°C for 5 min; 30 cycles at 95°C for 30 s, 55°C for 2 min, and 72°C for 4 min; and finally at 72°C for 10 min. When confirmed as coagulase-positive, multiplex PCR was performed to identify *S. aureus*, *S. intermedius* and *S. hyicus* according to Sasaki et al. (2010). Other strains were identified by amplifying the *sod*A gene, and through DNA sequencing using Sanger method (Silva et al., 2014).

The SEs (SEA-SEE, SEG-SEIJ, SEIK-SEIQ, SER-SET, SEIU, SEIV and SEIX), hemolysins (alpha, beta, delta, gamma component A, B and C and gamma-variant hemolysin), Panton-Valentine leukocidin (PVL), exfoliative toxins (ETA, ETB and ETD) and toxic shock syndrome toxin (TSST-1) genes were assessed by PCR. Primers used in this study are shown in Table 1.

Single PCR was initially performed for genes and positive (extracted DNA from strains belonging to Hygiene and Dairy Laboratory collection, University of São Paulo) and negative controls were incorporate into each run. Next, primers were combined in the same reaction when possible depending on amplification characteristics e.g. annealing temperature, number of PCR cycles and concentration of $MgCl_2$. The multiplex reactions were as follows: 1X PCR Buffer, 1U GoTaq® Hot Start Polymerase (Promega Corporation, Madison, USA), $MgCl_2$ (Promega Corporation, Madison, USA) concentration was variable, 10 pmol of each primer (synthesis by Sigma-Aldrich®, São Paulo, Brazil), 200 µM deoxynucleotides (Promega Corporation, Madison, USA), template DNA (approximately 40 ng) and ultrapure water to bring the final reaction volume to 25 µL. Genes that were not incorporate into multiplex PCR, were amplified by uniplex PCR using 2.5 mM of $MgCl_2$ and thermally cycled at 94°C for 5 min, 30 cycles at 94°C for 2 min, 48°C for 1 min, and 72°C for 1 min, and then once at 72 °C for 10 min.

*agr* type was developed according to Shopsin et al. (2003) and the amplification of *spa* region was carried out following the website http://www.ridom.com/, the repeats were identified for *spa* types detection after sequencing by Sanger method.

### Antibiotic resistance detection

Antibiotic resistance of each isolate was tested using the agar diffusion method following the Clinical and Laboratory Standards Institute guidelines (Clinical Laboratory Standards Institute, 2015). The antibiotics tested included penicillin, cefoxitin, oxacilin, erythromycin, clindamycin, chloramphenicol, ciprofloxacin, vancomycin, tobramycin, tetracycline and gentamicin. The *tet*K, *tet*L, *tet*M (Gómez-Sanz et al., 2010), *ant(4')*-Ia (van de Klundert et al., 1993), *erm*A, *erm*B, *erm*C (Gómez-Sanz et al., 2010), *mec*A (Moon et al., 2007) and *mec*C (Cuny et al., 2011) genes were detected by PCR, and Staphylococcal Cassette Chromosome *mec* (SCC*mec*) types I to V in methicillin-resistant *S. aureus* were identified as described by Kondo et al. (2007).

## RESULTS

Of the total isolates, 46 (93.9%) were confirmed as

**Table 1.** Primers sequences used to amplify virulence factors.

| Gene | Oligonucleotide sequence (5´→3´) | bp | Reference |
|---|---|---|---|
| *sea* | TTGGAAACGGTTAAAACGAA<br>GAACCTTCCCATCAAAAACA | 120 | Johnson et al. (1991) |
| *seb* | TCGCATCAAACTGACAAACG<br>GCAGGTACTCTATAAGTGCC | 478 | Johnson et al. (1991) |
| *sec* | GACATAAAAGCTAGGAATTT<br>AAATCGGATTAACATTATCC | 257 | Johnson et al. (1991) |
| *sed* | CTAGTTTGGTAATATCTCCT<br>TAATGCTATATCTTATAGGG | 317 | Johnson et al. (1991) |
| *see* | AGGTTTTTTCACAGGTCATCC<br>CTTTTTTTTCTTCGGTCAATC | 209 | Mehrotra et al. (2000) |
| *seg* | AAGTAGACATTTTTGGCGTTCC<br>AGAACCATCAAACTCGTATAGC | 287 | Omoe et al. (2002) |
| *seh* | GTCTATATGGAGGTACAACACT<br>GACCTTTACTTATTTCGCTGTC | 213 | Omoe et al. (2002) |
| *sei* | GGTGATATTGGTGTAGGTAAC<br>ATCCATATTCTTTGCCTTTACCAG | 454 | Omoe et al. (2002) |
| *selj* | CATCAGAACTGTTGTTCCGCTAG<br>CTGAATTTTACCATCAAAGGTAC | 142 | Nashev et al. (2004) |
| *selk* | TAGGTGTCTCTAATAATGCCA<br>TAGATATTCGTTAGTAGCTG | 293 | Omoe et al. (2005) |
| *sell* | CACCAGAATCACACCGCTTA<br>CTGTTTGATGCTTGCCATTG | 240 | Cremonesi et al. (2005) |
| *selm* | ATCATATCGCAACCGCTGAT<br>TTCAGTTTCGACAGTTTTGTTGTC | 626 | Ote et al. (2011) |
| *seln* | ATGAGATTGTTCTACATAGCTGCAAT<br>AACTCTGCTCCCACTGAAC | 680 | Ote et al. (2011) |
| *selo* | AAATGATTCTTTATGCTCCG<br>AAAGCACATTGTCATGGTGA | 300 | Ote et al. (2011) |
| *selp* | TGATTTATTAGTAGACCTTGG<br>ATAACCAACCGAATCACCAG | 396 | Omoe et al. (2005) |
| *selq* | AATCTCTGGGTCAATGGTAAGC<br>TTGTATTCGTTTTGTAGGTATTTTCG | 122 | Omoe et al. (2005) |
| *ser* | GGATAAAGCGGTAATAGCAG<br>GTATTCCAAACACATCTAAC | 166 | Omoe et al. (2005) |
| *ses* | CCCCGGATCCGATGAATCTAGACCTAAAATAG<br>CCCCGTCGACTTATTGGGAATAAAC | 794 | Ono et al. (2008) |
| *set* | CCCCGGATCCGATTCTCGTGAAGGTTTAAAAG<br>CCCCGTCGACCTATTTTTCCATATATATATC | 671 | Ono et al. (2008) |
| *selu* | ATGGAGTTGTTGGAATGAAGT<br>TTTTTGGTTAAATGAACTTCTACA | 796 | Fischer et al. (2009) |
| *selv* | GCAGGATCCGATGTCGGAGTTTTGAATCTTAGG<br>TAACTGCAGTTAGTTACTATCTACATATGATATTTCGACATC | 720 | Thomas et al. (2009) |
| *selx* | AGCAGACGCGTCAACACAAA<br>ACTTGTTCAATGTCATTAACACTTTTCAC | 612 | Wilson et al. (2011) |
| *hla* | CTGATTACTATCCAAGAAATTCGATTG<br>CTTTCCAGCCTACTTTTTTATCAGT | 209 | Jarraud et al. (2002) |
| *hlb* | GTGCACTTACTGACAATAGTGC<br>GTTGATGAGTAGCTACCTTCAGT | 309 | Jarraud et al. (2002) |

**Table 1.** Contd.

| | | | |
|---|---|---|---|
| *hld* | AAGAATTTTTATCTTAATTAAGGAAGGAGTG<br>TTAGTGAATTTGTTCACTGTGTCGA | 111 | Jarraud et al. (2002) |
| *hlg* | GTCAYAGAGTCCATAATGCATTTAA<br>CACCAAATGTATAGCCTAAAGTG | 535 | Jarraud et al. (2002) |
| *hlg-v* | GACATAGAGTCCATAATGCATTYGT<br>ATAGTCATTAGGATTAGGTTTCACAAAG | 390 | Jarraud et al. (2002) |
| *eta* | ACTGTAGGAGCTAGTGCATTTGT<br>TGGATACTTTTGTCTATCTTTTTCATCAAC | 190 | Jarraud et al. (2002) |
| *etb* | CAGATAAAGAGCTTTATACACACATTAC<br>AGTGAACTTATCTTTCTATTGAAAAACACTC | 612 | Jarraud et al. (2002) |
| *etd* | AACTATCATGTATCAAGG<br>CAGAATTTCCCGACTCAG | 376 | Yamaguchi et al. (2002) |
| *tst* | TTCACTATTTGTAAAAGTGTCAGACCCACT<br>TACTAATGAATTTTTTTATCGTAAGCCCTT | 180 | Jarraud et al. (2002) |
| *pvl* | ATCATTAGGTAAAATGTCTGGACATGATCCA<br>GCATCAAGTGTATTGGATAGCAAAAGC | 443 | Lina et al. (1999) |

coagulase-positive through amplification of *coa* gene. The species observed were *S. aureus* (42 strains, 85.7% of isolates), *S. hyicus* (4, 8.2%), *S. xylosus* (2, 4.1%) and *S. chromogenes* (1, 2.0%) (Table 2).

In multiplex PCR optimization, a total of 11 multiplex PCR (Table 3) to detect virulence genes (*sea, seb, sec, sed, see, seg, seh, sei, selj, selk, sell, selm, seln, selo, selp, selq, ser, selu, pvl, tst, hla, hlb, hld, hlg* and *hlg-v*) were performed. Multiplex PCR for 25 genes were evaluated across 11 reactions, which permitted optimization of the analyses and reducing costs.

Forty-two isolates (85.7% of isolates) were positive for one or more enterotoxin gene. The enterotoxin genes observed were *seh* (59.2%) and *selx* (57.1%) followed by *seg* (51.0%), *ser* (46.9%), *selu* (38.8%), *sell* (24.5%), *selo* (18.4%), *seln* and *selp* (6.1% each one), *seb*, *selj*, *selk* and *selm*, (4.1% each one) and *selq* (2,0%). *sea*, *sec*, *sed*, *see*, *sei*, *ses*, *set* and *selv* genes were not detected. In this study, 30 profiles were observed across 49 isolates. Among the profiles identified in this study, *seg+seh+ser+seu+selx,* was the most abundant (10.2% of strains), followed by *seg+seh*, *seg+seh+sem+seo+ser+seu+selx*,*seg+seh+seo+ser+seu +selx*, *seh*, *seh+seo+ser+selx*, *seh+ser+selx* and *sel* (4.1% each profile). In seven isolates, enterotoxin genes were not identified. All hemolysin genes were detected, that is *hla* (38.8%), *hlb* (55.1%), *hld* (32.7%), *hlg* (42.9%) and *hlg-v* (53.1%). The presence of all hemolysins was the most frequent profile (28.6%), and 19 of the strains (38.7%) did not carry hemolysin genes. Genes encoding exfoliative toxins, *pvl* and *tst* were not identified.

Regarding antibiotic resistance, isolates were resistant to penicillin (69.4% of isolates), cefoxitin (8.2%), erythromycin, chloramphenicol, tetracycline (4.1% to each antibiotic), tobramycin, clindamycin, oxacilin (2.0% to each antibiotic). One isolate demonstrated intermediate resistance to gentamicin, erythromycin, clindamycin, while all strains were sensitive to vancomycin and ciprofloxacin. Across all strains, three isolates were multi-drug resistant. Herein, *erm*A, *erm*C, *tet*K and *tet*M genes were detected (Table 2). Of the isolates resistant to cefoxitin (4 isolates, *spa* type t605 isolated from farm A) only 2 isolates were positive for *mec*A, one was identified as SCC*mec* type IVa and another was non-typeable. In addition, the methicillin-resistant *S. aureus* (MRSA) strains belong to *spa* typing t605 and *agr* type II, and the absence of a novel *mec*A homologue was observed. In *spa* typing detected across 42 *S. aureus* (Table 2), the type most frequent was t605 (83.3%), also it was present on all farms, followed by t267 (9.5%), t521 (4.8%) and t9129 (2.4%). The *agr* types detected were I (11.9%) and II (88.1%).

## DISCUSSION

In this study, in a limited group of staphylococci were identified and *S. aureus* was the dominant species; however, CNS was also present. In addition, several virulence factor genes were identified in the majority of isolates by multiplex PCR as well as antibiotic resistance to one or more antibiotics tested by diffusion method. Regarding SEs, the importance of *seh,* and *selx* genes corresponding to SEH and SEIX is emphasized due their high incidence, while low frequency or absence of classical SEs were observed. The *mec*A positive isolates detected were *spa* type 605, and *agr* type II, which were also identified in the majority isolates.

Herein, high frequency of *S. aureus* was detected, this species has been identified as the primary pathogen associated with mastitis (Ote et al., 2011; Silva et al., 2014), and previous studies have identified a high

**Table 2.** Species, *spa* typing, virulence factor genes and antibiotic resistance genes detected by farm.

| Farm | Species (n) | *spa* type (n) | *agr* type (n) | Enterotoxin gene (n) | Hemolysin gene (n) | Antibiotic resistance gene (n) |
|---|---|---|---|---|---|---|
| A | *S. aureus* (28) | t605 (28) | II (28) | *seb* (2)<br>*seg* (18)<br>*seh* (18)<br>*selk* (2)<br>*sell* (5)<br>*selm* (2)<br>*seln* (6)<br>*selq* (1)<br>*ser* (13)<br>*selu* (13)<br>*selx* (17) | *hla* (10)<br>*hlb* (17)<br>*hld* (10)<br>*hlg* (15)<br>*hlg-v* (15) | *mec*A (2) |
| | *S. hyicus* (2) | | | | | *erm*A (1)<br>*erm*C (1) |
| | *S. xylosus* (2) | | | *sell* (1)<br>*selp* (1) | | *tet*K (2)<br>*tet*M (1) |
| B | *S. aureus* (10) | t605 (5)<br>t267(4)<br>t9129 (1) | I (5)<br>II (5) | *seg* (7)<br>*seh* (7)<br>*selj* (1)<br>*sell* (2)<br>*seln* (2)<br>*selo* (1)<br>*ser* (8)<br>*selu* (6)<br>*selx* (9) | *hla* (7)<br>*hlb* (8)<br>*hld* (4)<br>*hlg* (4)<br>*hlg-v* (9) | *erm*C (1)<br>*tet*M (1) |
| | *S. hyicus* (2) | | | *seh* (1)<br>*selj* (1)<br>*sell* (2)<br>*selp*(1) | | |
| C | *S. aureus* (4) | t605 (2)<br>t521 (2) | II (4) | *seh* (3)<br>*sell* (2)<br>*seln* (1)<br>*selo* (2)<br>*ser* (2)<br>*selx* (2) | *hla* (2)<br>*hlb* (2)<br>*hld* (2)<br>*hlg* (2)<br>*hlg-v* (2) | |
| | *S. chromogenes* (1) | | | *selp* (1) | | |

frequency of this pathogen in Brazil (Silva et al., 2013; Lange et al., 2015). Giannechini et al. (2002) also detected high frequency of *S. aureus*, and low frequency of *S. hyicus* coagulase-positive among isolates from sub-clinical mastitis cases. The *coa* gene amplification also showed that the minority of the isolates belonged to CNS; which are capable of causing opportunistic mastitis (Moon et al., 2007). Lange et al. (2015) reported *S. chromogenes* at a frequency of 38.5%, which highlights the importance of coagulase-negative strains; however, in this study, the detection of CNS was low. *S. xylosus*, coagulase-negative, were also detected, within this species there are strains that can potentially be hazardous, and they are related to animal opportunistic infections (Dordet-Frisoni et al., 2007).

The low frequency of classical SEs is in agreement with a previous study in which *S. aureus* associated with bovine mastitis were analyzed (Ote et al., 2011). In this study, classical SEs were not identified in *S. chromogenes*, *S. xylosus* and *S. hyicus*; however, classical SEs have been reported in these species (Park et al., 2011). Among the other SEs, the frequency of *seh* was highly detected. SEH has emetic activity and staphylococcal food poisoning associated with *S. aureus*

**Table 3.** Conditions of the multiplex PCR optimized in this study.

| Set | Genes | Concentrated $MgCl_2$ (mM) | Condition of PCR[a] |
|---|---|---|---|
| A | *sea* + *sec* | 2.0 | 94°C - 2 min<br>54°C - 1 min 30 cycles<br>72°C - 1 min |
| B | *seb* + *selk* | 2.0 | 94°C - 2 min<br>55°C - 1 min 30 cycles<br>72°C - 1 min |
| C | *sed* + *seh* | 2.0 | 94°C - 2 min<br>55°C - 1 min 30 cycles<br>72°C - 1 min |
| D | *see* + *selq* | 2.0 | 94°C - 2 min<br>54°C - 1 min 30 cycles<br>72°C - 1 min |
| E | *seg* + *selu* | 2.0 | 94°C - 2 min<br>54°C - 1 min 30 cycles<br>72°C - 1 min |
| F | *sei* + *selm* + *selo* | 1.5 | 94°C - 2 min<br>54°C - 1 min 30 cycles<br>72°C - 1 min |
| G | *selj* + *sell* | 3.0 | 94°C - 2 min<br>64°C - 2 min 35 cycles<br>72°C - 1 min |
| H | *seln* + *selp* + *ser* | 3.0 | 94 °C - 30 s<br>58 °C - 30 s 35 cycles<br>72 °C - 1 min |
| I | *pvl* + *tst* | 3.0 | 94°C - 30 s<br>55°C - 30 s 30 cycles<br>72°C - 1 min |
| J | *hla* + *hlb* + *hld* | 2.0 | 94°C - 30 s<br>63°C - 30 s 30 cycles<br>72°C - 1 min |
| K | *hlg* + *hlg-v* | 2.0 | 94°C - 30 min<br>48°C - 30 s 30 cycles<br>72°C - 1 min |

[a]94°C/5 min for initial denaturation and 72°C/7 min for extension final.

carrying the *seh* gene has been reported (Jorgensen et al., 2005; Argudín et al., 2010). Considering the potential of SEH to cause foodborne disease, strains from our collection that carry the *seh* gene should be tested forenterotoxin protein expression in further investigations.

The staphylococcal enterotoxin-like toxin X (SEIX) also demonstrated a high frequency. The *selx* gene is encoded in the core genome of *S. aureus*, which explains the frequency of *selx*. However, its emetic activity has not yet been tested (Hu and Nakane, 2014). In addition, it is

suggested in this case to further study allelic diversification. Other genes (*seb*, *seg*, *sej* and *ser*) that encode for SEs with emetic activity were detected; it shows that milk quality control needs to be strict in order to avoid the pathogen or significant count of it, and consequently the possibility of milk contamination with SEs. *sek*, *sel*, *sem*, *sen*, *seo*, *sep, seq* and *seu* were detected; however, these have not exhibited emetic activity in primate models or emetic activity has not been tested for some genes. Several SEs profiles were identified, this finding demonstrates the high distribution of SEs genes in the species studied; for example, 32 superantigenic toxin genotypes were observed across 166 isolates (69 food poisoning isolates, and 97 healthy human nasal swab isolates) in the study performed by Omoe et al. (2005). All hemolysin genes were identified, *hla*, *hlb*, *hld* and *hlg*AC were also detected by Ote et al. (2011), and they identified frequencies between 78.6 and 100% in strains. In this study, hemolysin gene frequencies were between 32.7 and 55.1%. The most prevalent was *hlb*, which is in agreement with other study that assessed isolates from raw milk products (Morandi et al., 2009). Genes encoding exfoliative toxins, *pvl* and *tst* were not identified; previously Ote et al. (2011) identified *eta* and *tst* genes in isolates associated with bovine mastitis.

Regarding antibiotic resistance, penicillin resistance is commonly detected in *Staphylococcus* spp. (Moon et al., 2007; Gómez-Sanz et al., 2010), and this was demonstrated in the present study. Silva et al. (2013) did not detect resistance to erythromycin in their isolates, although they detected one strain of *S. aureus* with resistance to chloramphenicol. Erythromycin and tetracycline resistance genes were observed (Table 2); these genes have been detected in *Staphylococcus* sp. (Silva et al., 2014; Gómez-Sanz et al., 2010). It is important to highlight that all isolates were tested for the presence of *mec*A and *mec*C genes as well as other resistance genes. These results on *mec*A, and SCC*mec* type are in line with Silva et al. (2014), where they assessed methicillin-resistant coagulase-negative staphylococci in milk from cows with mastitis in Brazil. Herein, SCCmec type I to V was investigated due to the availability of positive controls, further studies to assess types I to XI are necessary due to their importance in methicillin resistance. Meanwhile, the absence of a novel *mec*A homologue could be expected because it is of rare occurrence (Cuny et al., 2011). The absence of *mec* genes in cefoxitin and oxacillin resistant strains can indicate the potential presence of modified *S. aureus* (MODSA); MODSA possesses a modification of its penicillin-binding proteins (PBPs), which is different of classical mechanism of MRSA (Bhutia et al., 2012).

Four *spa* types were detected, and on the farm A only one *spa* type (t605) was observed. This suggests that the *spa* type t605 is common and it can be endemic in the region causing subclinical bovine mastitis. The t605 type was initially detected in Austria, France, Germany, Netherlands, Norway, Spain, Sweden and United Kingdom, and represents 0.1% of relative global frequency of *spa* type occurrences in accordance with the website, http://www.ridom.com (http://spaserver.ridom.de - data collected on June 2015). Other studies in Brazil also detected this *spa* type in strains isolated from milk from bovine and others animals (Aires-de-Sousa et al., 2007; Silva et al., 2013). On the other hand, the *spa* type t127 were the most detected by Silva et al. (2013). The *agr* types detected were I and II, which were also detected in a previous study with isolates from bovine mastitis (Silva et al., 2013).

## Conclusion

The majority of isolates were identified as *S. aureus*. Other isolates also identified were *S. hyicus*, *S. xylosus* and *S. chromogenes*. The majority virulence factor genes identified using multiplex PCR, in total eleven different multiplex reactions were successfully optimized and applied in this study. The most isolates carried virulence factor genes, *seh*, and *sel*x were the most detected among SEs. Hemolysins genes were widely identified, presenting several profiles as well as SEs. Antibiotic resistance was widely detected for penicillin; in addition, MRSA strains were observed which presents a concern to public health. The most prevalent *spa* type was t605, which suggests that this could be an endemic *spa* type in the herds sampled. In summary, data regarding molecular variability, and antibiotic resistance for a small group of staphylococci isolated from mastitic milk was shown, which confirms that more studies should be completed to identify and understand strains/clones in specifics regions, and thus to help prevent *Staphylococcus* infection in dairy cows.

## Conflict of interest

The authors declare that there is no conflict of interest.

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## REFERENCES

Aarestrup FM, Dangler CA, Sordillo LM (1995). Prevalence of coagulase gene polymorphism in *Staphylococcus aureus* isolates causing bovine mastitis. Can. J. Vet. Res. 59(2):124-128.

Aires-de-Sousa M, Parente CESR, Vieira-da-Motta O, Bonna ICF, Silva DA, Lencastre H (2007). Characterization of *Staphylococcus aureus* isolates from buffalo, bovine, ovine, and caprine milk samples collected in Rio de Janeiro State, Brazil. Appl. Environ. Microb. 73(12):3845-3849.

Argudín MA, Mendonza MC, Rodicio MR (2010). Food poisoning and *Staphylococcus aureus* enterotoxins. Toxins (Basel) 2(7):1751-1773.
Bhutia KO, Singh TS, Biswas S, Adhikari L (2012). Evaluation of phenotypic with genotypic methods for species identification and detection of methicillin resistant in *Staphylococcus aureus*. Int. J. Appl. Basic Med. Res. 2(2):84-91.
Clinical Laboratory Standards Institute (2015). Performance Standards for Antimicrobial Susceptibility Testing. Wayne (PA): Clinical and Laboratory Standards Institute/NCCLS; 2015 [Twenty-fifth Informational Supplement, M100–S25].
Cremonesi P, Luzzana M, Brasca M, Morandi S, Lodi R, Agnellini D, Caramenti G, Moroni P, Castiglioni B (2005). Development of a multiplex PCR assay for the identification of *Staphylococcus aureus* enterotoxigenic strains isolated from milk and dairy products. Mol. Cell Probes 19(5):299-305.
Cuny C, Layer F, Strommenger B, Witte W (2011). Rare Occurrence of Methicillin-Resistant *Staphylococcus aureus* CC130 with a Novel *mec*A Homologue in Humans in Germany. PLoS One 6:1-5.
Dordet-Frisoni E, Dorchies G, de Araujo C, Talon R, Leroy S (2007). Genomic Diversity in *Staphylococcus xylosus*. Appl. Environ. Microbiol. 73(22):7199-7209.
Fischer A, Francois P, Holtfreter S, Broeker B, Schrenzel J (2009). Development and evaluation of a rapid strategy to determine enterotoxin gene content in *Staphylococcus aureus*. J. Microbiol. Methods 77(2):184-190.
Giannechini R, Concha C, Rivero R, Delucci I, López MJ (2002). Occurrence of clinical and sub-clinical mastitis in dairy herds in the west littoral region in Uruguay. Acta Vet. Scand. 43(4):221-230.
Gómez-Sanz E, Torres C, Lozano C, Fernández-Pérez R, Aspiroz C, Ruiz-Larrea F, Zarazaga M (2010). Detection, molecular characterization, and clonal diversity of methicillin-resistant *Staphylococcus aureus* CC398 and CC97 in Spanish slaughter pigs of different age groups. Foodborne Pathog. Dis. 7(10):1269-1277.
Hu DL, Nakane A (2014). Mechanisms of staphylococcal enterotoxins-induced emesis. Eur. J. Pharmacol. 722:95-107.
Jarraud S, Mougel C, Thioulouse J, Lina G, Meugnier H, Forey F, Nesme X, Etienne J, Vandensesch F (2002). Relationships between *Staphylococcus aureus* genetic background, virulence factors, *agr* groups (alleles), and human disease. Infect. Immun. 70(2):631-641.
Johnson WM, Tyler SD, Ewan EP, Ashton FE, Pollard DR, Rozee KR (1991). Detection of genes for enterotoxins, exfoliative toxins, and toxic shock syndrome toxin 1 in *Staphylococcus aureus* by the polymerase chain reaction. J. Clin. Microbiol. 29(3):426-430.
Jorgensen HJ, Mathisen T, Lovseth A, Omoe K, Qvale KS, Loncarevic S (2005). An outbreak of *Staphylococcus* food poisoning cause by enterotoxin H in mashed potato made with raw milk. FEMS Microbiol. Lett. 252(2):267-272.
Kondo Y, Ito T, Ma XX, Watanabe S, Kreiswirth BN, Etienne J, Hiramatsu K (2007). Combination of multiplex PCRs for Staphylococcal Cassette Chromosome *mec* type assignment: rapid identification system for *mec*, *ccr*, and major differences in junkyard regions. Antimicrob. Agents Chemother. 51(1):264-274.
Lange CC, Brito MAVP, Reis DRL, Machado MA, Guimarães AS, Azevedo AL, Salles ÉB, Alvim MC, Silva FS, Meurer IR (2015). Species-level identification of staphylococci isolated from bovine mastitis in Brazil using partial 16S rRNA sequencing. Vet. Microbiol. 176(3-4):382-388.
Lina G, Piémont Y, Godail-Gamot F, Bes M, Peter MO, Gauduchon V, Vandenesch F, Etienne J (1999). Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. Clin. Infect. Dis. 29(5):1128-1132.
Mehrotra M, Wang G, Johnson WM (2000). Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1, and methicillin resistance. J. Clin. Microbiol. 38(3):1032-1035.
Moon JS, Lee AR, Kang HM, Lee ES, Kim MN, Paik YH, Joo YS, Koo HC (2007). Phenotypic and genetic antibiogram of methicillin-resistant staphylococci isolated from bovine mastitis in Korea. J. Dairy Sci. 90(3):1176-1185.
Morandi S, Brasca M, Andrighetto C, Lombardi A, Lodi R (2009). Phenotypic and genotypic characterization of *Staphylococcus aureus* strains from Italian dairy product. Int. J. Food Microbiol. 2009:1-7.
Nashev D, Toshkova K, Salasia SI, Hassan AA, Lämmler C, Zschöck M (2004). Distribution of virulence genes of *Staphylococcus aureus* isolated from stable nasal carriers. FEMS Microbiol. Lett. 233(1):45-52.
Omoe K, Hu DL, Takahashi-Omoe H, Nakane A, Shinagawa K (2005). Comprehensive analysis of classical and newly described staphylococcal superantigenic toxin genes in *Staphylococcus aureus* isolates. FEMS Microbiol. Lett. 246(2):191-198.
Omoe K, Ishikama M, Shimoda Y, Hu DL, Ueda S, Shinagawa K (2002). Detection of *seg, seh* and *sei* genes in *Staphylococcus aureus* isolates and determination of the enterotoxin productivities of *S. aureus* isolate harboring *seg*, *seh* or *sei* genes. J. Clin. Microbiol. 40(3):857-862.
Ono HK, Omoe K, Imanishi K, Iwakabe Y, Hu DL, Kato H, Saito N, Nakane A, Uchiyama T, Shinagawa K (2008). Identification and characterization of two novel staphylococcal enterotoxins, types S and T. Infect. Immun. 76(11):4999-5005.
Ote I, Taminiau B, Duprez JN, Dizier I, Mainil JG (2011). Genotypic characterization by polymerase chain reaction of *Staphylococcus aureus* isolates associated with bovine mastitis. Vet. Microbiol. 153(3-4):285-292.
Park JY, Fox LK, Seo KS, McGuire MA, Park YH, Rurangirwa FR, Sischo WM, Bohach GA (2011). Detection of classical and newly described staphylococcal superantigen genes in coagulase-negative staphylococci isolated from bovine intramammary infections. Vet. Microbiol. 147(1-2):149-154.
Rajic-Savic N, Katic V, Velebit B, Colovic S (2015). Characteristics of Enterotoxigenic Coagulase Positive Staphylococci isolated from bovine milk in cases of subclinical mastitis. Procedia Food Sci. 5:250-253.
Sasaki T, Tsubakishita S, Tanaka Y, Sakusabe A, Ohtsuka M, Hirotaki S, Kawakami T, Fukata T, Hiramatsu K (2010). Multiplex-PCR method species identification of coagulase-positive staphylococci. J. Clin. Microbiol. 48(3):765-769.
Shopsin B, Mathema B, Alcabes P, Said-Salim B, Lina G, Matsuka, Martinez J, Kreiswirth BN (2003). Prevalence of *agr* specificity groups among *Staphylococcus aureus* strains colonizing children and their guardians. J. Clin. Microbiol. 41(1):456-459.
Silva NCC, Guimarães FF, Manzi MP, Budri PE, Gómez-Sanz E, Benito D, Langoni H, Rall VL, Torres C (2013). Molecular characterization and clonal diversity of methicillin susceptible *Staphylococcus aureus* in milk of cows with mastitis in Brazil. J. Dairy Sci. 96(11):6856-6862.
Silva NCC, Guimarães FF, Manzi MP, Gómez-Sanz E, Gómez P, Araújo-Júnior JP, Langoni H, Rall VL, Torres C (2014). Characterization of methicillin-resistant coagulase-negative staphylococci in milk from cows with mastitis in Brazil. Antonie van Leeuwenhoek 106(2):2270-233.
Thomas D, Dauwalder O, Brun V, Badiou C, Ferry T, Etienne J, Vandenesch F, Lina G (2009). *Staphylococcus aureus* superantigens elicit redundant and extensive human Vbeta patterns. Infect. Immun. 77(5):2043-2050.
van de Klundert, JAM, Vliegenthart JS (1993). PCR detection of genes for aminoglycoside-modifying enzymes. In. Persing, DH, Smith, TF, Tenover, FC, White, TJ. Diagnostic molecular microbiology. Washington. Am. Soc. Microbiol. 1993:547-552.
Wilson GJ, Seo KS, Cartwright RA, Connelley T, Chuang-Smith ON, Merriman JA, Guinane CM, Park JY, Bohach GA, Schlievert PM, Morrison WI, Fitzgerald JR (2011). A novel core genome-encoded superantigen contributes to lethality of community-associated MRSA necrotizing pneumonia. PLoS Pathogens 7:1-16.
Yamaguchi T, Nishifuji K, Sasaki M, Fudaba Y, Aepfelbacher M, Takata T, Ohara M, Komatsuzawa H, Amagai M, Sugai M (2002). Identification of the *Staphylococcus aureus etd* pathogenicity island which encodes a novel exfoliative toxin, ETD, and EDIN-B. Infect. Immun. 70(10):5835-5845.

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# Diversity and distribution of the endophytic fungal community in eucalyptus leaves

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***Eucalyptus*** **species are among the most widely grown and most economically valuable trees worldwide. In Brazil, eucalyptus is of major environmental and economic importance because it generates thousands of jobs and offers an alternative to using wood from native forests. In forest ecosystems, plant shoots are considered a common habitat for various microorganisms, and plants of the family Myrtaceae are an important source of fungal biodiversity. However, very little is known about the diversity and microbial distribution in eucalyptus leaves. This study aimed at showing the diversity and distribution of endophytic fungi in the leaves of eucalyptus plants aged 18 and 72 months. The leaves were collected at the onset of the rainy period, during the rainy period, and during the dry period. Diversity was measured using DNA extraction, 18S rRNA subunit amplification, denaturing gradient gel electrophoresis (DGGE), and sequencing of eluted bands. The endophytic fungal community was affected by plant location. Differences observed in the distribution of the phylogenetic groups found in the upper, middle and lower thirds of the tree canopy indicate that the endophytic community distribution in eucalyptus is dependent on leaf position. The age of the plants affected the diversity of endophytic fungi in *Eucalyptus* "urograndis". Phylogenetic analysis showed that the phyla Basidiomycota and Ascomycota dominate in the environments studied. The description of endophytic fungal diversity in this important forest species is an important step for assessing this genetic resource in the search for metabolites and processes that can contribute to improving plant development.**

**Key words:** Diversity, endophytic fungi, denaturing gradient gel electrophoresis (DGGE), sequencing.

## INTRODUCTION

*Eucalyptus* are the most widely used trees in planted forests, due to their growth characteristics, their adaptability to climate conditions and distinct soil types, and the value of their wood (FAO, 2015). *Eucalyptus* wood can be used in several ways, including the production of paper and cellulose, panels and

**Table 1.** Georeferencing of the sites planted with eucalyptus forests, predominant soil class, year of the first planting and mean forest productivity ($m^3$ $ha^{-1}$)/rotation.

| Site/project | Latitude (w) | Longitude (s) | Altitude (m) | Soil class | Plantation/eucalyptus planting | Productivity ($m^3$ $ha^{-1}$)/ rotation |
|---|---|---|---|---|---|---|
| I (Catas Altas) | 43° 24′ 54″ | 19° 57′ 32″ | 750 | LAd1 | 1970 | 340 |
| II (Santa Bárbara) | 43° 24′ 28″ | 20° 4′ 30″ | 750 | LAw2 | 1989 | 340 |

mechanically processed wood, and in the metalworking industry as plant charcoal (IBA, 2013). Additionally, *Eucalyptus* may also provide a profitable source of lignocellulose for energy production and advanced biofuels (Rockwood et al. 2008). In Brazil, *Eucalyptus grandis* x *Eucalyptus urophylla* hybrid ("urograndis" eucalyptus) plants exhibit uniform growth and high cellulose production, characteristics that have driven the growth of planted forests since the 1990s (Iglesias-Trabado and Wilstermann, 2008).

Plant shoots are a common habitat for various microorganisms (Vandenkoornhuyse et al., 2015), and interactions with these microorganisms are important in maintaining the equilibrium of the biogeochemical cycles, gas flows and other determinant processes in ecosystems (Lindow and Brandl, 2003). Endophytes can confer beneficial effects on the plant: protection against invading pathogens and herbivores, or via antibiosis or induced resistance and plant growth. They can still confer the host plant greater tolerance to salinity and drought (Hardoim et al., 2015). Thus, the agronomic and environmental significance of the microorganisms that inhabit plant shoots may be reflected in the adaptability of plant populations and also in crop quality and productivity (Turner et al. 2013). On the global scale, fungal diversity is greater in tropical forests, where terrestrial plant diversity is also greater; however, the true scale of associated endophytic diversity is still not well known (Luo et al., 2014).

Endophytic fungi are present on terrestrial plants and are especially abundant and diverse on the leaves of several tropical (Arnold, 2005) and subtropical trees as compared to other climate zones (Banerjee, 2011). However, multiple patterns have already been reported (Zhang and Yao, 2015), which means that fungal diversity patterns in plants are complex. These microorganisms are considered important components of global biodiversity (Arnold, 2005). The endophytic fungi may affect plant adaptability and evolution in their environment (Vandenkoornhuyse et al., 2015).

Characterizing the microbial community associated with eucalyptus plants in Brazilian commercial forests, in addition to providing a greater understanding of plant-microorganism interactions, is extremely important for maximizing the productivity and optimizing the management of crops that are significant to the Brazilian economy. The use of culture-dependent methods in diversity studies allows for the assessment of only a small fraction of this diversity (Torsvik and Ovreas, 2002). Microbial diversity can also be studied by analyzing the total DNA of the microbiota extracted directly from the plant for processing using electrophoretic techniques (Oliveira et al., 2013). Studies using these techniques have contributed to a better understanding of the microbial community structure and have led to new perspectives and advances in ecological studies (Hoshino and Matsumoto, 2007; Oliveira et al., 2013; Da Silva et al., 2014). Thus, the present study aimed at evaluating the composition and diversity of the endophytic fungal community in eucalyptus leaves at the onset of the rainy period and during the rainy and dry periods.

## MATERIALS AND METHODS

### Study sites

The two study sites are forests belonging to the Celulose Nipo-Brasileira company (CENIBRA) planted with *Eucalyptus* "urograndis" located in the municipalities of Catas Altas (Site 1 – Catas Altas I Project) and Santa Bárbara (Site 2 – Serra do Pinho Project), Minas Gerais, Brazil. The forest in Catas Altas is currently in the seventh farming cycle, first implemented in December 1970, and the forest in Santa Bárbara is currently in the third farming cycle, first implemented in December 1989. The mean productivity at the two sites is 340 $m^3$ $ha^{-1}$/six-year rotation (Table 1), and the soils are highly weathered, with an active, undulating, and strongly undulating relief and with yellow Oxisol as the most representative soil class.

The natural vegetation of these sites was a semideciduous seasonal forest, which was replaced with coffee crops and pastures. Subsequently, those crops were replaced with eucalyptus (CENIBRA). The plants were 18 months old in Catas Altas (Site 1) and 72 months old in Santa Bárbara (Site 2) at the time of sampling.

At both sites, the climate type, according to the Köppen classification, is the mesothermal Cwa (Köppen, 1948), with a dry winter and rain in the summer (Table 2). The mean maximum, average and minimum temperatures in recent years were 26.4, 16.9 and 21.6°C, respectively; the relative humidity was 67%, and

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**Table 2.** Rainfall recorded within the region studied (Catas Altas/Santa Bárbara).

| Year | JAN | FEB | MAR | APR | MAY | JUN | JUL | AUG | SEP | OCT | NOV | DEC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | mm$^3$ | | | | | | | | | | | |
| 2011 | 243 | 123.4 | 329.5 | 93.7 | 21.3 | 8.9 | 1.8 | 1 | 8.9 | 231.4 | 431 | 664.2 |
| 2012 | 328.3 | 28.7 | 166.1 | 21.8 | 138.6 | 16.5 | 0 | 5.3 | 36.8 | - | - | - |

the rainfall and water deficit were 122.9 and 15.3 mm, respectively (CENIBRA, Gaspar meteorological station.

**Sampling**

Leaves were sampled from selected plants that were 18 (Catas Altas) and 72 (Santa Bárbara) months old and had average height of 6.0 and 18.0 m, respectively. The trees were localized in a sub-area of 81 m$^2$ (approximately 8 trees), with a spacing of 3.33 x 3 m between trees. After the tree harvest, the leaves in three regions [upper (UPP), middle (MID) and lower (LOW)] of the canopy were sampled. To ensure a more representative sample from the whole canopy, leaves were collected from proximal, median and distal parts of the stem in each region (in triplicate and mixed on composite samples).

The samples were collected at the onset of the rainy period–ORP (October, 2011), during the rainy period– RP (December 2012), and during the dry period– DP (April 2012). Approximately, 50 leaves without symptoms of disease (150 g) were collected separately from the upper, middle and lower thirds of the canopy. The samples were placed in a box containing ice for transport to the Laboratory of Microbial Ecology (Laboratório de Ecologia Microbiana - LEM) of the Department of Microbiology (Departamento de Microbiologia), Federal University of Viçosa (Universidade Federal de Viçosa), Minas Gerais, Brazil. In the LEM, the samples were stored and vacuum-packed at -20°C for approximately one month before being processed for a diversity analysis of their filamentous fungi using independent cultivation methodology.

**Surface sterilization of the eucalyptus leaves**

Surface sterilization of eight healthy leaves from each third of the sampled tree crowns was performed after the leaves were washed under running water and distilled water. Next, the material was immersed twice in distilled water and phosphate buffer (0.05 mmol·L$^{-1}$), pH 7, immersed in 70% ethanol (v/v) for one minute, kept in a container filled with sodium hypochlorite (5%) + 0.05% (v/v) Tween-80 for five minutes, and then immersed for 30 s in 70% ethanol (v/v) before being immersed again in sodium hypochlorite + Tween-80 for 15 min (Miguel et al., 2013, 2016; Oliveira et al., 2013). This process was repeated once. This sterilization/washing was performed to reduce the surface DNA. Finally, the leaves were placed in sterile distilled water and individually placed into tubes containing 10 mL of R2A culture medium (Reasoner and Gelrdreich, 1985) and incubated at 28°C for 72 h. Aliquots (100 µL) of the final wash water were transferred into Petri dishes containing agar-R2A, which were then incubated at 28°C for 72 h to demonstrate the absence of microbial growth (Oliveira et al., 2013).

**Metagenomic DNA extraction from leaves**

Leaves sampled from each third of the crowns were surface sterilized, incubated in R2A medium, ground in liquid nitrogen, macerated, and transferred into 2.0-mL polypropylene or microcentrifuge tubes. Extraction buffer [(2% (p/v) cetyl trimethyl ammonium bromide (CTAB), 1.4 mol·L$^{-1}$ NaCl, 20 mmol·L$^{-1}$ EDTA, 100 mmol·L$^{-1}$ Tris–HCl, pH 8.0, and 1 g of polyvinylpyrrolidone, and 0.2% (v/v) β-mercaptoethanol] was added to the tubes containing the ground samples. Next, 1000 µL of extraction buffer, 0.5 g of 106 µm beads, 50 µL of 4% sodium dodecyl sulfate (SDS) and 400 µL of phenol-chloroform (1:24) were added to the tubes. The mixture was stirred in a homogenizer for 10 min and placed in a water bath at 60°C for 10 min. The tubes were centrifuged at 15,000 *g*, and the supernatant was transferred into tubes containing 400 µL of phenol-chloroform, followed by an additional centrifugation at 15,000 *g* for 5 min. The DNA was precipitated by mixing the supernatant with 0.6 volumes of isopropanol, followed by centrifugation at 15,000 *g* for 20 min. The DNA pellet was washed twice in 70% ethanol and resuspended in 100 µL of sterile Milli-Q water after drying under a laminar flow hood. The concentration and purity of the extracted DNA were confirmed via optical density at 260 and 280 nm (NanoDrop® ND-1000, Thermo Fisher Scientific, Inc.).

**Analysis of the endophytic fungal diversity**

Denaturing gradient gel electrophoresis (DGGE) and nested PCR were used to examine the endophytic fungal diversity of leaves. In the first PCR, the total DNA was used as a template to amplify the V1-V9 region of the fungal 18S rRNA gene. The oligonucleotide primers NS1 (May et al., 2001) and EF3 (Oros-Sichler et al., 2006) were used for the first reaction. The resulting fragments were used as templates for a second PCR, and the V7-V8 region was amplified using the primers FF390 and FR1GC (Vainio and Hantula 2000), which contain a GC clamp incorporated into the oligonucleotide's forward region.

The first PCR was performed in a final volume of 25 µL, containing 5 µL of GoTaq Flex® Reaction Buffer, 200 µM dNTPs, 2.0 U of GoTaq Flex DNA polymerase, 3.0 mM magnesium chloride, 0.16 µM NS1 primer, 0.16 µM EF3 primer, approximately 50 ng of total DNA and sterile deionized water (Milli-Q). The amplifications were performed under the following conditions: initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 47°C for 1 min, and extension at 72° C for 2 min, and a final extension at 72°C for 10 min. The second PCR was performed using 1.0 µL of the first reaction as the template, and the primers NS1 and EF3 were replaced with the pair FF390/FR1GC. The reaction conditions were as follows: initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension for 1 min at 72°C, and a final extension at 72°C for 10 min.

The resulting amplicons were subjected to DGGE (DCode System, Bio-Rad Inc., California). A mixture of DNA from pure cultures of *Nocardioides thermolilacinus*, *Bacillus cereus*, *Streptomyces setonii*, *Clavibacter michiganensis*, *Pectobacterium carotovorum*, *Pseudomonas putida*, *Pseudomonas syringae*, *Xanthomonas vesicatoria* and *Ralstonia solanacearum* was used as an external marker to facilitate normalization of the gels in the

BioNumerics® software, version 7.1 (Applied Maths, Kortrijk, Belgium). The PCR products were loaded in an 8% polyacrylamide gel (w/v) (37.5:1, acrylamide – N,N'-methylenebisacrylamide) (Sigma) with a denaturing gradient using 1X TAE as the buffer (40 mmol·$L^{-1}$ Tris–HCl, pH 8, 20 mmol·$L^{-1}$ acetic acid, 1 mmol·$L^{-1}$ EDTA, pH 8.0), 0.09% (v/v) TEMED (N,N,N',N'-tetramethylenediamine) and 0.7% (w/v) ammonium persulfate. The denaturing gradient was optimized at 35 to 55% urea/formamide (100% denaturant contains 7 mol·$L^{-1}$ urea and 40% (v/v) formamide). Electrophoresis was performed in 1X TAE buffer at 60 V for 20 h at a constant temperature of 60°C. The DNA fragments in the gel were stained for 20 min in 1X TAE buffer containing 1X SYBR Gold dye (Invitrogen, Carlsbad, California, USA), and the gel images were recorded using Molecular Imaging System L-pix Chemi equipment (Loccus Biotechnology, São Paulo, SP, Brazil).

To analyze the endophytic fungal community, individual bands that showed better signal under UV light (300 nm) were excised from the polyacrilamide gels, eluted into polypropylene tubes containing 30 µL of sterile Milli-Q water, and kept overnight at 4°C. A 7-µL aliquot of the eluate from each band was used as a template for PCR with the oligonucleotide primers FF390 and FR1 (without GC clamp). The 132 amplicons obtained from PCRs was visualized on an agarose gel (0.8% w/v) stained with Gel Red® 1000X, and images were obtained using L-pix Chemi (Loccus Biotechnology, São Paulo, São Paulo, Brazil). The 65 amplicons obtained from these reactions containing 100 ng/µL were sequenced by Macrogen, Inc. Korea, and the sequences obtained were compared with those available in the GenBank database (NCBI). For each sequence, an identity search was performed with the BLASTn algorithm (Basic Local Alignment Search Tool) (http://www.ncbi.nlm.nih.gov/BLAST) for nucleotides (Altschul et al. 1990). The sequences reported in this study have been submitted to GenBank under the accession numbers KU663411 to KU663476.

The DGGE band profiles were compared using BioNumerics® software, version 7.1 (Applied Maths, Kortrijk, Belgium). The fungal richness variable was estimated using the program based on a binary matrix, in which the presence of one band corresponding to an operational taxonomic unit (OTU) was encoded as one (1) and its absence as zero (0). The structure of this community was evaluated based on the Dice similarity coefficient and the unweighted pair group method with arithmetic mean (UPGMA). The richness and diversity analyses were performed using the software PAST (Hammer et al., 2001), in which diversity is estimated using the Shannon index, and statistical analyses were performed in Minitab version 15 (Minitab, 2006) (Minitab Inc., State College, Pennsylvania, USA) using Tukey's test at 5% probability. The correlation of the endophytic fungal communities in Catas Altas and Santa Bárbara at the onset of the rainy period, during the rainy period and the dry period determined by DGGE was determined using Principal Component Analysis (PCA) in Canoco software (version 4.5, Biometris, Wageningen, Netherlands). Rarefaction curves were calculated using Analytic Rarefaction 1.3 software (http://strata.uga.edu/software/anRareReadme.html).

### Phylogenetic analysis

The obtained sequences after sequencing were compared with those from the NCBI Nucleotide database using the BLAST algorithm (Altschul et al., 1990). The 18S rRNA sequences that were distinct from each other in the database and sharing more than 97% identity were imported with Mega 6.0 and aligned using ClustalW.

The alignments were manually adjusted, and a phylogenetic analysis was performed using the neighbor-joining method (Saitou and Nei, 1987). The phylogenetic distance was computed using the p-distance method, and the robustness of the resulting trees and the statistical significance levels of the interior nodes were obtained by bootstrap analysis with 1000 replicates, and the values above 50% were shown.

## RESULTS

The protocol for DNA extraction and 18S rRNA gene amplification resulted in amplicons with distinct electrophoretic migration patterns in DGGE, allowing the evaluation of the endophytic fungal diversity in the leaves of eucalyptus (Figure 1).

The electrophoretic patterns obtained by DGGE showed more intense bands in the same relative positions (same location in the gels) and OTUs distincts were detected in the leaves analyzed. The presence of lower- and higher-intensity OTUs indicates that the nested PCR provided the resolution necessary for the diversity analyses (Figure 1). This resolution (Carmona et al., 2012) was interpreted as a single band after electrophoresis on acrylamide gel. The DNA fragments in the bands excised from different positions in the DGGE gel were identified as belonging to the phyla Basidiomycota and Ascomycota. The band-excision technique was useful in assessing the endophytic fungal diversity of eucalyptus in the present study (Figure 1).

Analysis of the fungal 18S rRNA gene fragments present in the leaves revealed distinct fungal communities with respect to the cultivation sites (Catas Altas and Santa Bárbara). DGGE allowed the detection of differences between the endophytic communities in eucalyptus farmed in Catas Altas and Santa Bárbara (Figures 2 and 3). The comparative analysis between the two areas showed a smaller number co-occurring groups in relation to the analysis of individual areas. The highest bootstrap (98%) corresponded to samples from Santa Bárbara at the top of the canopy at the beginning of the rainy season (Figure 3).

In the eucalyptus leaves collected within the Catas Altas region, UPGMA analysis generated five distinct groups, where the highest similarity value (52.3%) corresponded to the collection performed at the onset of the rainy period in leaves from the lower portion of the tree canopy (Figure 2A). The highest similarity found within the Santa Bárbara region was 55.3% during the dry period, also from the lower portion of the tree canopy (Figure 2B). The occurrence of common OTUs (23 and 22) in the eucalyptus leaves is independent of the sampling period. Other OTUs exhibit distinct distribution profile between the sampling periods, such as a higher incidence of specific OTUs during the rainy period in Catas Altas, whereas this occurred during the dry period at the Santa Bárbara location (Figure 4). The Shannon diversity indices within Catas Altas ranged from 2.56 to 3.02, and the richness indices ranged from 13 to 21 (Table 3). In Santa Bárbara, the variation was smaller, with diversity indices ranging from 2.09 to 2.4 and richness indices ranging from 7.5 to 11.3 (Table 3).

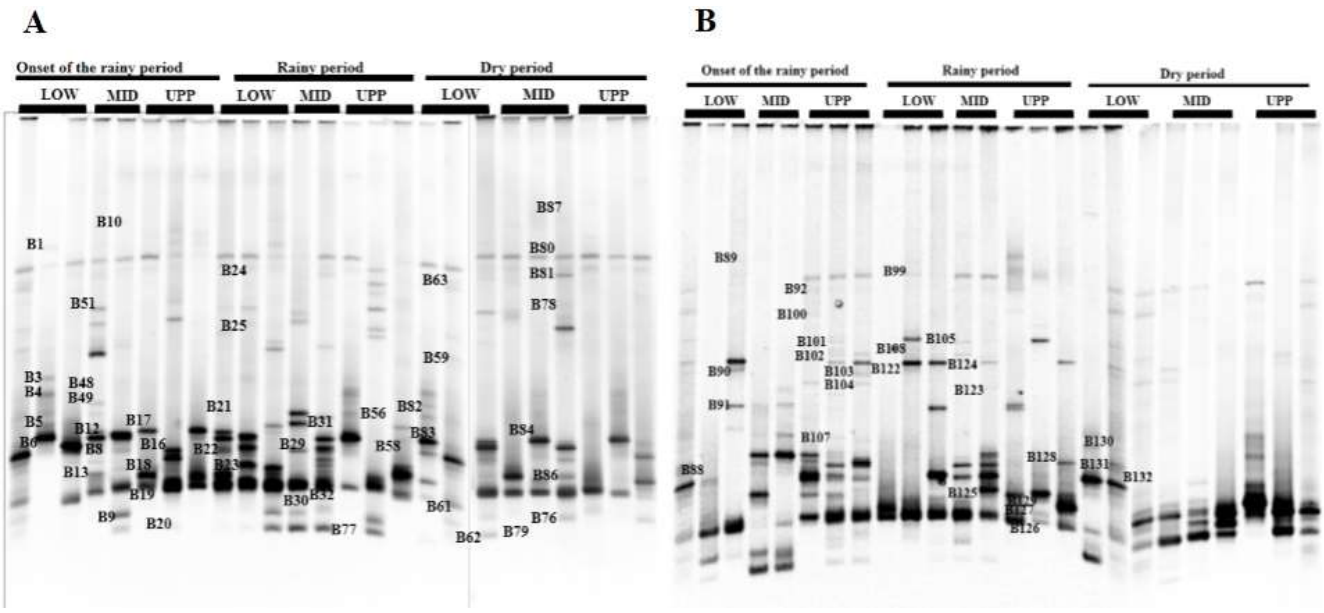


**Figure 1.** DGGE electrophoretic patterns of the endophytic fungal community extracted from leaves of the lower, middle and upper thirds (in triplicate) of the tree canopy of eucalyptus grown at distinct sites: (A) 18-month-old trees grown in the municipality of Catas Altas, (B) 72-month-old trees grown in the municipality of Santa Bárbara. LOW: leaves from the lower third of the tree canopy; MID: leaves from the middle third of the tree canopy; UPP: leaves from the upper third of the tree canopy. The samplings were performed at the onset of the rainy period, during the rainy period, and during the dry period. Letter B combined with Arabic numbers indicates the band excision location. The leaf samples collected from the middle part of the crown at the beginning of the rainy season and the rainy season from both locations were analyzed in duplicate because the amount of DNA extracted from the third sample was insufficient for analysis.

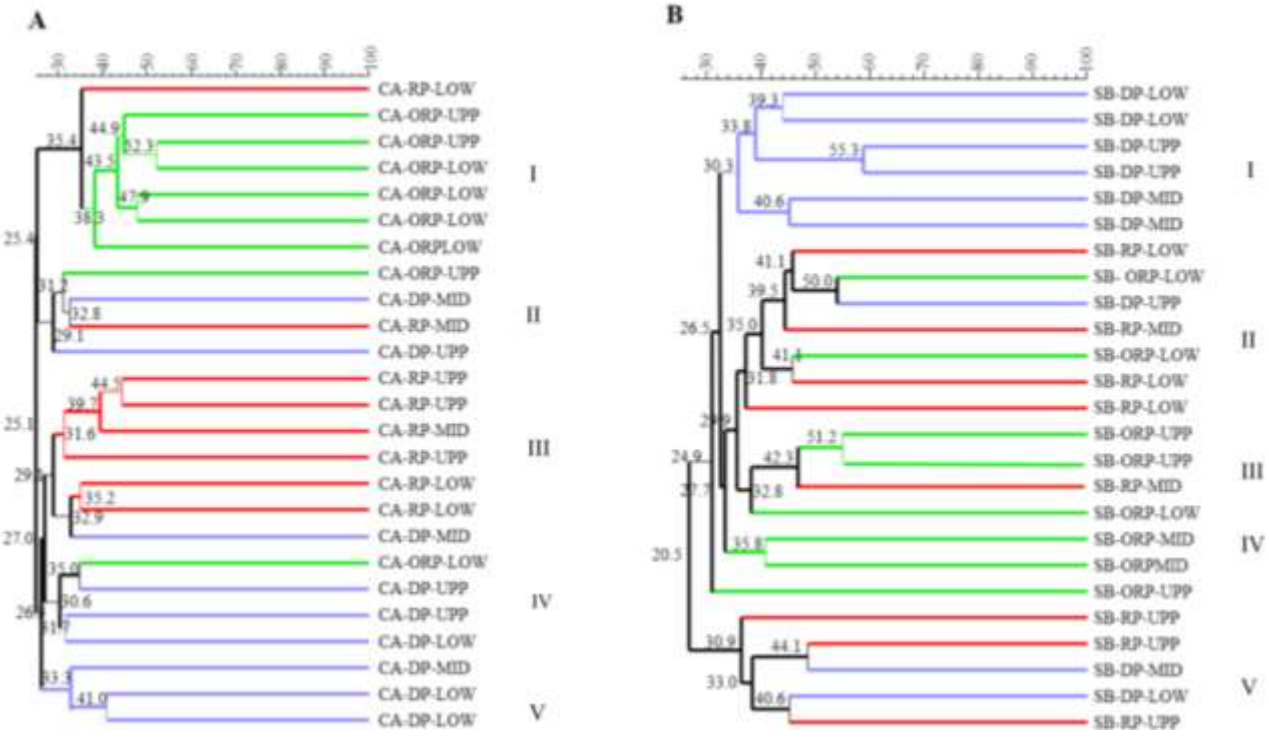


**Figure 2.** Cluster analysis and similarity indices obtained from the DGGE electrophoretic pattern of the endophytic fungal community extracted from leaves from the lower, middle and upper thirds of the tree canopy of eucalyptus. **(A)** 18-month-old trees grown in the municipality of Catas Altas. **(B)** 72-month-old trees grown in the municipality of Santa Bárbara. ORP: sampling performed at the onset of the rainy period; RP: sampling performed during the rainy period; DP: sampling performed during the dry period.

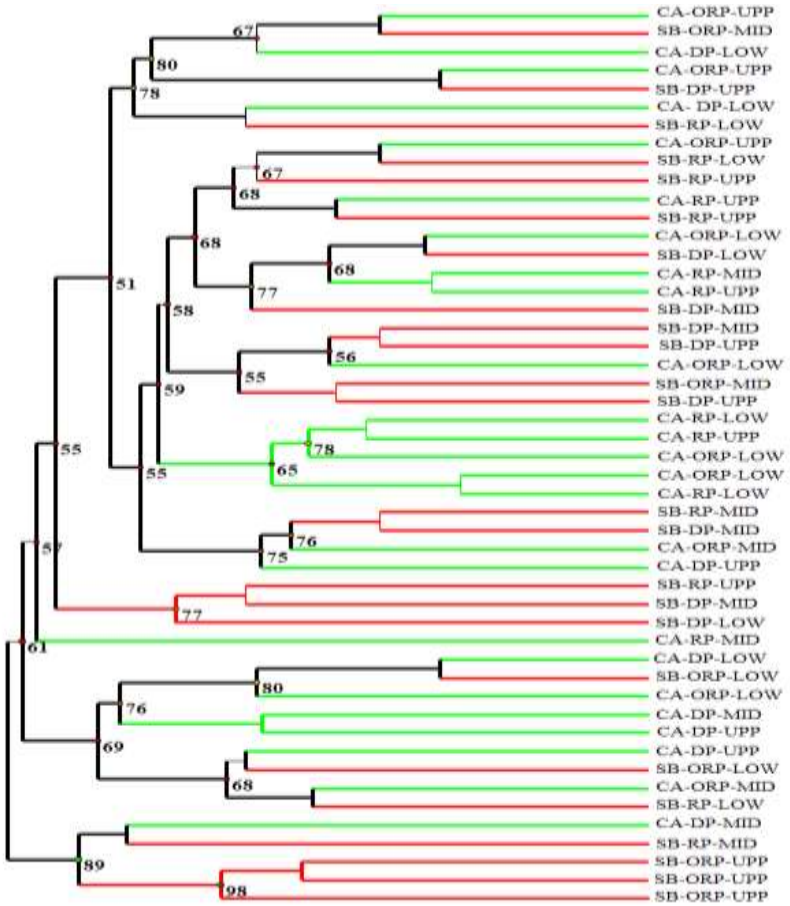


**Figure 3.** Cluster analysis and similarity indices obtained from the DGGE electrophoretic patterns of the endophytic fungal samples extracted from leaves of the lower, middle, and upper thirds of the tree canopy of eucalyptus grown at Catas Altas (CA) (18-month-old trees) and Santa Bárbara (SB) (72-month-old trees). ORP: onset of the rainy period; RP: rainy period; DP: dry period. LOW: lower portion of the canopy; MID: middle portion of the canopy; UPP: upper portion of the canopy.

Although, there are variations in Shannon diversity index and richness in Catas Altas and Santa Bárbara individually, the difference between them is not significant according to the Tukey test at 5% probability. However, when comparing the averages of these indices between the two areas, Catas Altas shows higher diversity than Santa Bárbara according to the Tukey test at 5% probability (Table 3). The first and second axis of the principal component analysis (PCA) explained 25.1 and 22.6% of the variation in the community of endophytic fungi in Catas Altas and Santa Bárbara, respectively (Figure 5).

The endophytic fungal distribution in eucalyptus leaves in the Catas Altas region differs depending on the position of the leaves in the tree canopy and between the rainy and dry periods. At this site, 14 species were identified; the greatest number of species was found at the onset of the rainy period (Table 4).

The endophytic community of the Santa Bárbara leaves comprises seven species (Table 4), which are mostly the same as those found in Catas Altas. However, *Anomoloma albolutescens*, *Rhodotarzetta rosea* and *Rhizoctonia solani* were exclusive to Santa Bárbara (Table 4).

Although, leaf position and seasonality did not affect the diversity and richness of endophytic fungi (Table 3), these factors affected the endophytic fungal distribution of Catas Altas more than that of Santa Bárbara (Table 4). The highest endophytic prevalence in Catas Altas was found at the onset of the rainy period (Table 4), whereas in Santa Bárbara, it was found during the rainy period (Table 4). At the latter site of the fungal species identified by sequencing, only *Boletus rubropunctus* was found in more than one third of the tree canopy and during more than one of sampling period (Table 4). In eucalyptus, there are differences in the colonization and persistence

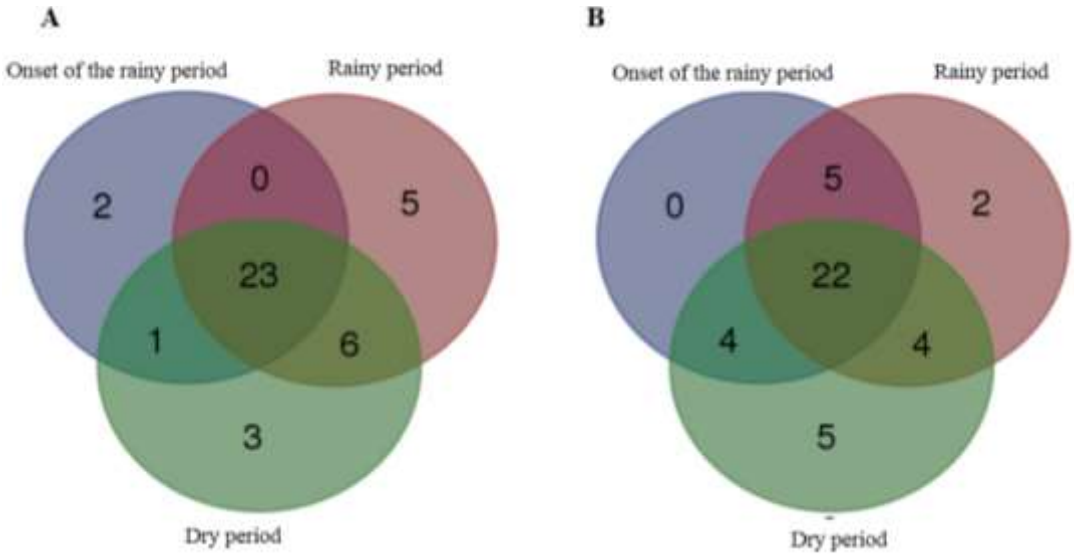


**Figure 4.** OTU distribution by Venn diagram in eucalyptus leaves at the onset of the rainy period, during the rainy period, and during the dry period. (A) OTU distribution in leaves collected at Catas Altas (18-month-old trees). (B) OTU distribution in leaves sampled at Santa Bárbara (72-month-old trees).

**Table 3.** Richness and diversity of endophytic fungi at the onset of the rainy period, during the rainy period, and the dry period in leaves of the upper, middle and lower thirds of the tree canopy of eucalyptus in 18 and 72-month-old plants grown at Catas Altas (CA) and Santa Bárbara (SB).

| Study sites | Indices | Onset of the rainy period | | | Rainy period | | | Dry period | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Third of the eucalyptus canopy | | | | | | | | |
| | | Lower | Middle | Upper | Lower | Middle | Upper | Lower | Middle | Upper |
| CA | Richness | $17.30^{Aa}$ | $15.50^{Aa}$ | $15.70^{Aa}$ | $13.00^{Aa}$ | $19.50^{Aa}$ | $15.70^{Aa}$ | $18.30^{Aa}$ | $21.00^{Aa}$ | $16.00^{Aa}$ |
| SB | Richness | $11.30^{Ba}$ | $7.50^{Ba}$ | $11.00^{Ba}$ | $10.00^{Ba}$ | $9.50^{Ba}$ | $8.70^{Ba}$ | $9.30^{Ba}$ | $9.70^{Ba}$ | $8.30^{Ba}$ |
| CA | Diversity | $2.84^{Aa}$ | $2.73^{Aa}$ | $2.75^{Aa}$ | $2.56^{Aa}$ | $2.89^{Aa}$ | $2.75^{Aa}$ | $2.90^{Aa}$ | $3.02^{Aa}$ | $2.76^{Aa}$ |
| SB | Diversity | $2.35^{Ba}$ | $2.20^{Ba}$ | $2.40^{Ba}$ | $2.30^{Ba}$ | $2.20^{Ba}$ | $2.15^{Ba}$ | $2.22^{Ba}$ | $2.24^{Ba}$ | $2.09^{Ba}$ |

Uppercase letters in richness in columns indicate significant differences between means. Uppercase letters in diversity in columns indicate significant differences between means. The same letters in either richness or diversity in rows indicate no significant difference between the means. All comparisons used the Tukey test at 5% probability.

of endophytic fungi as a function of seasonality (Table 4), and Basidiomycota is the fungal phylum that predominates in eucalyptus leaves (Table 4). Phylogenetic analysis of the sequences revealed that they all belong to the phyla Basidiomycota and Ascomycota, forming distinct clades (Figure 6). Most of the groupings formed exhibited bootstrap values above 70, which are considered moderate to strong (Schneider, 2007). These findings indicate the robustness of the analysis. In Catas Altas, bands 1 (B1), 4 (B4), B8 (B8), 25 (B25), 62 (B62) and 87 (B87) formed the groupings with the greatest phylogenetic support with bootstrap values greater than 80, with most of them between 98 and 100 (Figure 6). These bootstrap values are considered strong (Schneider, 2007) and indicate the robustness of the phylogenetic analysis.

The OTUs from the amplicons extracted from the bands that corresponded to leaves collected within the Santa Bárbara region formed groups with the greatest bootstraps for bands 99 (B99) and 101 (B101), whose bootstrap values were 97 and 99, respectively. The OTUs were grouped with high phylogenetic support into two distinct clades, both belonging to the phylum Ascomycota. Phylogenetic tree support is ensured by a value of 99 for the outermost node (Figure 6), although some of the bootstrap values could be considered moderate and low.

The rarefaction curve calculated for the samples from the beginning of the rainy season, rainy season and dry season of Catas Altas and Santa Bárbara tended to reach a plateau, showing that the number of OTUs screened in the fungal community of both areas was

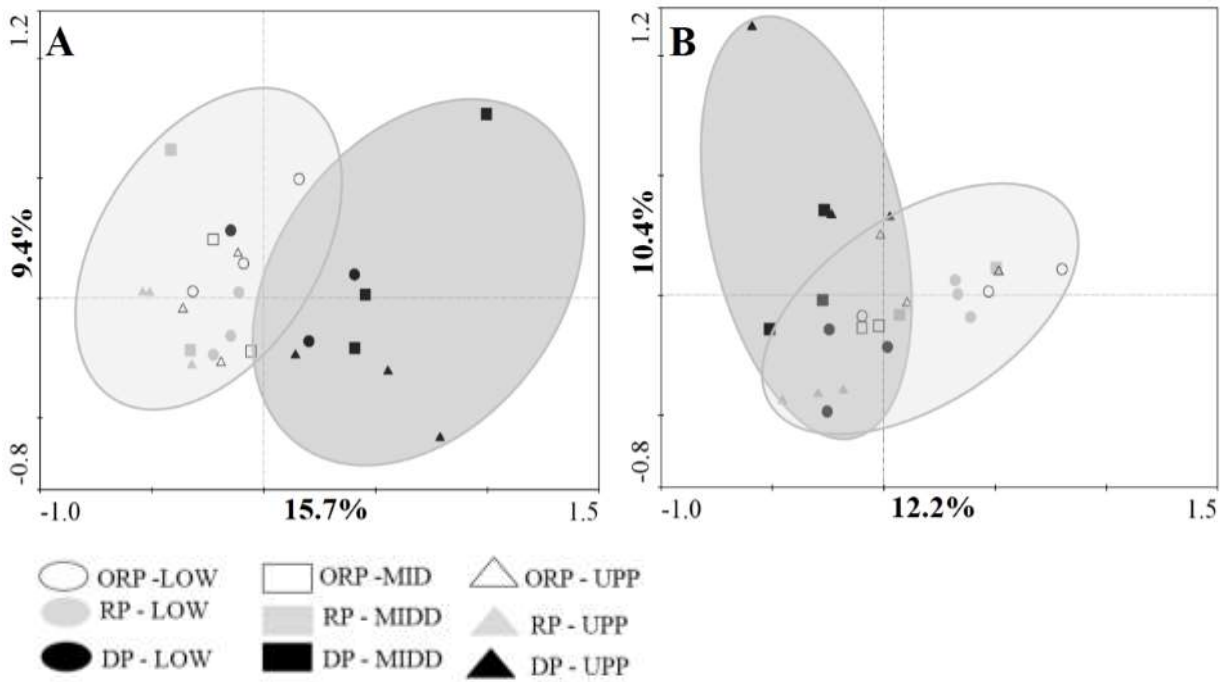


**Figure 5.** Principal component analysis (PCA) based on PCR-DGGE profiles of the 18S rRNA gene from plants samples from *Eucalyptus* "urograndis" from (A) Catas Altas and (B) Santa Bárbara at the onset of the rainy period (ORP), during the rainy (RP) and dry periods (DP) in the lower portion of the canopy (LOW); the middle portion of the canopy (MID) and the upper portion of the canopy (UPP).

**Table 4.** Distribution, identity, e-value and NCBI accession number for each endophytic fungal species identified by sequencing the 18S rRNA gene at the onset of the rainy period, during the rainy and dry periods in leaves of the lower, middle and upper thirds of the tree canopy in 18- and 72-month-old eucalyptus plants grown at Catas Altas (CA) and Santa Bárbara (SB).

| Study sites | Sampling | Canopy thirds | Identification | Identity (%) | e-value | Accession/NCBI |
|---|---|---|---|---|---|---|
| CA | Onset of the rainy period | Lower | *Laetisaria fuciformis* | 99 | $1\ e^{-153}$ | AY293139.1 |
| | | | *Trametes versicolor* | 99 | $1\ e^{-143}$ | KM222266.1 |
| | | | *Yarrowia lipolytica* | 99 | $2\ e^{-155}$ | JQ698926.1 |
| | | | *Malassezia restricta* | 99 | $3\ e^{-61}$ | AAYY01000016.1 |
| | | | *Pachylepyrium carbonicola* | 99 | $5\ e^{-142}$ | HQ832428.1 |
| | | | *Fusarium solani* | 99 | $7\ e^{-157}$ | KM2222302.1 |
| | | Middle | *Fusarium solani* | 99 | $7\ e^{-157}$ | KM2222302.1 |
| | | | *Pachylepyrium carbonicola* | 97 | $6\ e^{-126}$ | HQ832427.1 |
| | | | *Malassezia restricta* | 99 | $3\ e^{-61}$ | AAYY01000016.1 |
| | | | *Acidomyces acidothermus* | 98 | $4\ e^{-137}$ | JQ172747.1 |
| | | Upper | *Malassezia restricta* | 99 | $3\ e^{-61}$ | AAYY01000016.1 |
| | | | *Knufia petricola* | 98 | $6\ e^{-137}$ | KC988739.1 |
| | | | *Fusarium solani* | 99 | $7\ e^{-157}$ | KM2222302.1 |
| | Rainy period | Lower | *Aspergillus glaucus* | 100 | $1\ e^{-153}$ | AY083218.1 |
| | | Middle | *Pachylepyrium carbonicola* | 97 | $6\ e^{-126}$ | HQ832428.1 |

**Table 4.** Contd.

| | | | | | | |
|---|---|---|---|---|---|---|
| | | Upper | *Pachylepyrium carbonicola* | 97 | 6 e $^{-126}$ | HQ832428.1 |
| | | | *Malassezia restricta* | 99 | 3 e $^{-61}$ | AAYY01000016.1 |
| | | | *Marasmius alliaceus* | 98 | 2 e $^{-146}$ | NG_013179.1 |
| | | Lower | *Pachylepyrium carbonicola* | 97 | 6 e $^{-126}$ | HQ832428.1 |
| | | | *Coniophora puteana* | 95 | 48 e $^{-115}$ | GU187631.1 |
| | | | *Sistotrema brinkmannii* | 98 | 2 e $^{-146}$ | KM232435.1 |
| | Dry period | Middle | *Coniophora puteana* | 97 | 3 e $^{-145}$ | GU187631.1 |
| | | | *Boletus rubropunctus* | 98 | 5 e $^{-142}$ | FJ480426.1 |
| | | | *Microdochium nivale* | 95 | 2e $^{-149}$ | AF548077.1 |
| | | | *Pachylepyrium carbonicola* | 97 | 6 e $^{-126}$ | HQ832428.1 |
| | | Upper | *Pachylepyrium carbonicola* | 97 | 6 e $^{-126}$ | HQ832428.1 |
| | | | *Boletus rubropunctus* | 98 | 5 e $^{-142}$ | FJ480426.1 |
| | | | *Coniophora puteana* | 97 | 3 e $^{-145}$ | GU187631.1 |
| | Onset of the rainy period | Middle | *Anomoloma albolutescens* | 97 | 8e $^{-145}$ | GU187618.1 |
| | | | *Malassezia restricta* | 98 | 1e $^{-60}$ | AAYY01000016.1 |
| | | | *Boletus rubropunctus* | 97 | 3e $^{-144}$ | FJ480426.1 |
| SB | Rainy period | Lower | *Boletus rubropunctus* | 97 | 3e $^{-144}$ | FJ480426.1 |
| | | | *Rhodotarzetta rosea* | 97 | 4e $^{-147}$ | DQ646550.1 |
| | | | *Pachylepyrium carbonicola* | 97 | 6e $^{-126}$ | HQ832428.1 |
| | | Middle | *Boletus rubropunctus* | 97 | 3e $^{-144}$ | FJ480426.1 |
| | | Upper | *Microdochium nivale* | 97 | 2e $^{-149}$ | AF548077.1 |
| | | | *Rhizoctonia solani* | 97 | 1e $^{-143}$ | D85644.1 |
| | Dry period | Middle | *Boletus rubropunctus* | 97 | 3e $^{-144}$ | FJ480426.1 |

sufficient to reveal most of the sequence types within the community and to reasonably describe the diversity of group (Figure 7).

## DISCUSSION

The diversity of endophytic fungi in the eucalyptus leaves, as determined by nested PCR and DGGE, demonstrates the appropriateness of this approach in evaluating the endophytic fungal diversity in eucalyptus leaves (Figures 1, 2 and 3). Notably, this method was developed more than 20 years ago (Muyzer et al., 1993) and has been an efficient method for microbial diversity studies in several environments, such as in soil (Bresolin et al., 2010), in plants (Oliveira et al., 2013; Miguel et al., 2016) and in animals (Kittelmann et al., 2012). The different intensities of the bands in the electrophoretic profile of DGGE were interpreted as different community structures.

The DGGE analysis using UPGMA provides current fingerprinting patterns that can be measured quickly (Fromin et al., 2002) and result in dendrograms that graphically show the similarities between samples (Laplante and Derome, 2011). The endophytic fungi were distributed into five distinct groups via UPGMA analysis (Figure 2), where the highest similarity value (52.3%) corresponded to leaves sampled from the lower third of the tree canopy at the onset of the rainy period in the Catas Altas region. This finding indicates changes in the endophytic fungal distribution due to seasonality and leaf position (Figure 5). This change is less pronounced in older leaves from the Santa Bárbara region, where the lowest number of distinct clades was found (Figure 2B).

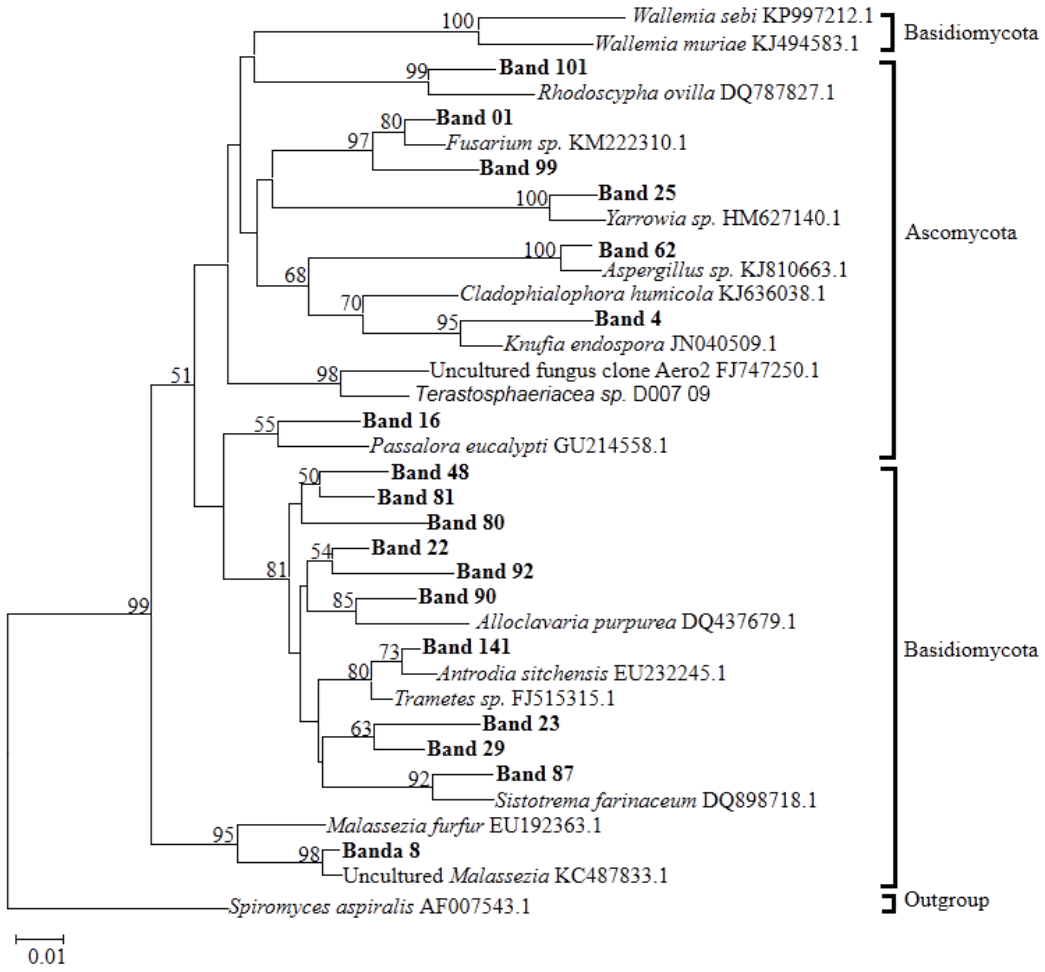


**Figure 6.** Phylogenetic tree constructed with the neighbor-joining method using fungal 18S rRNA gene sequences identified in the leaves of 18- and 72-month-old eucalyptus plants grown at Catas Altas and Santa Bárbara, respectively. Bootstrap values above 50% are shown.

The highest similarity value at the Santa Bárbara site was 55.3%, occurring in the lower third of the tree canopy during the dry period (Figure 2B).

The distribution of most OTUs during the three sampling periods of the eucalyptus leaves (23 and 22) is similar; however, specific OTUs exist, reflecting the differences in endophytic fungal community structure. Additionally, the OTUs also exhibit distinctions in the rainy and dry periods, such as higher incidence during the rainy period in Catas Altas, in contrast to Santa Bárbara, where the highest incidence occurred during the dry period (Figure 4). The variation in the Shannon diversity indices and the richness indices in Catas Altas, which were between 2.56 and 3.02, and between 13 and 21 (Table 3), respectively, and in Santa Bárbara, where these values were lower, with diversity between 2.09 and 2.4 and richness between 7.5 and 11.3 (Table 3), were interpreted to indicate that the location within the tree canopy and seasonality are not factors that significantly affect diversity (Figure 4). This interpretation is attributed to the fact the average Shannon and richness indices do not differ significantly according to the Tukey test at 5% probability (Table 3). However, when these rates are compared between the fungal communities of Catas Altas and Santa Bárbara, the higher average for Catas Altas indicates that the age of the plants influences the diversity (Table 3). Species diversity is measured in terms of richness and uniformity, and the most common and extensively used index is Shannon-Wiener (H'); typical values range from 1.5 to 3.5 (Gazis and Chaverri, 2010). More diverse communities tend to exhibit more distinct species (Ghimire et al., 2010), which explains the discrepancy in the diversity indices in leaves from the Catas Altas and Santa Bárbara sites (Table 3).

Seasonality, although it did not influence the diversity (Table 3), modulated the distribution of endophytic fungi in Catas Altas more than that in Santa Bárbara, enabling best groups and the distinction between the rainy periods (beginning of the rainy season and the rainy season) and section (Figure 5). This distinction can be attributed to the

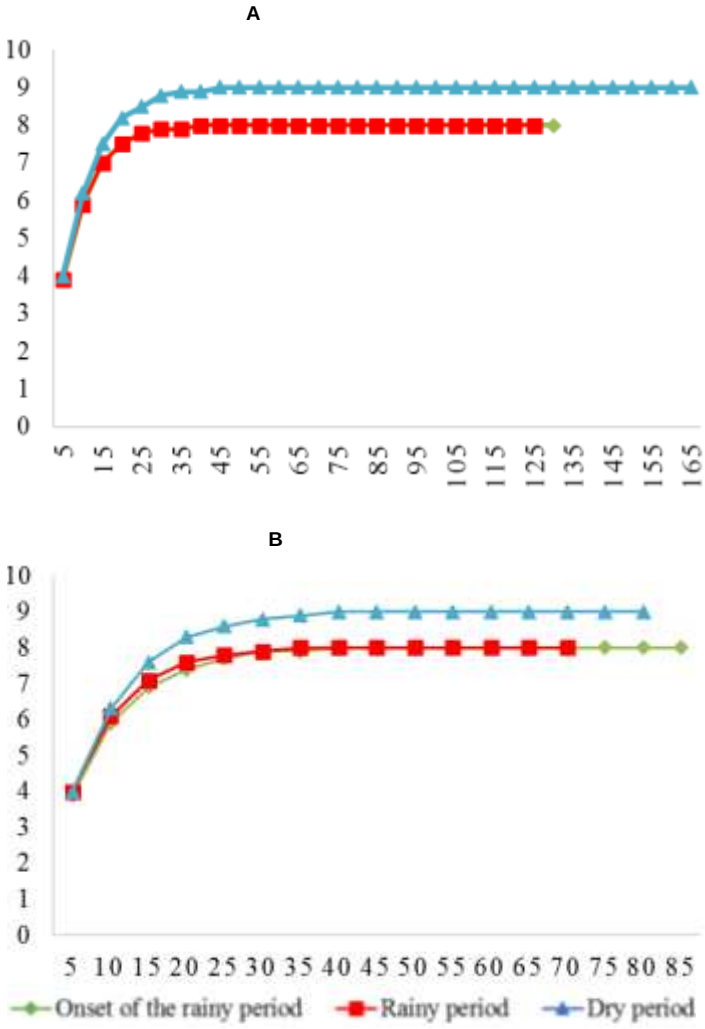


**Figure 7.** Rarefaction curves indicating OTUs based on the amplification of 18S rRNA gene diversity. (A) Catas Altas and (B) Santa Bárbara at the onset of the rainy period, during the rainy period, and during the dry period.

different species found in each of these areas (Table 4). Seasonality shapes endophytic fungal diversity in eucalyptus, which can be observed based on the presence of common and site-specific OTUs in Catas Altas and Santa Bárbara (Figure 4 and Table 3). Seasonality can also affect the gain, loss, latency, or persistence of a given microbial species in the community (Ghimire et al., 2010). Although, the functional significance of these changes in microbial community structure due to seasonality has not been demonstrated, some authors report that plants are affected by factors such as antagonism among fungi, as well as abiotic variables that can affect the host plant and thus shape the dynamics of the associated microbiota (Ghimire et al. 2010).

Endophytic colonization is usually affected by the ontogeny of the leaves (Arnold and Herre, 2003). Variations in diversity and abundance observed in this study may be associated with the nutritional and defense properties at each developmental stage of these organs (Sanchez-Azofeifa et al., 2012). In this study, the largest differences in endophytic fungal diversity among the plants from Catas Altas (younger) and Santa Bárbara (older) can be attributed to plant age (Table 3). In addition, other variables, such as cultivation and rotation cycles (Ellouze et al., 2014), nutrient and sugar levels in the leaves, and other characteristics (Lang et al., 2011), may together have affected the differences between the diversity indices at the two sites. Notably, the forest in Catas Altas is currently in the seventh farming cycle, first implemented in December 1970, and the forest in Santa Bárbara is currently in the third farming cycle, first implemented in December 1989. The crop and rotation cycles can affect the fungal community of the soil

(Ellouze et al., 2014) and, consequently, endophytic colonization, considering that leaves contain many endophytic microorganisms that originate from the soil (Sprent and Defaria, 1988; Hardoim et al., 2008; Van Der Lelie et al., 2009). Additionally, other factors may also contribute to differences in the endophytic communities, such as changes in leaf physiology and the presence of chemical substances, such as phenolic compounds, that can limit the richness of microbial species. As these compounds are natural inhibitors of fungal colonization, especially by representatives of the phylum Ascomycota, including *Aspergillus* (Banso and Rai, 2008) and *Fusarium* (Kaur et al., 2011). An equally likely explanation is the simple absence of these taxa in the older plants from Santa Bárbara.

The differences in the distribution of endophytic fungi in the upper, middle and lower thirds of the tree canopy (Table 4) may indicate that endophytic colonization depends on the site of the plant sampled. The species, *Pachylepyrium carbonicola* and *Malassezia restricta* (Table 4), which are present in Catas Altas, and *Boletus rubropunctus* (Table 4), which is present in Santa Bárbara, can occupy multiple micro-habitats within the plants, indicating more generalist behavior (Table 4). Factors such as altitude, moisture content and canopy density, among others, are reported to affect the level of plant infection (Qi et al., 2012).

The most commonly observed endophytic fungi in eucalyptus farmed in Catas Altas and Santa Bárbara were *Fusarium solani*, *Malassezia restricta*, *Pachylepyrium carbonicola* and *Boletus rubropunctus* (Table 4). The high identity of the sequences obtained with those in the NCBI database (Table 4) was the criterion used to confirm these species as belonging to the phyla Ascomycota and Basidiomycota (Figure 6). Although, many of the species identified are pathogenic to some plants, they were endophytic in the present study. Notably, disinfection of the surfaces of healthy leaves without symptoms of infection was confirmed by the absence of fungal growth in R2A inoculated with the final rinse water. The strong dominance of some fungal groups (Table 4) indicates that they can play a relevant role in plant physiology. Fungal species can produce a wide variety of growth regulators, such as gibberellins (GAs), abscisic acid (ABA), and auxins (IAA) (You et al., 2012), and they can also confer tolerance to adverse biotic and abiotic factors (Hubbard et al., 2014).

Endophytic microorganisms can colonize plants via wounds at lateral root emergence sites (Hallman et al., 1997) or by releasing hydrolytic enzymes (Robl et al., 2013) that allow them to enter and colonize the plants (Hallman et al., 1997).

*Fusarium* species are most commonly isolated as pathogens from plants at all latitudes (Zakaria and Ning, 2013) rather than as endophytes in tropical plants (Vega et al., 2010; Zakaria and Ning, 2013). Fungi of the genus, *Marasmius* are recognized by the production of secondary metabolites that inhibit the growth of *Escherichia coli* (Rosa et al., 2003). This group has already been described as endophytic (Ngieng et al., 2013). However, according to the literature, there have been no reports of endophytism for the species *Marasmius alliaceus*. The genus, *Boletus* contains species that are described as endophytic in the leaves of *Pinus* sp. (Arnold et al., 2007). However, the species, *Boletus rubropunctus* is reported here as endophytic for the first time.

The presence of endophytic fungi in leaves reported here expands the understanding of endophytic colonization in eucalyptus. The description of endophytic fungal diversity in this important forest species is an important step in accessing this genetic resource in the search for metabolites and processes that can contribute to improving plant development.

## Conclusions

DGGE was efficient at assessing the diversity and distribution of endophytic fungi in eucalyptus. Using the DNA fragments in the bands excised from different positions of the DGGE gel was a satisfactory strategy for assessing the endophytic fungal diversity of eucalyptus in the present study. The age of plants affected the diversity of endophytic fungi in *Eucalyptus* “urograndis”. The leaf position and seasonality affected the endophytic fungal distribution of Catas Altas more than that of Santa Bárbara. The phyla Basidiomycota and Ascomycota are predominant components of the endophytic fungal microbiota in eucalyptus.

## Conflict of Interests

The authors have not declared any conflict of interests.

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## REFERENCES

Altschul S, Gish W, Miller W, Myers E, Lipman D. (1990). Basic local alignment search tool. J. Mol. Biol. 215(3):403-410.

Analytic Rarefaction 1.3 software. Available online: http://strata.uga.edu/software/anRareReadme.html (accessed on 24 March 2016).

Arnold AE (2005). Diversity and Ecology of fungal endophytes in tropical forests. Current Trends in Mycological Research (S. Deshmukh, ed.). Oxford & IBH Publishing Co. Pvt. Ltd., New Delhi: pp. 49-68.

Arnold AE, Henk DA, Eells RL, Lutzoni F, Vilgalys R (2007). Diversity and phylogenetic affinities of foliar fungal endophytes in loblolly pine inferred by culturing and environmental PCR. Mycologia 99(2):185-206.

Arnold AE, Herre EA (2003). Canopy cover and leaf age affect colonization by tropical fungal endophytes: ecological pattern and process in *Theobroma cacao* (Malvaceae). Mycologia 95(3):388-398.

Banerjee D (2011). Endophytic fungal diversity in tropical and subtropical plants. Res. J. Microbiol. 6(1):54.

Banso DS, Rai M (2008). Antifungal activity of essential oils from Indian medicinal plants against human pathogenic *Aspergillus fumigatus* and *A. niger*. World J. Med. Sci. 3(2):81-88.

Bresolin JD, Bustamante MMC, Krüger RH, Silva MRSS, Perez KS (2010). Structure and composition of bacterial and fungal community in soil under soybean monoculture in the Brazilian cerrado. Braz. J. Microbiol. 41(2):391-403.

Carmona M, Sepúlveda D, Cárdenas C, Nilo L, Marshall SH (2012). Denaturing gradient gel electrophoresis (DGGE) as a pow erful novel alternative for differentiation of epizootic ISA virus variants. PloS One 7(5):e37353.

Da Silva MDCS, De Almeida PT, Moreira BC, Carolino M, Cruz C, Bazzolli DMS, Silva CC, Kasuya MCM (2014). Nitrogen-fixing bacteria in *Eucalyptus globulus* plantations. PloS One 9(10):e111313.

Ellouze W, Esmaeili TA, Bainard LD, Yang C, Bazghaleh N, Navarro-Borrell A, Hanson K, Hamel C (2014). Soil fungal resources in annual cropping systems and their potential for management. BioMed Res. Int. 2014:1-15.

FAO (2015). Global Forest Resources Assessment. Progress tow ards sustainable Forest Management.

Fromin N, Hamelin J, Tarnaw ski S, Roesti D, Jourdain-Miserez K, Forestier N, Teyssier-Cuvelle S, Gillet F, Aragno M, Rossi P (2002). Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. Environ. Microbiol. 4(11):634-643.

Gazis R, Chaverri P (2010). Diversity of fungal endophytes in leaves and stems of w ild rubber trees (*Hevea brasiliensis*) in Peru. Fungal Ecol. 3(3):240-254.

Ghimire SR, Charlton ND, Bell JD, Krishnamurthy YL, Craven K (2011). Biodiversity of fungal endophyte communities inhabiting sw itchgrass (*Panicum virgatum* L.) grow ing in the native tallgrass prairie of northern Oklahoma. Fungal Divers. 47(1):19-27.

Hallman J, Quadt-Hallmann A, Mahaffee WF, Kloepper JW (1997). Bacterial endophytes in agricultural crops. Can. J. Microbiol 43(10):895-914.

Hammer Ø, Harper DAT, Ryan PD (2001). PAST: Paleontological statistics softw are package for education and data analysis. Palaeontol. Electronica 4(1):9.

Hardoim PR, Overbeek LSV, Berg G, Compant AMPS, Campisano A, Döring M, Sessitsche A (2015) The Hidden World w ithin Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes. Microbiol. Mol. Biol. Rev. 79(3):293-320.

Hardoim PR, Overbeek LSV, Elsas KDV (2008). Properties of bacterial endophytes and their proposed role in plant grow th. Trends Microbiol. 16(10):463-471.

Hoshino YT, Matsumoto N (2007). Changes in fungal community structure in bulk soil and spinach rhizosphere soil after chemical fumigation as revealed by 18S rDNA PCR-DGGE. Soil Sci. Plant Nutr. 53(1):40-55.

Hubbard M, Germida JJ, Vujanovic V (2014). Fungal endophytes enhance w heat heat and drought tolerance in terms of grain yield and second-generation seed viability. J. Appl. Microbiol. 116(1):109-122.

Iglesias-Trabado G, Wilstermann D (2008). *Eucalyptus universalis*. Global cultivated eucalypt forests map. Indústria Brasileira de árvores – IBA, v. 1, 2014. 100 p.

Kaur S, Singh HP, Batish DR, Kohli RK (2011). Chemical characterization and allelopathic potential of volatile oil of *Eucalyptus* tereticornis against *Amaranthus viridis*. J. Plant. Interact. 6(4):297-302.

Kittelmann S, Naylor GE, Koolaard JP, Janssen PH (2012). A Proposed Taxonomy of Anaerobic Fungi (Class *Neocallimastigomycetes*) Suitable for Large-Scale Sequence-Based Community Structure Analysis. PLoS One 7(5):e36866.

Köppen W (1948). Climatology. México. Fund of Economic Culture. 140p.

Lang C, Polle A (2011). Ectomycorrhizal fungal diversity, tree diversity and root nutrient relations in a mixed Central European forest. Tree Physiol. 31(5):531-538.

Laplante K, Derome N (2011). Parallel changes in the taxonomical structure of bacterial communities exposed to a similar environmental disturbance. Ecol. Evol. 1(4):489-501.

Lindow SE, Brandl MT (2003) Microbiology of the phyllosphere. Appl. Environ. Microbiol. 69(4):1875-1883.

Luo J, Walsh E, Naik A, Zhuang W, Zhang K, Cai L, Zhang N (2014). Temperate pine barrens and tropical rain forests are both rich in undescribed fungi. PloS One 9(7):e103753.

May LA, Smiley B, Schmidt MG (2001). Comparative denaturing gradient gel electrophoresis analysis of fungal communities associated w ith whole plant corn silage. Can. J. Microbiol. 47(9):829-841.

Miguel PSB, De Oliveira MNV, Delvaux JC, de Jesus GL, Borges AC, Tótola MR, Neves JCL, Costa MD (2016). Diversity and distribution of the endophytic bacterial community at different stages of Eucalyptus grow th. Antonie van Leeuw enhoek 109(6):755-771. .

Miguel PSB, Delvaux JC, Oliveira MNV, Monteiro LCP, Freitas FS, Costa MD, Tótola MR, Moraes CA, Borges AC (2013). Diversity of endophytic bacteria in the fruits of *Coffea canephora*. Afr. J. Microbiol. Res. 7(7):586-594.

Minitab I (2006). MINITAB statistical softw are. Version: Release, 15.

Muyzer G, De Waal EC, Uitterlinden AG (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59(3):695-700.

Ngieng NS, Zulkharnain A, Roslan HA, Husaini A (2013). Decolourisation of synthetic dyes by endophytic fungal flora isolated from Senduduk plant (*Melastoma malabathricum*). ISRN Biotechnology 2013(2013):1-7.

Oliveira MNV, Santos TMA, Vale HMM, Delvaux JC, Cordero AP, Ferreira AB, Miguel PSB, Tótola MR, Costa MD, Moraes CA, Borges AC (2013). Endophytic microbial diversity in coffee cherries of *Coffea arabica* from southeastern Brazil. Can. J. Microbiol. 59(4):221-230.

Oros-Sichler M, Gomes NCM, Neuber G, Smalla K (2006). A new semi-nested PCR protocol to amplify large 18S rRNA gene fragments for PCR-DGGE analysis of soil fungal communities. J. Microbiol. Methods 65(1):63-75.

Qi F, Jing T, Zhan Y (2012). Characterization of endophytic fungi from *Acer ginnala* Maxim in an artificial plantation: media effect and tissue-dependent variation. PLoS One 7(10):e46785.

Reasoner DJ, Geldreich EE (1985). A new medium for the enumeration and subculture of bacteria from potable w ater. Appl. Environ. Microbiol. 49(1):1-7.

Robl D, Da Delabona P, Mergel CM, Rojas JD, Dos Costa P, Pimentel IC, Vicente VA, Pradella JGC, Padilha G (2013). The capability of endophytic fungi for production of hemicellulases and related enzymes. BMC Biotechnology 13(94):1-12.

Rockw ood DL, Rudie AW, Ralph SA, Zhu JY, Winandy JE (2008). Energy product options for *Eucalyptus* species grow n as short rotation w oody crops. Int. J. Mol. Sci. 9(8):1361-1378.

Rosa LH, Machado KMG, Jacob CC, Capelari M, Rosa CA, Zani CL (2003). Screening of brazilian basidiomycetes for antimicrobial activity. Mem. Inst. Osw aldo Cruz 98(7):967-974.

Saitou N, Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4(4):406-425.

Sanchez-Azofeifa A, Oki Y, Fernandes GW, Ball RA, Gamon J (2012). Relationships betw een endophyte diversity and leaf optical properties. Trees 26(2):291-299.

Schneider H (2007). Métodos de análise filogenética: um guia prático [Methods for phylogenetic analysis: a practical guide]. Ribeirão Preto: Holos Editora e Sociedade Brasileira de Genética.

Sprent JI, Defaria SM (1988). Mechanisms of infection of plants by nitrogen-fixing organisms. Plant Soil 110(2):157-165.

Ter Braak CJF, Smilauer P (2002). Canoco for Window s version 4.5.

Biometris–Plant Research International, Wageningen.
Torsvik V, Ovreas L (2002). Microbial diversity and function in soil: From genes to ecosystems bacteria. Curr. Opin. Microbiol. 5(3):240-245.
Turner TR, James EK, Poole PS (2013). The plant microbiome. Genome Biol. 14(209):1-10.
Vainio EJ, Hantula J (2000). Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. Mycol. Res. 104(8):927-936.
Van Der Lelie D, Taghavi S, Monchy S, Schwender J, Miller L, Ferrieri R, Rogers A, Wu X, Zhu W, Weyens N, Vangronsveld J, Newman L (2009). Poplar and its bacterial endophytes: coexistence and harmony. Crit. Rev. Plant. Sci. 28(5):346-358.
Vandenkoornhuyse P, Quaiser A, Duhamel M, Le Van A, Dufresne A (2015). The importance of the microbiome of the plant holobiont. New Phytol. 206(4):1196-1206.
Vega FE, Simpkins A, Aime MC, Posada F, Peterson SW, Rehner A, Infante F, Castillo A, Arnold EA (2010). Fungal endophyte diversity in coffee plants from Colombia, Hawai'i, Mexico and Puerto Rico. Fungal Ecol. 3(3):122-138.
You YH, Kang SM, Choo YS, Lee JM (2012). Fungal diversity and plant growth promotion of endophytic fungi from six halophytes in Suncheon Bay. J. Microbiol. Biotechnol. 22(11):1549-1556.
Zakaria L, Ning CH (2013). Endophytic *Fusarium* spp. from Roots of Lawn Grass (*Axonopus compressus*). Trop. Life Sci. Res. 24(2):85-90.
Zhang T, Yao YF (2015). Endophytic Fungal Communities Associated with Vascular Plants in the High Arctic Zone Are Highly Diverse and Host-Plant Specific. PloS One 10(6):e0130051.

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*Short Communication*

# Dual-path platform (DPP) and enzyme-linked immunosorbent assay (ELISA): Change the sequence of the tests does not change the number of positive dogs for canine visceral leishmaniasis

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**The Brazilian Ministry of Health determined in 2012 that the official protocol for diagnosis of Canine Visceral Leishmaniasis (CVL) would be the Dual-Path Platform (DPP) for screening, followed by enzyme-linked immunosorbent assay (ELISA) for confirmation. This study evaluated serum samples from 426 dogs from a region in northern Brazil. All samples were tested according to the Official Protocol and the sequence inverting (ELISA followed DPP). Regardless of the protocol adopted, prevalence (14.7%) has not changed. The approach using ELISA followed by DPP state that, the number of positive animals in screening was higher compared to the official protocol. Screen the ELISA test could be more appropriate.**

**Key words:** Canine visceral leishmaniasis, Dual-Path Platform (DPP), enzyme-linked immunosorbent assay (ELISA), tocantins.

## INTRODUCTION

Canine visceral leishmaniasis (CVL) is a potentially fatal disease caused by the intracellular protozoan parasite *Leishmania infantum*, which is endemic in South and Central America, Mediterranean basin and parts of Asia. Dog is the most important reservoir host, and infection is maintained by transmission between dogs by phlebotomine sandfly species (Quinnell and Couternay, 2009).

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From an epidemiological point of view, the canine disease is more important than the human disease because, besides being more prevalent, it has large numbers of asymptomatic dogs with parasites in the dermis, and has the potential of transmitting the parasite to sand-fly (Laurenti et al., 2013).

Recently, to improve accuracy in the diagnosis of CVL in Brazil, the Visceral Leishmaniasis Control and Surveillance Program (VLCSP) has recommended the immunochromatographic rapid test comprising rK26 and rK39 recombinant antigens, the Dual-Path Platform (DPP; Bio- Manguinhos/Fiocruz, Rio de Janeiro, Brazil), for the screening of *L. infantum*-infected dogs and enzyme-linked immunosorbent assay (ELISA) to confirm the positive results (Ministério da Saúde, 2011). In this sense, the present study aimed to carry out the first seroepidemiological survey for CVL in the city of Gurupi, Tocantins, northern Brazil between 2013 and 2015. For this, we used the Brazilian official protocol (DPP and ELISA), and the reversal order in serologic techniques, investigating whether changing the protocol could change the animals positive rate.

## MATERIALS AND METHODS

The present study consist a cross-sectional survey carried out in Gurupi (latitude 11° 43' 45"S, longitude 49° 04' 07"W, altitude 287 m), a municipality located in the southwest of Tocantins, Brazil.

For random sampling calculation, we used official data expected prevalence of 20%, 95% confidence interval (95% CI) and maximum acceptable error of 0.05, totaling 246 samples. Furthermore, 10% samples were added, amounting to 271 samples. However, more samples were collect over a period of time, reaching 426 blood samples from asymptomatic and symptomatic dogs between September 2013 and November 2015.

Each sample was tested using two approaches, the first using the protocol recommended by the Brazilian Ministry of Health, and the second, reversing the order of the tests. The first protocol used DPP CVL rapid test (Bio-Manguinhos/Fiocruz) for screening and ELISA (Canine Leishmaniasis EIE Kit, Biomanguinhos/Fiocruz) as a confirmatory test. This protocol used serum for serological tests while both protocols followed the manufacturer's instructions. The second protocol used ELISA (Canine Leishmaniasis EIE Kit) for screening and DPP CVL rapid test for confirmation.

The cut-off of the EIE Kit was defined based on the manufacturer's instructions, which consider the mean of the optical density of the negative controls multiplied by two. Statistical analysis was performed using Stata software (version 11.0; Stata Corp, College Station, TX). The prevalence rates indicated by DPP and ELISA were estimated using 95% CI.

## RESULTS AND DISCUSSION

In the first approach, following the Brazilian Ministry of Health protocol, of the 426 serum samples evaluated by both methods, 112 (26.29%) were positive in DPP and from this initial screening, 63 (56.23%) were positive by ELISA. While in the second proposal, out of the 426 samples screened in the ELISA test, 136 (31.92%) were positive, and from this screening, 63 (46.32%) samples were positive to purified protein derivative (PPD) test. For both protocols, the prevalence was 14.7%, with no differences in the final number of positive animals in the two serologic techniques (Figure 1). Sensitivity and specificity were 82.3 and 92.8% at DPP test and 85 and 92.3% in the ELISA test, respectively.

Official data indicate that, the city of Gurupi has an intense transmission rate of CVL, with a prevalence of 23% in 2013 and 23.5% in 2014 (official unpublished data). These results are favored by the climate of the region and the constant degradation of native areas housing construction and agricultural activities. The rates of positive animals found in an urban area in the State of Pernambuco (Brazil), has an overall seroprevalence which was 40.3% (Dantas-Torres e Brandão-Filho 2006). However, the results found in this study, is in line with the average in Brazil, ranging from 5.9 to 51.35% (Franca-Silva, 2003; Monteiro et al., 2005; Morais et al., 2013). It notes that, the current official protocol has to be implemented in 2012. The sensitivity of the DPP test depends on the clinical condition of the animal. However it is known that, the DPP is more sensitive when used in symptomatic dogs, and lower the income in asymptomatic animals (Grimaldi et al., 2012).

In a previous state developed in other regions of Brazil, this was bought for the first time to change the protocol for diagnosis of CVL. A survey was conducted with 1226 dogs, followed by a cohort study using 447 dogs. Results showed that the protocol using DPP and ELISA detected a higher prevalence (8.1%) of infected dogs than the protocol using ELISA and IFAT (prevalence, 6.2%). However, regardless of the test sequence (DPP followed by ELISA or ELISA followed by DPP), the number of positive animals is the same in both tests (Coura-Vital et al., 2014). Positive serum samples for *Ehrlichia canis*, *Babesia canis*, *Toxoplasma gondii*, *Neospora caninum* and *Trypanosoma cruzi* were tested using three serological methods ELISA, indirect immunofluorescent antibody test (IFAT) and Kalazar Detect™, for CVL. Of the 57 dog samples tested, 24 (42.1%) tested positive using one of the three serological methods: 10/57 (17.5%) for ELISA, 11/57 (19.3%) for IFAT and 3/57 (5.3%) for Kalazar Detect™. Results demonstrated that the presence of other infectious agents may lead to cross-reactivity on leishmaniasis serological tests. (Zanette et al., 2014). Moreover, in another study using DPP and ELISA, cross-reactivity was obtained with only *Babesia* (Laurenti et al., 2014).

Among DPP using and ELISA for screening of dogs in endemic areas, the DPP have advantages by being easy and practical easier to handle, with the result been ready in 15 min after blood collection. Further, laboratory

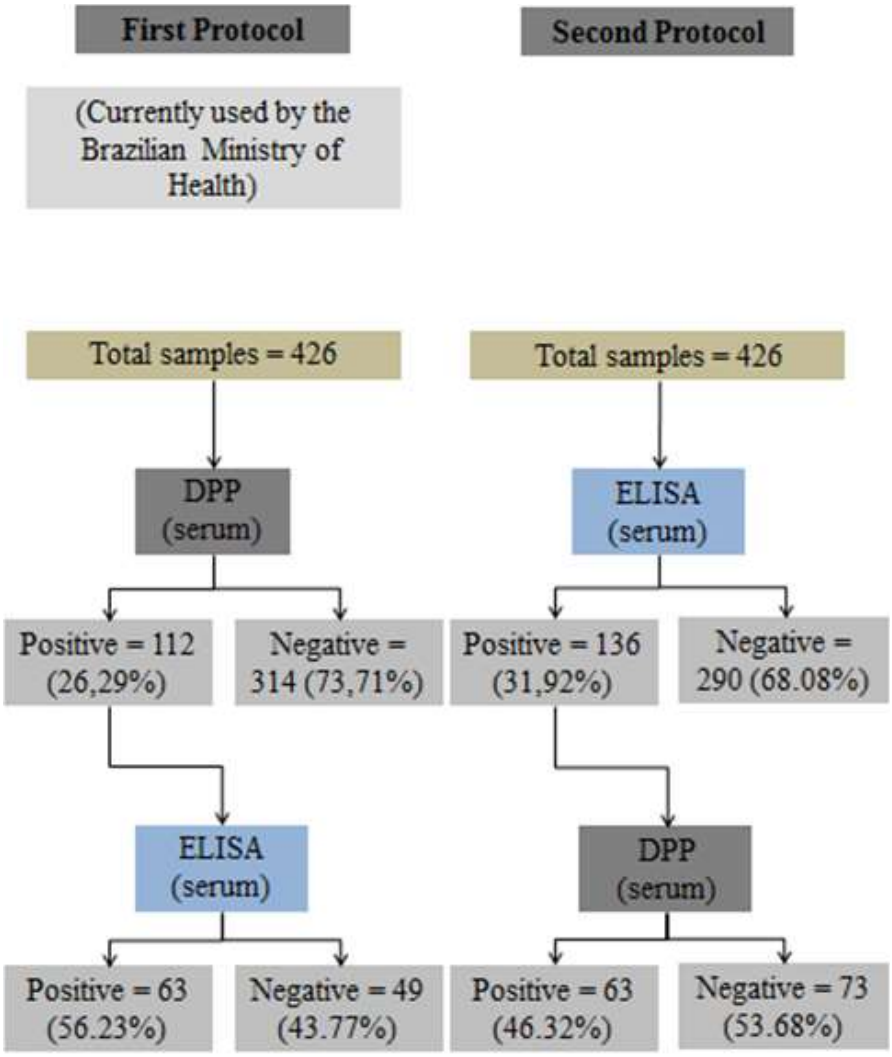


**Figure 1.** Drawing of two evaluations protocols with samples of 426 dog area with intense transmission of CVL. Left, the official protocol used by the Brazilian Ministry of Health, Right, the protocol with reversing the order of serologic tests.

equipment is not necessary for diagnosis. On the other hand, if the animal is positive, spend more time in collecting more samples to be sent to, the Central Public Health Laboratories (LACENS). As the ELISA detects more positive animals in screening, it is interesting that in areas of high prevalence and incidence, the ELISA will be used for screening and DPP for confirmation, given that there was no difference in the final number of animals positive.

## Conflict of Interests

The authors have not declared any conflict of interests.

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## REFERENCES

Coura-Vital W, Ker HG, Roatt BM, Aguiar-Soares RD, Leal GG, Moreira N, Oliveira, LA, de Menezes Machado EM, Morais MH, Corrêa-Oliveira R, Carneiro M, Reis AB (2014). Evalution of change in canine diagnosis protocol adopted by the visceral leishmaniasis control program in Brazil and a new proposal for diagnosis. Plos One. 7(3):e91009.

Dantas-Torres F. de Brito MEF, Brandão-Filho SP (2006). Seroepidemiological survey on canine leishmaniasis among dogs from an urban area of Brazil. Vet. Parasitol. 140:54-60.

Franca-Silva JC, da Costa RT, Siqueira AM, Machado-Coelho GL, da Costa CA (2003). Epidemiology of canine visceral leishmaniosis in the endemic area of Montes Claros Municipality, Minas Gerais State, Brazil. Vet Parasitol. 111:161-173.

Grimaldi GJr, Teva A, Ferreira AL, dos Santos CB, Pinto IS (2012). Evaluation of a novel chromatographic immunoassay based on Dual-Path Platform technology (DPP(R) CVL rapid test) for the serodiagnosis of canine visceral leishmaniasis. Trans. R Soc. Trop. Med. Hyg. 106:54-59.

Laurenti MD, Leandro Jr MVS, Tomokane TY, De Lucca HRL, Aschar M, Souza CSF, Silva RM, Marcondes M, da Matta VLR (2014). Comparative evaluation of the DPP® CVL rapid test for canine serodiagnosis in area of visceral leishmaniasis. Vet. Par. 205:444-450.

Laurenti MD, Rossi CN, da Matta VL, Tomokane TY, Corbett CE, Secundino NF, Pimenta PF, Marcondes M. Asymptomatic dogs are highly competent to transmit Leishmania (Leishmania) infantum chagasi to the natural vector. Vet. Parasitol. 23:296-300..

Ministério da Saúde (2011). Esclarecimento sobre substituição do protocolo Diagnóstico da leihsmaniose visceral canina; Nota técnica conjunta nº 01/2011, CGDT-CGLAB/DEVIT/SVSE/MS.

Monteiro EM, da Silva JC, da Costa RT, Costa DC, Barata RA, de Paula EV. Visceral leishmaniasis: a study on phlebotomine sand flies and canine infection in Montes Claros, State of Minas Gerais. Rev. Soc. Bras. Med. Trop. 38:147-152.

Morais NA, Sousa MG, Meireles LR, Kesper JrN, Umezaw a ES (2013). Canine visceral leishmaniasis and Chagas dsease among dogs in Araguaína, Tocantins. Rev. Bras. Parasitol. Vet. 22:225-229.

Quinnell RJ, Couternay O (2009). Transmission, reservoir hosts and control of zoonotic visceral leishmaniasis. Parasitology. 136:1915-1934.

Zanette MF, Lima VM, Laurenti MD, Rossi CN, Vides JP, Vieira RF, Biondo AW, Marcondes M. Serological cross-reactivity of Trypanosoma cruzi, Ehrlichia canis, Toxoplasma gondii, Neospora caninum and Babesia canis to Leishmania infantum chagasi tests in dogs. Ver. Soc. Bras. Med. Trop. 47:105-107.


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