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ABSTRACT

A collection of more than 200 yeasts and 200 lactic acid bacteria (LAB) was assembled throughout a four-year experimentation in Ivory Coast, Cameroon and Brasil, from spontaneous cocoa bean fermentation processes. The strains isolated were assessed for their antifungal activity against six fungi isolated from both fermented and dried cocoa beans, belonging to *Aspergillus* and *Penicillium* genera. It has been generally accepted that fungi are not beneficial to cocoa bean and chocolate quality. Their presence during fermentation, drying and storage has been linked to flavor defects and health risk due to the production of mycotoxins. Thus, the aim of this study was to select yeast and LAB strains able to limit fungal growth, to be used as a mean of biological control during cocoa production. An initial screening was carried out by using the overlay method where the plates, overlaid with malt extract soft agar inoculated with fungal spore suspension, were examined for inhibition zone around LAB and yeast streaks. Then, the most active strains were studied in inhibition test in 96-well microplates where mould growth was measured by microplate reader at 490 nm. The nature of their antifungal strenght (organic acid and/or proteins) was also evaluated. The most promising candidates as biological agents to be used in future field trials, belonged to the species *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Saccharomyces cerevisiae* and *Candida ethanolica* and their antifungal strength was attributed mainly to

organic acid production (for LAB) and proteinaceous compounds (for yeasts) or to their synergic effect.

Keywords: mould contamination; bioprotection; *Lactobacillus fermentum*; *Lactobacillus plantarum*; *Saccharomyces cerevisiae*; cocoa fermentation.

1. INTRODUCTION

Microbial fermentation and drying of cocoa beans are essential processes to initiate the formation of cocoa flavor precursors. The physical and chemical characteristics of cocoa beans make it a substrate suitable for microbial growth and the microorganisms involved follow a similar dynamic described by many authors (Schwan & Wheals, 2004). The initial acidity of the pulp surrounding the beans and the low oxygen levels support colonization by yeast population. Then, in coincidence with their decline, lactic acid bacteria (LAB) reach the maximum peak of growth. Finally, these too decrease and are replaced by acetic bacteria, which are favored by the presence of ethanol and increased aeration conditions at temperatures ranging from 37 °C to 50 °C or more. Aerobic spore-forming bacteria and filamentous fungi are recurrent in the later stages of fermentation and are mainly associated to the production of off-flavors in fermented cocoa beans. In particular, it has been generally accepted that fungi are not beneficial to cocoa bean and chocolate quality, and the so-called “moldy” defect refers to their contamination of the inside of the beans as result of shell fracture, germination-damage or insect attack (Schwan, Pereira & Fleet, 2014).

During fermentation, the growth of filamentous fungi is affected by high amounts of alcohol and lactic and acetic acids produced by yeasts and bacteria, respectively. Low pH, microaerophilic conditions and high temperatures limit their growth which is confined to well-aerated parts of the fermenting cocoa bean mass (Copetti et al., 2014), where the most frequently present species are *Aspergillus fumigatus* and *Mucor* sp. able to such

environmental conditions. On the contrary, xerophilic fungi belonging to the genera *Aspergillus*, *Penicillium*, *Mucor* and *Fusarium* can become dominant during drying, when yeast and bacteria growth ceases due to low water activity (0.85). During storage, if the conditions are not optimal (i.e. high humidity), fungal spores, produced during drying, can be determinant for cocoa bean spoilage. Besides spore germination, the possible concomitant production of mycotoxins has been already well documented in cocoa; aflatoxin produced by *Aspergillus flavus*, ochratoxin generally associated to *Penicillium* spp. and citrinin to *Penicillium citrinum* are the most commonly found (Copetti et al., 2014).

LAB and yeasts, during fermentation, limit spontaneously fungal growth thanks to substrate competition and antifungal metabolite production. The inhibition mechanism of weak organic acids, as lactic and acetic acids, mainly produced by bacteria, it has not been completely elucidated although it seems to involve cytoplasm pH decrease with consequent failure of proton motive force (Corsetti, Perpetuini & Tofalo, 2012). Moreover, different authors (Lavermicocca et al., 2000; Strom et al., 2002) have attributed antifungal activity to phenyllactic acid (PLA), and some studies focused on the correlation of PLA production with antifungal strength (Cortés-Zavaleta, 2014). Antifungal activity by yeasts is mainly attributed to alcohol production and nutrient competition (La Penna, M., Nesci, A. & Etcheverry, M., 2004; Spadaro & Droby, 2016). Other metabolites such as carbon dioxide, ethanol, reuterin, diacetyl, proteinaceous compounds or low-molecular weight peptides, and a combination of these factors, have been also hypothesized as responsible for their antifungal strength (Bianchini, A. & Bullerman, L. B., 2010).

Therefore, LAB and yeasts represent a potential source of microorganisms able to control mould growth. The aim of this study was, thus, to select LAB and yeast strains to be used in application of fungal biological control during cocoa bean fermentation processes. The nature of their inhibition strength was also investigated.

2. MATERIALS and METHODS

2.1 Strain collection

A microbial collection was created throughout a four-year experimentation in Ivory Coast, Cameroon and Brasil. More than 200 yeasts and 200 lactic acid bacteria (LAB) were isolated from spontaneous Forastero cocoa bean fermentation processes, carried out in both heaps and boxes, and identified in previous researches (Visintin et al., 2016; Visintin et al., 2017). The most abundant species were *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, *Candida ethanolica* and *Hanseniaspora uvarum* among yeasts, and *Lactobacillus fermentum* and *Lactobacillus plantarum* among LAB. LAB and yeast strains were stored in de Man, Rogosa and Sharpe (MRS) broth (Biolife, Milan, Italy) e Yeast extract-Peptone-Dextrose (YPD, Biolife), respectively, added with 30% glycerol and kept at -80 °C.

Six mould strains were used as target to test the antifungal activity of the aforementioned yeast and LAB strains. They had been isolated from cocoa beans, in a previous study, at the end of spontaneous fermentation or during drying, and identified by ITS-PCR according to Glass & Donaldson (1995) ITS1 and ITS4 primer design. The selected moulds were chosen for their recurrent presence in fungal community of cocoa beans and for their potentiality to produce mycotoxin; they were *Aspergillus flavus* (ST2A), *Penicillium citrinum* (M6E1TS), *Penicillium griseufulvum* (strains M2BT2 and S2TC), *Aspergillus niger* (DsfAn) and *Aspergillus fumigatus* (DsfAf). Mould strains were stored on Malt extract agar (Biolife) slants and kept at 4 °C.

2.2 Overlay method

An initial screening to check the antifungal activity of yeast and LAB strains was carried out by using the “overlay method” described by Magnusson & Schnürer (2001). Briefly, starting from microbial cultures stored at -80 °C, LAB and yeasts were propagated twice, at 30 °C for 18 h, in MRS broth and YPD, respectively and, subsequently, streaked on the same media added of 15 g/L agar and incubated at 30 °C for 24 h. Then, the plates were overlaid with 10 mL of Malt extract soft agar (Biolife) previously inoculated with 10^4 conidia/mL of the target moulds. Conidia suspensions were prepared as follows: filamentous fungi were

streaked on Malt extract agar plates and incubated at 25 °C; after 5 days the conidia were harvested from mycelium surface with Ringer solution (Oxoid, Milan, Italy) and counted by means of Burker chamber. The suspensions were then diluted to obtain the requested conidial concentration.

The overlaid plates were finally examined, after 5 days at 25 °C, for mould growth inhibition zone around LAB and yeast streaks; the antifungal activity was evaluated as strong (++), weak (+), or absent (-) on the basis of the transparency of the inhibition area (Figure 1).

2.3 Preparation of cell-free culture supernatants

For the analysis described in sections 2.4 and 2.5, cell-free culture supernatants (CFCSs) were used and prepared as follows: LAB and yeasts strains were propagated twice, at 30 °C for 18 h, in MRS broth and YPD, respectively, starting from the cultures stored at -80 °C. Then, the cells were removed from broths by centrifugation (13,200 rpm, 5 min) and supernatants filtered by 0.20 µm pore size Millipore filters to obtain CFCSs. Finally, CFCSs pH values were checked for each strain and they ranged from 4.0 to 4.2 for LAB strains and from 4.6 to 4.8 for yeast strains.

2.4 Microplate inhibition analysis

After the evaluation of the results obtained by overlay analysis, 25 LAB and 16 yeast strains were selected among the whole collection for their stronger inhibition towards the six target moulds. In addition, their antifungal activity was evaluated by growth inhibition test in 96-well microplates where mould growth was measured by microplate reader at 490 nm (Synergy HT, Biotek, Vermont, USA). One-hundred forty microliters of LAB and yeast CFCSs were added with 10 µL of conidia suspension (10^4 conidia/mL) prepared as described in section 2.2. The microplates were then incubated for 72 h at 30 °C and the absorbances measured at 490 nm. The optical density values obtained from mould growth in CFCSs were compared to values found in control wells, where 10 µL of conidial suspension (10^4 conidia/mL) were inoculated in fresh MRS broth and YPD without addition

of LAB and yeast CFCSs (positive control, PC), respectively. Blank wells (B) with sterile fresh MRS broth and YPD were also set up and absorbance measurements subtracted to the ones obtained from conidia inoculated wells. All samples were analysed in triplicate.

In addition, the nature of LAB and yeast antifungal strenght (organic acid and/or proteinaceous metabolites) was also studied. For each strain, the following samples were set up in 96-well microplates, and absorbance values compared to evaluate mould growth extent: (1) 10 µL of conidia suspension (10^4 conidia/mL) inoculated in CFCSs; (2) 10 µL of conidia suspension (10^4 conidia/mL) inoculated in CFCSs added with NaOH 1 M or 0.1 M to reach pH 5.5; (3) 10 µL of conidia suspension (10^4 conidia/mL) inoculated in CFCSs added with NaOH 1 M or 0.1 M to reach pH 5.5 and with 10 µl of proteinase K (10 mg/ml)(Sigma, Milan, Italy); (4) PC samples; (5) B samples. Samples (2) and (3) were set up in order to neutralize mould growth inhibition due to organic acids, (2) and (3), and proteinaceous metabolites (3) produced by LAB and yeast strains. The microplates were incubated for 72 h at 30 °C and the absorbance values measured at 490 nm. All samples were set up in triplicate.

2.5 Detection of PLA by LC-MS/MS

The CFCSs of the 25 LAB and 16 yeast strains were submitted to assessment of PLA by liquid chromatography-mass spectrometry (LC-MS/MS). PLA standard was provided as (±)-2-hydroxy-3-phenylpropanoic acid by Aldrich (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) at concentration >98%.

For the characterization and quantification of PLA by LC-MS/MS, a UPLC Dionex Ultimate 3000 (Thermo Fisher Scientific Inc., Walthman, MA, USA) equipped by an electrospray ionization (ESI) interface with a tridimensional ion trap mass spectrometer LCQ Fleet (Thermo Fisher Scientific Inc., Walthman, MA, USA) was used. The UV detector, inserted in-line after the analytical column and before the ESI interface, was set at 215 nm and 271 nm, with bandwidth 4 nm and data collection rate of 5 Hz for both lecture channels. The

analytical column was C18 phase with high retention for polar analyte modification, Luna Omega Polar C18, length 150 mm, 2.10 mm ID, 1.6 µm particle size, 100 Å pore size (Phenomenex, Torrance, CA, USA). Eluent A was methanol and eluent B was MQ grade water, both containing 0.1% formic acid. Methanol and formic acid were in LC-gradient grade. The elution was performed by changing the mobile phase composition as follows: from zero to 1 min A:B:C ratio of 1:99:0, from 1 to 20 min A:B:C ratio 100:0:0. A cleaning phase with a ramp from 20 to 21 min with C eluent, and the maintenance of C eluent at 35% (A:B:C 65:0:35) for 4 min were carried out. Then, the restoration of the original column condition occurred: from 25 to 26 min the eluent ratio became A:B:C 1:99:0 and this condition was kept for 4 min. The temperature was 75 °C and elution flux 0.400 mL/min for the whole analysis. Samples without any treatment or dilution were filtered in a 0.45 µm pore size PTFE syringe and a portion of 5 µl was directly injected in the LC system.

For MS analyses, the ESI interface was used with the following setting: capillary temperature 275 °C, capillary voltage -18 V, spray voltage 5 kV, tube lens -80.65 V, sheath gas flow rate (arb) 10, aux gas flow rate (arb) 5. The elution time was from 6 to 15 min (temporal range including PLA elution) when two different events of 30 ms were applied in negative polarity ionization in mass range from 50.00 to 200.00 m/z.

2.6 Statistical analysis

ANOVA test was run in order to explore for statistically different anti-mould activity (evaluated as % of inhibition detected by OD_{490nm} measurements) between yeast and LAB. The comparison was run independently for each mould type (see Figure 2) as well as pooling together the results of all moulds tested in order to investigate the average inhibitory activity. In addition, in order to compare strains and select the ones with a greater anti-moulds activity (regardless of the nature of the inhibition), ANOVA was used; Duncan test was then applied in order to further group the strains in subsets, allowing the identification of the best ones for each tested mould. The same approach was used to analyse data from the 16 yeast

strains. The best inhibitory strains were selected by following ANOVA classification: best performing LAB were identified as those able to inhibit at least 4 mould with a % of inhibition higher than 99.0%; in the other hand, for the yeasts, the cut-off chosen was a percentage of inhibition higher than 90% against at least 4 moulds out of 6.

Then, in order to investigate the nature of antifungal activity, the inhibition percentages obtained at each different growth condition were compared. The comparison was done for each strain, tested by triplicate analyses, using ANOVA followed by the Duncan post-hoc test. Considering the groups CFCS (1), $pH_{5.5}$ (2), and $pH_{5.5}$ + pK (3) if significant differences were among all of them, then the nature was classified as due to organic acid production added to the synthesis of anti-microbial proteinaceous compounds; if group 1 was significantly different from 2 and 3 (which were non different), then the nature of the inhibition was related to organic acid production; if group 1 was not significantly different from 2 but from 3, then the activity was solely related to the anti-microbial proteinaceous compounds produced.

Finally, ANOVA test was applied in order to compare the average PLA produced by the two groups of microorganisms: LAB and yeasts.

All statistical analyses were performed using SPSS vs 12.1 for windows and significances were assessed using $p<0.05$

3. RESULTS

3.1 Mould growth inhibition detected by overlay analysis and microplate reader

The results obtained by interpreting the overlay method led to the selection, among the whole collection, of 25 LAB and 16 yeast strains showing the best performance in terms of antifungal strength. In particular, strains strongly active (++) at least versus 5 out of the 6 moulds used as target were selected. Moreover, LAB and yeast strains unable to inhibit (-) even one of the 6 moulds were excluded. As shown in Table 1, 6 LAB and 11 yeasts showed

strong inhibition power (++) versus all the 6 target moulds; the remaining selected strains were strongly active (++) against 5 out of the 6 moulds and moderately inhibited (+) the growth of 1 out of the 6 moulds.

The percentages of mould growth inhibition, measured on absorbance data by microplate reader, are also reported in Table 1. The percentages were calculated on OD_{490nm} values obtained by mould growth in CFCS compared to PC and subtracted of B values (see 2.4 section). These data were not totally in agreement with the results obtained by overlay method. In a few case, for example, microbial strains showing 100.0% of mould inhibition by microplate reader, exhibited weak inhibition (+) on plate by overlay method. On the contrary, relatively low inhibition values of 8.9% and 10.3% were registered in strains classified as strongly active (++) on plates. The two methods gave the same results in LAB and yeast strains not inhibiting mycelium proliferation by overlay method (-) and showing 0.0% of inhibition by microplate reader (data not shown).

Percentage data were further processed in order to compare the overall antifungal strength of LAB and yeast strains. Based on statistical analyses, on average, LAB expressed 32.4% more inhibitory capacity than yeasts (figure 2). Precisely, LAB showed a mean value of mould growth inhibition of 88.5% compared to 56.1% found in yeasts. Moreover, the behaviour of the two microbial groups was also significantly different considering separately each target mould, as reported in figure 2. LAB showed from a minimum of 17.4% (vs DsfAn strain) to a maximum of 39.0% (vs S2TC strain) more of inhibitory activity compared to yeasts.

3.2 Comparison among strains and strain selection

The ANOVA analyses on percentage values of mould growth inhibition obtained by microplate reader allowed to group the 25 LAB and 16 yeast strains into subsets significantly diverse for each target mould (data not shown). This approach enabled to set a cut-off to

select LAB (N=10) and yeast (N=4) strains with the best performance in terms of antifungal activity (Table 2 and 3).

3.3 Nature of antifungal activity

The nature of antifungal activity showed by LAB and yeast strains was hypothesized by comparing OD_{490nm} data measured by microplate reader. In particular, the inhibition percentages of mould growth obtained in CFCS samples were compared with the percentages detected in CFCS samples at pH 5.5, with or without the addition of proteinase K, as described in 2.4 and 2.6 sections. In Table 2 and 3, the data referring to the best performant 4 yeasts and 10 LAB strains are reported in details, and different letters (a/b/c) show significant differences, within the data of the same row, relative to mould growth inhibition in the three different conditions analysed. The results were analysed and interpreted, as described in section 2.6, and, in Table 4, it has been reported the percentage of samples for which mould growth inhibition, by the 10 LAB and 4 yeast strains selected, was due to pH decrease by organic acid production (oa), or to proteinaceous molecules with antifungal activity (pm) or, finally, to a synergic effect of the two factors oa + pm. Moreover, the data were compared with the overall results referred to the 25 LAB and 16 yeast strains initially selected. By comparing singularly the two factors oa and pm, LAB antifungal activity seemed mainly due to acid organic production, as it was observed from 31.7% to 36.7% of samples (Table 4). On the contrary, yeast activity was explained mainly with proteinaceous metabolites production (26.0%), especially in the samples (37.5%) referred to the 4 selected yeast strains. In both microbial groups, the synergic inhibitory effect of the two factors (oa + pm) overcame each factor evaluated singularly in most samples (from 43.3% to 48.7% and 49.0% for LAB and yeasts, respectively) with the exception of the ones related to the 4 selected yeast strains (20.8%) where the exclusive contribution of proteinaceous metabolites prevailed (Table 4). In a few samples, the percentage of mould growth inhibition

did not vary at the three different conditions (oa/pm/oa + pm) and no hypothesis could be stated on the nature of antifungal strenght.

3.4 PLA production

The amount of microbial PLA production was calculated as mean values among the 25 LAB strains and among the 16 yeasts, separately. On average, LAB showed a significantly higher production (0.24 mM) compared to yeast strains (0.11 mM). Moreover, PLA production was analysed separately in the 10 LAB and 4 yeasts selected for their best performance in terms of antifungal activity. The 4 yeast strains produced, on average, 0.14 mM PLA compared to 0.022 mM produced by the other 12 strains. Regarding LAB, the difference in PLA production was not so markedly different. The 10 LAB strains selected showed a production, on average, of 0.25 mM compared to 0.23 mM of the 15 remaining. Results were only descriptive and statistical analyses were not performed due to the limited and unequal sample size.

4. DISCUSSION

Filamentous fungi may contaminate many stages in cocoa processing. Poor practices are widely reported and they may affect and amplify the risk of fungal infections. To minimize this problem, the Codex Alimentarius Commission (2013) recommended keeping separated healthy and damaged pods and, the latter, should not be stored longer than one day before opening and fermenting. Moreover, fermentation should be not extended beyond 7 days and, during drying, efforts should be made to protect cocoa beans from humidity at night and during raining. Finally, considering the hygroscopic nature of dried cocoa beans, their moisture content should be checked during storage and kept below 8%. So many stages at risk of contamination make the improvement of the practices, in cocoa processing, difficult to implement satisfactorily. Thus, the use of antifungal microbial strains to be inoculated in fermenting cocoa bean masses has been taken in consideration as a putative application of

biological control. In this regard, cocoa spontaneous fermentations could be a reservoir of antifungal LAB and yeast strains to be selected for their use as antifungal adjunct or starter cultures (Pereira et al., 2012).

Mould contamination during cocoa fermentation has been linked, besides flavour defects, to health risks due to the production of mycotoxins, especially ochratoxin synthesized by *A. niger*, *Aspergillus carbonarius* and *Aspergilus ochraceus*. In our study, we chose, as target moulds, species recurrent in cocoa fermentation environments (Schwan, Pereira & Fleet, 2014) and, specifically, strains that had been isolated during fermentation and drying in our previous experimentations (Visintin et al., 2016; Visintin et al., 2017). The target fungi chosen are all potential mycotoxin producers. In addition to *A. niger* aforementioned, *A. flavus* and *A. fumigatus* are able to synthesized aflatoxin (Klingelhofer et al., 2018) and gliotoxin (Dolan et al., 2005), respectively; while the production of patulin is associated to *P. griseofulvum* (Dombrink-Kurtzman & Blackburn, 2005), and of citrinin to *P. citrinum* (Heperkan et al., 2009).

The LAB and yeast strains used in the present study showed, in general, a fairly good antifungal potentiality. *A. flavus* ST2A and *A. niger* AnDsf were the more resistant moulds as reported by other authors who highlighted the different robustness of *Aspergillus* sp., especially *A. niger*, compared to *Penicillium* sp. (Gerez et al., 2009; Hassan & Bullerman, 2008). Considering the initial 400 isolate collection, LAB and yeast strains showed high variability in terms of antifungal strength, also at intraspecific level. The importance to operate a selection at strain level has been discussed by other authors (Gerez et al. 2010) and validates the need to start from an initial high number of isolates to reach an accurate and successful selection. In our studies, LAB showed higher antifungal activity compared to yeasts. Starting from more than 200 isolates, 25 LAB strains belonging to *L. fermentum* (21) and *L. plantarum* (4) species were initially selected, and, finally, 10 of them (8 *L. fermentum* and 2 *L. plantarum*) were judged as potentially successful strains. Both *L. plantarum* and *L.*

fermentum species, from different environment, have already been described as able to limit fungal growth (Lavermicocca et al. 2000; Strom et al. 2002) and the results obtained in the present study confirm their strength. Regarding yeasts, more than 200 isolates were submitted to preliminary screening and 16 strains belonging mainly to *S. cerevisiae* were selected. Four of them (3 *S. cerevisiae* and 1 *C. ethanolica*) were, then, judged as the best performant yeast strains.

An initial strain selection was based on a qualitative screening. Then, a quantitative analysis based on absorbance lectures at microplate reader led to more detailed results. In few samples, the two methods did not reach comparable results. This was probably due to the different growth conditions. The quantitative analysis was carried out in liquid media that probably allowed an easier diffusion of the compounds responsible for antifungal activity, compared to agar medium used for qualitative overlay method. Moreover, the media employed for the growth of the target moulds were different for the two methods and this may have affected their sensitive or resistance to LAB and yeast strains. Therefore, if in vitro experimentation allow to manage huge number of strains and, thus, operate an initial screening, in vivo trials have to be carried out to confirm the results and to evaluate the real potential of each of the selected strains (Dalié, Deschamps & Richard-Forget, 2010). Twenty kilograms of cocoa beans are considered to be the critical mass for resembling real cocoa fermentation conditions. Thus, the performance of the 10 LAB and 4 yeast selected strains will be tested in 20 kg cocoa bean fermentations before being validated in field experimentation.

Many authors have described mould inhibitory activity in different microorganisms, especially LAB; however, the nature of the antifungal compounds have been investigated to a lesser extent. The difficult in elucidating their mechanism of action is due, probably, to the synergistic effects of different compounds. Some hypothesis and evidences have been reported to explain this phenomenon. Mixture of organic acids seem to be responsible for

antifungal activity in microbial strains, and Dal Bello and colleagues (2007) and Strom and colleagues (2002) highlighted the major contribute of PLA in *L. plantarum* strains. Other authors (Schnurer & Magnussoon, 2007; Vermeulen, Ganzle & Vogel, 2006) have also identified PLA as one of the most effective antifungal compounds and showed that its production is strain-dependent. Lavermicocca, Valerio & Visconti (2000) reported that fungicidal activity by *L. plantarum* strains, in a synthetic medium, against 19 moulds, was due to about 60 mM PLA production. The 10 LAB strains selected in our study showed a production, on average, of 0.25 mM which is much less than the threshold indicated by Lavermicocca and partially justify their fungistatic more than fungicidal action. In agreement with Cortés-Zavaleta and colleagues (2014) who studied the production of PLA in 13 LAB strains (included *L. plantarum* and *L. fermentum*) highlighting that the amount of PLA ranged from 0.021 mM to 0.275 mM, our results confirm the hypothesis that PLA can hardly be considered the only compound related to antifungal activity. Despite our data highlight the important role, in antifungal strength, of organic acid production affecting mould inhibition in 31.7% of the samples analysed, nevertheless, in 15% of the samples, mould growth was limited by proteinaceous metabolites, and 43.3% of the samples showed mould inhibition due to the synergic effect of the two components. Moreover, in a few samples (10.0%), the nature of antifungal strength could not be related either to organic acids or to proteinaceous compounds confirming the idea of the existence of still many unknown aspects in this mechanism. The proteinaceous nature of some antifungal compounds, detected in LAB, has been also described but to a lesser extent compared to organic acids. For example, some authors described *Lactobacillus coryneformis* (Magnusson & Schnürer 2001) and *Lactotococcus lactis* (Roy et al., 1996) as antifungal proteinaceous metabolite producers.

LAB are not the only microorganisms able to produce PLA (Mu et al., 2012). Even if to a lesser extent, PLA production has been demonstrated also in propionic and coryneform bacteria, *Bacillaceae* and yeasts as *Geotrichum candidum*. In our study, we found that the

4 yeast strains selected for their higher inhibitory activity, produced, on average, 0.14 mM PLA compared to 0.022 mM produced by the other 12 strains. In addition, our results underline the important role of proteinaceous metabolites which limited mould growth in 37.5% of the samples analysed against the 12.5% for which mould growth was inhibited by organic acids. The antifungal activity of yeast has already been explained, in some species, as due to the production of killer proteins which are stable to pH values ranging from pH 3 to 5.5 and, thus, our experiment seem to confirm this hypothesis. For example, Santos and colleagues (2009) have characterized two killer toxins, PMKT and PMKT2, active against yeasts and moulds. Another mechanism studied in yeasts has been linked to the expression of some enzymatic proteins as proteases. For example, *Aureobasidium pullulans* was found to produce protease ALP5 efficient against moulds as *Penicillium expansum* and *Botrytis cinerea* (Banani et al., 2014).

5. CONCLUSIONS

Our collection of LAB and yeast isolates, obtained by previous studies on spontaneous cocoa bean fermentations, represented a precious reservoir of strains among which promising candidates have been selected to be used as biological agents to control mould contamination. They belong to *L. fermentum*, *L. plantarum*, *S. cerevisiae* and *C. ethanolica* species, well adapted to cocoa environment and, thus, potentially competitive in colonization of fermentation cocoa masses. New trials, in future, should include their use as adjunct or starter cultures in field experimentations where their antifungal strength should be further evaluated. In fact, the production of antifungal secondary metabolites can be affected by extrinsic factors and results can be conditioned by nutritional and abiotic agents in cocoa fermentations.

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Table 1. Growth inhibition of the six target moulds by antifungal LAB ad yeast strains detected by overlay analysis and microplate reader.

Antifungal strains		Mould strains											
		ST2A		DsfAf		DsfAn		M6E1TS		S2TC		M2BT2	
Species	**Code**	overlay	%	overlay	%	overlay	%	overlay	%	overlay	%	overlay	%
L. fermentum	LAPA34	++	88.1 (1.7)	++	99.4 (1.0)	+	85.5 (1.8)	++	99.8 (1.8)	++	100.1 (0.2)	++	99.6 (0.1)
L. fermentum	LAPA62	++	97.3 (1.7)	++	98.9 (0.9)	++	95.6 (0.7)	++	94.9 (2.5)	+	68.4 (3.2)	++	91.9 (2.3)
L. fermentum	LAPA64	++	85.9 (1.3)	++	100.5 (0.0)	+	48.8 (1.9)	++	100.4 (0.1)	++	100.7 (0.1)	++	100.9 (0.0)
L. fermentum	LAPP61	++	63.1 (0.7)	++	100.1 (0.1)	+	51.9 (4.0)	++	99.3 (1.3)	++	100.7 (0.2)	++	99.3 (0.6)
L. fermentum	LAPP62	++	55.8 (1.2)	++	100.8 (0.5)	+	37.3 (1.9)	++	100.3 (0.3)	++	100.3 (0.3)	++	100.4 (0.3)
L. fermentum	LAPP64	++	86.7 (0.8)	++	99.9 (0.1)	+	97.2 (3.8)	++	100.0 (0.5)	++	99.7 (0.3)	++	101.0 (0.4)
L. fermentum	LAPP65	++	87.5(1.0)	++	100.0 (0.0)	++	50.4 (1.5)	++	100.1 (0.0)	++	100.1 (0.0)	++	98.7 (0.9)
L. fermentum	LAPM34	++	68.1 (3.4)	++	100.1 (0.0)	+	45.8 (0.6)	++	100.1 (0.1)	++	100.1 (0.0)	++	80.1 (6.3)
L. fermentum	LAPM63	++	100.1 (1.5)	+	100.6 (0.1)	++	51.8 (1.1)	++	100.0 (0.0)	++	99.7 (0.1)	++	100.4 (0.2)
L. fermentum	LAPM65	++	53.8 (2.4)	++	100.1 (0.2)	+	37.3 (5.0)	++	100.1 (0.2)	++	100.3 (0.2)	++	98.8 (0.5)
L. fermentum	LAPD63	++	35.9 (4.2)	++	100.0 (0.0)	+	53.9 (0.2)	++	99.4 (0.2)	++	100.4 (0.1)	++	97.8 (1.7)
L. fermentum	LAPD65	++	100.0 (0.0)	++	100.1 (0.1)	+	51.8 (1.6)	++	97.9 (1.4)	++	100.7 (0.2)	++	98.5 (0.9)
L. fermentum	LAS02	++	91.2 (1.5)	++	100.0 (0.0)	++	53.0 (0.0)	++	100.1 (0.0)	++	99.5 (0.5)	++	98.8 (0.2)
L. fermentum	LAS03	++	73.8 (1.2)	++	100.3 (0.1)	+	52.7 (2.9)	++	99.6 (0.1)	++	100.2 (0.4)	++	98.8 (0.4)
L. fermentum	LAS04	++	56.1 (1.8)	+	100.2 (0.1)	++	42.1 (1.4)	++	100.5 (0.2)	++	98.9 (1.3)	++	98.8 (0.9)
L. fermentum	LAS05	++	74.9 (4.5)	++	100.9 (0.5)	++	49.4 (3.1)	++	101.0 (1.8)	++	100.5 (0.8)	++	100.2 (0.3)
L. fermentum	LAS13	++	55.5 (1.4)	++	100.0 (0.0)	+	54.6 (0.2)	++	100.2 (0.1)	++	96.1 (1.5)	++	93.5 (3.5)
L. fermentum	LAS24	++	48.9 (1.4)	++	100.1 (0.1)	+	62.9 (2.5)	++	99.5 (0.5)	++	98.4 (0.4)	++	98.6 (0.0)
L. fermentum	LAS25	++	65.3 (1.1)	++	101.3 (1.0)	++	62.5 (1.7)	++	100.8 (0.6)	++	100.5 (0.5)	++	100.6 (0.8)
L. fermentum	LAS62	++	50.3 (4.1)	++	100.0 (0.0)	+	54.5 (1.1)	++	100.1 (0.0)	++	100.3 (0.0)	++	95.3 (5.5)
L. fermentum	LAS65	++	98.2 (0.9)	++	94.2 (1.7)	+	94.6 (1.1)	++	91.3 (2.0)	++	70.7 (2.9)	++	93.5 (0.4)
L. plantarum	LA1B23	++	30.8 (3.8)	++	100.2 (0.0)	++	16.1 (1.0)	++	71.8 (7.1)	++	92.0 (2.6)	++	60.9 (7.1)
L. plantarum	LAUR64	++	99.7 (0.0)	++	100.2 (0.9)	++	99.9 (0.1)	++	99.8 (0.2)	++	99.8 (0.1)	++	100.1 (0.2)
L. plantarum	LARB63	++	35.0 (2.2)	++	100.1 (0.1)	+	47.1 (0.9)	++	100.7 (0.3)	++	100.1 (0.1)	++	89.4 (7.1)
L. plantarum	LARB65	++	100.9 (0.8)	++	100.3 (0.2)	+	88.4 (0.6)	++	99.6 (0.1)	++	99.6 (0.9)	++	97.6 (0.6)
S. cerevisiae	Li1B01	++	8.9 (3.4)	++	72.3 (4.3)	++	51.5 (4.3)	++	60.6 (2.3)	+	2.7 (0.5)	++	44.3 (4.7)
S. cerevisiae	Li1B02	++	54.9 (1.7)	++	61.5 (1.3)	++	48.3 (1.8)	++	58.1 (9.4)	++	54.3 (1.4)	++	51.9 (1.0)
S. cerevisiae	Li1B03	++	24.4 (2.0)	++	89.9 (1.9)	++	42.2 (0.7)	++	67.2 (6.0)	++	49.2 (1.5)	++	71.2 (0.8)
S. cerevisiae	Li1B05	++	37.3 (2.9)	++	48.6 (8.5)	++	37.8 (1.9)	++	37.8 (1.1)	++	15.8 (1.3)	++	33.7 (0.4)
S. cerevisiae	Li1B11	++	43.4 (5.9)	++	100.1 (0.4)	++	102.1 (1.8)	++	95.3 (4.8)	++	89.4 (0.8)	++	91.9 (0.6)
S. cerevisiae	Li1T23	++	44.3 (3.8)	++	100.7 (0.1)	++	21.2 (2.4)	++	96.4 (3.8)	++	97.6 (1.7)	++	100.6 (0.0)
S. cerevisiae	Li3B13	++	45.7 (1.6)	++	100.3 (0.1)	+	32.5 (2.2)	++	99.0 (1.4)	++	91.9 (1.4)	++	95.4 (0.8)
S. cerevisiae	Li3B14	++	40.6 (0.6)	++	100.1 (0.0)	++	21.7 (0.9)	++	77.9 (8.7)	++	93.7 (3.3)	++	55.9 (1.7)
S. cerevisiae	Li3B64	++	17.9 (0.3)	++	47.1 (6.0)	++	11.6 (3.9)	++	67.4 (2.3)	++	42.9 (0.1)	++	37.6 (1.6)
S. cerevisiae	Li3T12	++	19.1 (1.7)	++	33.1 (0.8)	++	17.8 (1.4)	++	14.9 (1.1)	++	19.2 (1.6)	+	12.2 (0.1)
S. cerevisiae	LIPD34	++	56.8 (4.8)	++	82.7 (1.3)	++	47.8 (1.8)	++	47.1 (5.5)	++	51.5 (0.6)	++	56.3 (0.4)
H. uvarum	LIS04	++	19.0 (2.2)	++	97.6 (0.1)	+	9.3 (0.6)	++	66.5 (1.3)	++	68.7 (1.0)	++	74.1 (4.1)
C. ethanolica	LIS62	++	62.3 (1.4)	++	99.7 (0.1)	++	99.6 (0.1)	++	99.9 (0.2)	++	99.3 (0.1)	++	97.0 (0.9)
C. ethanolica	LIPD61	++	22.1 (0.9)	++	33.1 (1.6)	++	14.1 (0.5)	++	18.9 (0.9)	++	36.0 (4.8)	++	10.3 (2.0)
T. delbrueckii	LIPM35	++	70.0 (2.0)	++	81.3 (0.9)	++	86.0 (0.9)	++	77.1 (1.9)	++	70.0 (0.7)	++	72.3 (3.6)
T. delbrueckii	LIUR31	+	19.8 (0.7)	++	92.4 (2.2)	++	53.4 (5.9)	++	76.9 (8.7)	++	48.8 (2.1)	++	65.8 (1.0)

overlay: referred to mould growth inhibition detected by overlay method described in section 2.2; result interpretation (++ and +) is explained in Figure 1;

%: referred to mould growth inhibition percentages obtained from OD_{490nm} measurements by microplate reader, described in section 2.4.

All the analysis have been made in triplicate and the values in brackets are the standard deviations.

Table 2. Mould growth inhibition, detected by OD_{490nm} measurements at microplate reader, in three different conditions (CFCS/$pH_{5.5}$/ $pH_{5.5}$ + pK), by 4 yeast strains selected for their best antifungal activity.

		Mould growth conditions		
	Moulds	CFCS	$pH_{5.5}$	$pH_{5.5}$ + pK
Li1B11	M6E1TS	95.0 (4.8)a	93.5 (1.9)a	75.1 (2.2)b
	M2BT2	91.9 (0.6)a	91.7 (0.8)a	87.5 (0.1)b
	DsfAn	102.1 (1.8)a	54.7 (1.6)b	35.2 (5.2)c
	DsfAf	100.2 (0.4)	100.1 (0.4)	100.1 (0.2)
	S2TC	89.4 (0.8)a	89.9 (3.2)a	84.2 (2.5)b
	ST2A	43.4 (5.9)	45.6 (7.1)	43.7 (8.4)
Li1T23	M6E1TS	96.4 (3.8)a	85.9 (3.2)b	82.2 (1.4)c
	M2BT2	100.6 (0.0)a	98.7 (0.0)a	85.5 (0.9)b
	DsfAn	21.2 (2.4)	24.1 (2.9)	26.6 (2.8)
	DsfAf	100.7 (0.1)a	100.7 (0.1)a	97.2 (2.4)b
	S2TC	97.6 (1.7)a	97.7 (0.8)a	70.5 (4.0)b
	ST2A	44.3 (3.8)a	37.6 (2.3)b	36.1 (1.2)b
Li3B13	M6E1TS	98.9 (1.4)a	97.9 (0.3)a	72.9 (2.7)b
	M2BT2	95.4 (0.8)a	82.3 (3.7)b	80.8 (0.7)b
	DsfAn	32.5 (2.2)a	21.2 (2.3)b	14.3 (3.1)c
	DsfAf	100.3 (0.1)a	100.7 (0.3)a	88.8 (0.9)b
	S2TC	91.9 (1.4)a	91.9 (0.0)a	72.4 (3.7)b
	ST2A	45.7 (1.6)a	35.7 (2.0)b	27.2 (2.8)c
LIS62	M6E1TS	99.9 (0.2)	100.1 (0.2)	98.9 (0.1)
	M2BT2	97.0 (0.9)a	90.5 (2.1)b	88.3 (1.8)c
	DsfAn	99.6 (0.1)	99.3 (0.3)	99.6 (0.0)
	DsfAf	99.7 (0.1)	99.5 (0.0)	99.4 (0.1)
	S2TC	99.3 (0.1)	98.7 (0.2)	99.6 (0.1)
	ST2A	62.3 (1.4)	42.3 (5.2)	26.1 (3.7)

CFCS: cell free yeast culture supernatants
$pH_{5.5}$: cell free yeast culture supernatants at pH 5.5
$pH_{5.5}$ + pK: cell free yeast culture supernatants at pH 5.5 and added with proteinase K
a/b/c: referred to significant different data analysed within each row

Table 3. Mould growth inhibition, detected by OD_{490nm} measurements at microplate reader, in three different conditions (CFCS/$pH_{5.5}$/ $pH_{5.5}$ + pK), by 10 LAB strains selected for their best antifungal activity.

		Mould growth conditions		
	Moulds	CFCS	$pH_{5.5}$	$pH_{5.5}$ + pK
LAS05	M6E1TS	101.0 (1.8)a	67.7 (1.6)b	66.0 (4.2)b
	M2BT2	100.2 (0.3)a	86.4 (0.2)b	56.4 (2.5)c
	DsfAn	49.4 (3.1)a	40.1 (2.4)b	38.8 (3.4)b
	DsfAf	100.9 (0.5)a	93.3 (1.1)b	89.3 (2.3)c
	S2TC	100.5 (0.8)a	60.1 (2.1)b	60.5 (1.2)b
	ST2A	74.9 (4.5)a	68.2 (2.9)b	58.7 (2.1)c
LAS25	M6E1TS	100.8 (0.6)a	69.3 (2.6)b	64.3 (2.6)c
	M2BT2	100.6 (0.8)a	63.9 (8.6)b	53.4 (2.2)c
	DsfAn	62.5 (1.7)a	43.5 (2.8)b	42.2 (0.9)b
	DsfAf	101.3 (1.0)a	78.8 (5.2)b	77.3 (3.3)b
	S2TC	100.5 (0.5)a	56.6 (2.6)b	55.7 (0.1)b
	ST2A	65.3 (1.1)a	61.2 (1.3)b	46.9 (0.8)c
LAPA34	M6E1TS	99.8 (1.8)a	81.1 (2.8)b	79.5 (1.5)b
	M2BT2	99.6 (0.1)a	83.2 (3.4)b	68.2 (1.8)c
	DsfAn	85.5 (1.8)	86.9 (3.8)	86.1 (3.3)
	DsfAf	99.4 (1.0)	99.0 (0.6)	98.8 (0.2)
	S2TC	100.1 (0.2)a	101.6 (0.8)a	70.2 (1.4)b
	ST2A	88.1 (1.7)a	83.3 (0.9)b	80.1 (1.6)c
LAPA64	M6E1TS	100.4 (0.1)a	65.1 (2.9)b	66.1 (2.8)b
	M2BT2	100.9 (0.0)a	57.3 (1.9)b	59.9 (2.8)b
	DsfAn	48.8 (1.9)	47.4 (0.6)	45.7 (3.5)
	DsfAf	100.5 (0.0)a	92.2 (0.9)b	90.3 (1.7)b
	S2TC	100.7 (0.1)a	60.8 (0.1)b	62.3 (2.3)b
	ST2A	85.9 (1.3)a	71.8 (2.3)b	66.2 (2.6)c
LAPP61	M6E1TS	99.3 (1.3)a	48.8 (2.6)b	47.6 (3.3)b
	M2BT2	99.3 (0.6)a	65.9 (3.0)b	63.9 (1.9)b
	DsfAn	51.9 (4.0)a	41.7 (3.4)b	35.4 (2.5)c
	DsfAf	100.1 (0.1)a	85.8 (1.8)b	79.9 (0.1)c
	S2TC	100.7 (0.2)a	59.3 (1.1)b	41.2 (1.0)c
	ST2A	63.1 (0.7)a	62.9 (1.6)b	62.9 (1.3)b

		Mould growth conditions		
	Moulds	CFCS	$pH_{5.5}$	$pH_{5.5}$ + pK
LAPP62	M6E1TS	100.3 (0.3)a	78.0 (2.3)b	66.2 (5.2)c
	M2BT2	100.4 (0.3)a	55.3 (1.4)b	53.7 (0.9)b
	DsfAn	37.3 (1.9)a	24.1 (1.9)b	19.3 (1.4)c
	DsfAf	100.8 (0.5)a	70.4 (3.9)b	40.3 (2.3)c
	S2TC	100.1 (0.3)a	80.2 (1.2)b	75.6 (0.9)c
	ST2A	55.8 (1.2)	55.5 (1.4)	54.8 (0.8)
LAPP64	M6E1TS	100.0 (0.5)a	99.9 (0.3)a	93.8 (0.9)b
	M2BT2	101.0 (0.0)a	89.6 (3.4)b	90.1 (1.8)b
	DsfAn	97.2 (3.8)	97.6 (0.2)	97.9 (1.7)
	DsfAf	99.9 (0.1)a	99.3 (0.9)a	96.1 (1.0)b
	S2TC	99.7 (0.3)a	98.9 (0.9)a	93.7 (1.2)b
	ST2A	86.7 (0.8)a	82.9 (2.1)a	78.3 (3.8)b
LAPM63	M6E1TS	100.0 (0.0)a	72.3 (4.3)b	73.4 (3.4)b
	M2BT2	100.4 (0.2)a	57.1 (1.2)b	31.5 (3.9)c
	DsfAn	51.8 (1.1)a	36.6 (0.9)b	30.1 (1.8)c
	DsfAf	100.6 (0.1)a	91.5 (4.4)b	72.4 (5.5)c
	S2TC	99.7 (0.1)a	66.7 (1.5)b	67.6 (0.5)b
	ST2A	100.1 (1.5)a	32.0 (3.7)b	9.6 (2.6)c
LAUR64	M6E1TS	99.8 (0.2)a	99.1 (0.9)a	91.9 (2.4)b
	M2BT2	100.0 (0.0)a	94.9 (1.9)b	88.7 (1.4)c
	DsfAn	99.9 (0.1)a	99.2 (1.0)a	95.2 (0.9)b
	DsfAf	100.2 (0.9)a	98.4 (0.3)a	93.3 (1.7)b
	S2TC	99.8 (0.1)a	57.0 (0.8)b	44.8 (3.9)c
	ST2A	99.7 (0.0)a	93.8 (3.2)b	37.6 (2.9)c
LARB65	M6E1TS	99.6 (0.1)a	93.7 (1.7)b	91.9 (2.7)b
	M2BT2	97.6 (0.6)a	84.5 (0.9)b	80.9 (0.8)c
	DsfAn	88.4 (0.6)	87.2 (0.2)	88.1 (0.5)
	DsfAf	100.3 (0.2)a	99.8 (0.2)a	95.5 (0.9)b
	S2TC	99.6 (0.9)a	70.3 (1.1)b	66.2 (0.5)c
	ST2A	100.9 (0.8)a	56.9 (2.1)b	52.7 (0.5)c

CFCS: cell free yeast culture supernatants
$pH_{5.5}$: cell free yeast culture supernatants at pH 5.5
$pH_{5.5}$ + pK: cell free yeast culture supernatants at pH 5.5 and added with proteinase K
a/b/c: referred to significant different data analysed within each row

Table 4. Percentage of samples where mould growth was affected by organic acid production (oa), or by the activity of proteinaceous metabolites (pm), or by the synergic effect of the two factors (oa + pm).

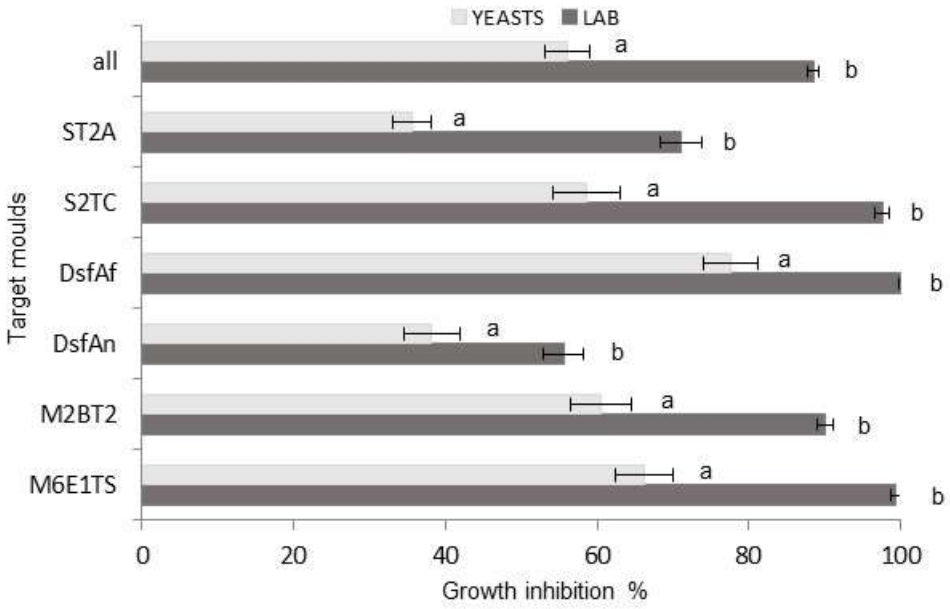
	Factors affecting antifungal strenght			
	oa	pm	oa + pm	not detected
LAB*	36.7%	9.3%	48.7%	5.3%
LAB**	31.7%	15.0%	43.3%	10.0%
YEASTS*	17.7%	26.0%	49.0%	7.29%
YEASTS**	12.5%	37.5%	20.8%	29.2%

* referred to 25 LAB and 16 yeasts strains selected by overlay analysis
** referred to 10 LAB and 4 yeasts strains selected by microplate reader experimentation
not detected: for a few samples it was not possible to hypothesize the nature of antifungal strength.

Figure 1. Interpretation of LAB and yeast antifungal activity by overlay method: strong (++), weak (+), absent (-) mould growth inhibition was determined on the transparency of the area around yeast and LAB streaks.
Figure 2. Overall antifungal strength of LAB and yeast strains towards each target mould. Percentage values of growth inhibition (see section 2.4) are mean values obtained by the total of 25 LAB and 16 yeast strains. a and b: referred to significantly different values when ANOVA was performed within each tested mould or pooling together all the growth inhibition observed regardless of tested mould (all category in the Y axes).

Figure 1

Figure 2


YEASTS
LAB
Target moulds
all
ST2A
S2TC
DsfAf
DsfAn
M2BT2
M6E1TS
a
b
0
20
40
60
80
100
Growth inhibition %