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Isolation, selection and evaluation of yeasts for use in fermentation of coffee beans by the wet process

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ABSTRACT

During wet processing of coffee, the ripe cherries are pulped, then fermented and dried. This study reports an experimental approach for target identification and selection of indigenous coffee yeasts and their potential use as starter cultures during the fermentation step of wet processing. A total of 144 yeast isolates originating from spontaneously fermenting coffee beans were identified by molecular approaches and screened for their capacity to grow under coffee-associated stress conditions. According to ITS-rRNA gene sequencing, *Pichia fermentans* and *Pichia kluyveri* were the most frequent isolates, followed by *Candida Candida glabrata*, *quercitrusa*, *Saccharomyces* sp., *Pichia guilliermondii*, *Pichia caribbica* and *Hanseniaspora opuntiae*. Nine stress-tolerant yeast strains were evaluated for their ability to produce aromatic compounds in a coffee pulp simulation medium and for their pectinolytic activity. *P. fermentans* YC5.2 produced the highest concentrations of flavor-active ester compounds (viz., ethyl acetate and isoamyl acetate), while *Saccharomyces* sp. YC9.15 was the best pectinase-producing strain. The potential impact of these selected yeast strains to promote flavor development in coffee beverages was investigated for inoculating coffee beans during wet fermentation trials at laboratory scale. Inoculation of a single culture of *P. fermentans* YC5.2 and co-culture of *P. fermentans* YC5.2 and *Saccharomyces* sp. YC9.15 enhanced significantly the formation of volatile aroma compounds during the fermentation process compared to un-inoculated control. The sensory analysis indicated that the flavor of coffee beverages was influenced by the starter cultures, being rated as having the higher sensory scores for fruity, buttery and fermented aroma. This demonstrates a complementary role of yeasts associated with coffee quality through the synthesis of yeast-specific volatile constituents. The yeast strains *P. fermentans* YC5.2 and *Saccharomyces* sp. YC9.15 have a great potential for use as starter cultures in wet processing of coffee and may possibly help to control and standardize the fermentation process and produce coffee beverages with novel and desirable flavor profiles.

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1. Introduction

Coffee is an important plantation crop grown in more than 50 developing countries and is one of the most popular non-alcoholic beverages consumed throughout the world (Selvamurugan et al., 2010). Its annual production has reached 8.4 million metric tons, with a turnover close to US $10 billion. Brazil is the leading producer of coffee, followed by Vietnam, Colombia, Indonesia and Mexico (FAO, 2013). Post-harvest processing of coffee cherries is carried out in producing countries using two processes, referred to as wet and dry (Pandey et al., 2000).

Wet processing is used mainly for arabica coffee: the ripe fruits are depulped and then submitted to 24–48 h of underwater tank fermentation and dried until a final water content of 10–12% (Avallone et al., 2001; Murthy and Naidu, 2012). The wet method is widely used in some regions, including Colombia, Central America and Hawaii (Vilela et al., 2010). In the dry processing, in contrast, entire coffee fruits are dried (in the sun) on platforms and/or on a floor without prior removal of the pulp (Silva et al., 2008). Brazil is the largest producer of coffees obtained by dry process; however, the wet process has increasingly been used as a way to improve the coffee quality (Borem, 2008; Gonçalves et al., 2008).

During wet processing, the ripe coffee fruits undergo a spontaneous fermentation, carried out by a complex microbiological process that involves the actions of microorganisms like yeasts, bacteria and filamentous fungi (Avallone et al., 2001; Silva et al., 2008). The

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fermentation is carried out to eliminate any mucilage still stuck to the beans and helps improve beverage flavor by producing microbial metabolites, which are precursors of volatile compounds formed during roasting (Mussatto et al., 2011). Yeasts are among the microorganisms most frequently isolated from fermenting coffee beans, but limited information is available regarding their effect on the development of coffee's taste characteristics (Evangelista et al., 2014). Surveys have shown that the most frequently occurring species during coffee processing are *Pichia kluyveri, Pichia anomala*, *Hanseniaspora uvarum*, *Saccharomyces cerevisiae*, *Debaryomyces hansenii* and *Torulaspora delbrueckii* (Masoud et al., 2004; Silva et al., 2008; Vilela et al., 2010). In addition, bacteria with pectinolytic activity belonging to the genera *Erwinia*, *Klebsiella*, *Aerobacter*, *Escherichia* and *Bacillus*, as well as a variety of filamentous fungi are often isolated (Avallone et al., 2001; Silva et al., 2012; Vaughn et al., 1958).

The use of selected yeasts is well known for other fermented foods and beverages, such as wines, to which as many as eight strains or more of *S. cerevisiae* may contribute to the one wine fermentation (Fleet, 2008). This was possible after many studies to understand the impact of individual yeast strains upon final product quality (Ciani et al., 2006; Erten, 2002; Fleet, 2003, 2008; Gao and Fleet, 1988; Mendoza et al., 2007). For coffee processing that reaches this level, further research is needed to increase the understanding of the microbial ecology, physiology and biochemistry of coffee fermentation and how this scientific knowledge contributes to the development of coffee beverage character. The exploration of the biodiversity of indigenous coffee yeast strains can be an important contribution to the understanding and selection of strains with specific phenotypes able to contribute to the final product quality (Masoud et al., 2004; Silva et al., 2012).

To the best of our knowledge, no previous studies have investigated the use of aromatic yeasts as starter cultures during the fermentation step of wet coffee processing and what impact such yeasts might have on coffee beverage flavor. Here we report an experimental approach to target identification and selection of indigenous coffee yeasts and their potential use as starter cultures with the aim of improving the flavor of coffee beverage processed by wet method. The on-farm implementation of these novel starter cultures is part of a patented process developed in our laboratory (Soccol et al., 2013).

2. Materials and methods

2.1. Spontaneous coffee fermentation and yeast isolation

A total of 150 kg of coffee cherries (*Coffea arabica* var. Mundo Novo) were manually harvested at the mature stage from a farm located in the city of Lavras, Minas Gerais State, Brazil, and mechanically depulped using a BDSV-04 Pinhalense depulper (Pinhalense, Sao Paulo, Brazil). Approximately 75 kg of depulped beans were then conveyed in a clear water stream to tanks and left to ferment for 48 h in accordance with local wet processing method. The environmental temperature was 23–30 °C (day-time temperature) and 11–15 °C (night-time temperature). Every 8 h, liquid fraction samples were withdrawn in triplicate from the middle depth of the tank fermentation, placed aseptically in sterile plastic bags and transferred to the laboratory in ice boxes. Ten milliliters of each sample was added to 90 ml sterile saline-peptone water, followed by serial dilutions. Yeasts were enumerated by surface inoculation on YEPG agar [1% yeast extract (Merck, São Paulo, Brazil), 2% peptone (Himedia, São Paulo, Brazil), 2% glucose (Merck) and 2.5% agar (Difco, São Paulo, Brazil); pH = 5.6] containing 100 mg/l chloramphenicol (Sigma, São Paulo, Brazil) and 50 mg/l chlortetracycline (Sigma) to inhibit bacterial growth. Plating was performed, in triplicate, with 100 μl of each dilution. Cultures were incubated at 30 °C for 4 days. According to the macroscopic indications (texture, surface, margin, elevation, and color), colonies of different types on YEPG medium were counted separately, and representatives isolated from different fermentation times were purified by repetitive streaking on YEPG agar. The purified isolates were stored at −80 °C in YEPG broth containing 20% (v/v) glycerol (Difco).

2.2. Identification of yeast isolates

The yeast DNA was extracted from the pure cultures according to the method described by Pereira et al. (2013). The 5.8S ITS rRNA gene region of yeast isolates was amplified using the primers ITS1 and ITS4 (Masoud et al., 2004). The obtained ITS-rRNA gene region of yeast isolates was digested by restriction endonucleases *Hae*III and *Msp*I, according to the manufacturer's instructions (Invitrogen, São Paulo, Brazil). The PCR products and restriction fragments were separated by gel electrophoresis on 0.7% (w/v) agarose gel, and stained with ethidium bromide (Sigma). The bands were then visualized by UV transilluminator and photographed. A size marker (Gene Ruler of 100 bp DNA Ladder Plus, Fermentans) was used as a reference. The patterns of Amplified rRNA gene Restriction Analysis (ARDRA) were clustered using BioNumerics Version 6.50 (Applied Maths, Sint-Martens-Latem, Belgium). Representative isolates were selected on the basis of genotypic groupings, and the 5.8S ITS rRNA gene region was sequenced using an ABI3730 XL automatic DNA sequencer. The sequences obtained were compared with sequences available in the GenBank database through a basic local alignment search tool (BLAST). The nucleotide sequences of representative isolates were deposited in the GenBank database under access numbers KF747750 to KF747757.

2.3. Pre-selection of isolates: evaluation of individual stress factors

All yeast species were evaluated for their ability to grow under stress conditions that occur during the wet fermentation of coffee beans according to the procedure described by Pereira et al. (2012). The isolates were transferred from YEPG plates to pre-culture 10 ml YEPG broth and incubated at 30 °C for 24 h, 120 rpm. Subsequently, 1 ml of the resulting yeast cultures were transferred to 50-ml Erlenmeyer flasks containing 10 ml YEPG broth and grown for 3 h at 120 rpm (until early exponential phase). At this point, cells were harvested and diluted in sterile water to Abs600 of 0.2. Spots of 3 μL were placed onto stress plates, which were incubated for at least 48 h at 30 °C.

The test medium used was composed of basal medium [0.05% yeast extract (Sigma), 0.3% (w/v) vitamin-free Casamino Acids (Difco) and 2.5% agar (Difco)] and 5% glucose (Merck). The basal medium without an added carbon source was used as a negative control. The heat stress plates were incubated at 25, 30, 37 or 43 °C. Plates with different glucose or fructose concentration were prepared by adjusting the sugar concentration of the basal medium to 15, 30 or 50% (w/w) hexose-equivalent; the sugar being added by sterile filtration. Ethanol, acetic acid and lactic acid stress plates were composed of basal medium with glucose supplemented with 6, 8 or 10% (v/w) ethanol (Sigma); 1, 2 or 3% (v/w) lactic acid (Sigma); 1, 2 or 3% (v/w) acetic acid (Sigma) (added aseptically). Acidic stress plates were composed of basal medium with glucose in the pH 2.0, 4.0, 6.0 or 8.0; pH adjustments were made with sterile 1 M H_2SO_4 or 1 M NaOH.

2.4. Formulation of coffee pulp simulation medium and micro-fermentation trials with pre-selected yeasts

A coffee pulp simulation medium was formulated containing 50% (v/v) fresh coffee pulp extract plus 2.0 g/l citric pectin (Sigma), 15 g/l fructose (Merck), 15 g/l glucose (Merck), 5.0 g/l yeast extract (Merk) and 5.0 g/l soya peptone (Oxoid); pH = 5.5. Citric pectin, which is present in coffee pulp, was added as energy sources for yeast, plus glucose and fructose, while fresh coffee pulp was added to ensure the availability of nitrogen, trace elements and growth factors naturally present during the wet fermentation of coffee beans. For the preparation of coffee pulp extract, 200 g of the coffee pulp and coffee peel from *C. arabica* var. Mundo

Novo was mixed with 1 L of sterile water in a blender for 5 min. The medium was sterilized for 15 min at 121 °C.

In preparing the inoculation cultures of pre-selected yeasts, cells from YEPG agar plates were transferred to glass tubes containing 10 ml YEPG broth and incubated with agitation (120 rpm) at 30 °C for 24 h. Subsequently, these cells were transferred to 200-ml Erlenmeyer flasks containing 90 ml YEPG broth and grown for 30 h and 120 rpm at 30 °C. After centrifuging cells and washing twice with sterile 0.1% peptone water, 250-ml Erlenmeyer flasks with 200 ml of coffee pulp simulation medium were inoculated with log 6 cells/ml of each yeast species. The fermentations were carried out in duplicate.

The growth of yeast at 48 h of fermentation was calculated by colony-forming unit through plating of tenfold serial dilutions of the samples in sterile 0.1% peptone water that was incubated at the appropriate fermentation temperature for 24 h. The volatile compounds produced after 48 h of fermentation in the headspace of the Erlenmeyer flasks were analyzed by gas chromatography as described in Section 2.5. Samples were withdrawn from the headspace with a 10-ml gas-tight syringe and injected into the gas chromatography apparatus.

2.5. Headspace analysis by gas chromatography

Headspace analysis of the volatile compounds was conducted by gas chromatography according to the method of Rossi et al. (2009). Aroma compounds were identified by comparing the peak retention times against those of authentic standards purchased from Sigma in a gas chromatograph (Shimadzu model 17A) equipped with a flame ionization detector at 230 °C. The standards used were 11 alcohols (ethanol, 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol, 1-decanol, 2-hexanol, 2-octanol, 2-methyl-1-butanol, n-butanol, 3-methyl-1-butanol), 12 esters (ethyl acetate, propyl acetate, ethyl laurate, ethyl propionate, ethyl isobutyrate, ethyl hexanoate, ethyl octonoate, diethyl succinate, isoamyl acetate, isobutyl acetate, n-butyl acetate, hexyl acetate), 5 ketones (2,3-butanedione, 2-pentanone, 2-hexanone, 2-octanone, 2-heptanone), 3 aldehydes (acetaldehyde, benzaldehyde, and 3-methyl-butanal) and 2 organic acids (acetic acid and caprylic acid). The operation conditions were as follows: a 30 m × 0.32 mm HP-5 capillary column, column temperature of 40 to 150 °C at a rate of 20 °C/min, injector temperature at 230 °C. Individual volatiles were expressed as μmol/l of headspace, as ethanol equivalent.

2.6. Polygalacturonase activity

Erlenmeyer flasks containing 200 ml of synthetic pectin medium [12% citric pectin (Sigma), 0.3% glucose (Merck), 1.0% KH2PO4 (Merck), 0.5% $MgSO_4.7H_2O$ (Merck), 0.68% $CaCl_2$ (Merck) and 1.0% $(NH4)_2SO_4$ (Merck)] were inoculated in triplicate with 4 log cells/ml of each pre-selected yeast strain. Cultures were incubated at 30 °C for 48 h at 120 rpm and sampled every 24 h. Yeast cells were removed by centrifugation (2060 × g, 20 min, 4 °C) and the cell free supernatants used for determination of polygalacturonase activity by measuring the increase in reducing sugars released from pectin dispersion using 3,5-Dinitrosalicyclic acid DNS (Miller, 1959). A 0.1 ml aliquot of the supernatant was added to 0.9 ml of 0.1% (w/v) of polygalacturonic acid (Sigma) in 1 M sodium acetate buffer (pH 5.0) and incubated in water bath at 50 °C for 15 min. The reaction was stopped by the addition of 1.0 ml DNS reagent. The mixture was boiled for 5 min and then cooled in an ice bath. Absorbance was read at 600 nm, with the optical density (OD600) determined using an appropriate calibration curve. One unit of polygalacturonase activity (U) was expressed as μmol of galacturonic acid released per min and μg total protein under assay conditions. The total protein was determined by the method of Bradford (1976), with bovine serum albumin (BSA) as the standard. The data was analyzed by Analysis of Variance (ANOVA), and the means were compared using Duncan's test.

2.7. Culture of selected yeasts in coffee pulp simulation medium

The influence of fermentation temperature on the growth and volatile compound production of *Pichia fermentans* YC5.2 was analyzed in pure and mixed cultures with the pectinolytic *Saccharomyces* sp. YC9.15. Triplicate fermentations were performed in Erlenmeyer flasks containing 200 ml of coffee pulp simulation medium at 15, 28 and 37 °C and 120 rpm for 48 h. The initial yeast cell concentration was of log 6 cells/ml with a 1:1 ratio of *P. fermentans* YC5.2 to *Saccharomyces* sp. YC9.15. Yeast growth was determined by surface inoculation on YEPG agar. Differentiation of the two yeast species on YEPG agar was done by colony morphology and testing of isolated colonies by a PCR assay using species specific primers that were developed in our laboratory (data presented in the supplemental material). The quantitative analysis of volatile compounds produced after 48 h of fermentation in the headspace of the Erlenmeyer flasks was done through gas chromatography as describe in Section 2.5. The data was analyzed by ANOVA and the means were compared using Duncan's test.

2.8. Wet fermentation with selected yeast cultures and sensory evaluation of coffee beverages

Freshly harvested coffee cherries (*C. arabica* var. Mundo Novo), obtained from a coffee farm located in Lavras, Minas Gerais State, Brazil, were mechanically depulped (BDSV-04 Pinhalense depulper) to obtain beans with mucilage. Laboratory fermentations were conducted in 6-l Erlenmeyer-flasks containing 1.5 kg of depulped beans and 3 l of fresh water. The coffee was fermented by inoculation of *P. fermentans* YC5.2 in pure or mixed cultures with *Saccharomyces* sp. YC9.15. The initial yeast cell concentration was 6 log cells/ml with a 1:1 ratio of *P. fermentans* YC5.2 to *Saccharomyces* sp. YC9.15. As a control, spontaneous process was allowed to ferment with indigenous microorganisms present in the coffee fruit. The fermentations were carried out in triplicate at 28 °C for 48 h to simulate the natural process performed on farms. At the end of the fermentation processes, the growth of the inoculated microorganisms was measured by counting viable cells and the volatile compounds in the headspace of the Erlenmeyer flasks were analyzed by gas chromatography as described in Section 2.5. The data was analyzed by ANOVA and the means were compared using Duncan's test.

The resulting parchment coffee was dried in a laboratory oven at 35–40 °C until a water content of 12% (wet basis) was achieved, then roasted at 140 °C for 30 min. The roasted coffee was sampled, added to boiling water at the rate of 2% (w/v) and infused for 5 min. The samples were evaluated by a panel of five experienced judges based on the flavor descriptors: fruity, buttery, caramel aroma, chocolate aroma, fermented aroma and acidic. A note, from 0 (low intensity) to 10 (high intensity), was attributed to each criterion. Results from the two inoculated samples were compared to the control using ANOVA and the means were compared using Duncan's test.

3. Results and discussion

3.1. Isolation and identification of yeast isolates

Yeast (2.7 log cfu/ml) was present at the beginning of the fermentation and grew to a maximum population of 7.15 log cfu/ml during the subsequent 40 h, followed by a drop to 5.2 log cfu/ml by 48 h (Fig. 1a). A total of 144 yeasts were isolated throughout the wet fermentation of coffee beans. Based on ARDRA-PCR profiles (see Fig. S1 in the supplemental material), eight groups were delineated from which representatives were identified to species level by sequencing. The main species found and their evolution throughout the fermentation are shown in Fig. 1b. *P. fermentans* (Accession No. KF747751) was the most frequently isolated species, followed by *P. kluyveri* (Accession No. KF747755), *Candida glabrata* (Accession No. KF747753) and *C. quercitrusa* (Accession No. KF747756). *Saccharomyces* sp. (Accession

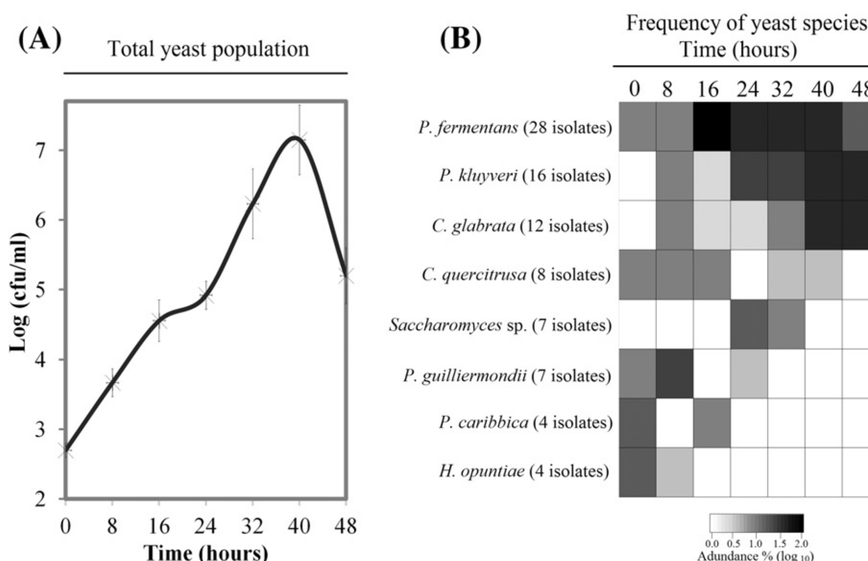

Fig. 1. Assessment of yeast diversity during the spontaneous coffee fermentation process. (A) Dynamic of total yeast population. Bars represent standard deviation. (B) Heat map showing the frequency and abundance of individual yeast species. Values in brackets are the number of isolates identified by ITS-rRNA gene sequencing. The heat map was generated by using Visual Basic for Applications (VBA) in conjunction with Microsoft® Office Excel Version 7.0.

No. KF747750) was detected at 24 and 32 h, while *P. guilliermondii* (accession no. KF747752), *Pichia caribbica* (accession no. KF747757) and *Hanseniaspora opuntiae* (accession no. KF747754) were generally isolated at the start of the fermentation. These yeast species are reported to be fermentative and have been found in soil, fruits and trees (Kurtzman, 1998). While *P. fermentans* was first isolated as the dominant yeast in spontaneously fermented coffee beans, *P. kluyveri* has been reported in other geographical areas and processing methods (Masoud et al., 2004; Silva et al., 2008). Species of the genus *Pichia* have been reported to inhibit ochratoxigenic filamentous fungi growth during coffee fermentation, acting as a possible biological control for the prevention of ochratoxin A in coffee (Masoud et al., 2005). Interestingly, the phylogenetic position of some isolates belonging to the genus *Saccharomyces* (e.g., the selected *Saccharomyces* sp. YC9.15) had less than 97% similarity to known species, which suggests the isolation of a new candidate species of the genus *Saccharomyces*.

3.2. Pre-selection of yeasts: growth/survival under stress conditions

A preliminary screening was carried out with all 144 isolates to test their capacity to grow under coffee-associated stress conditions. Table S1 in the supplemental material summarizes the results for growth/survival of yeast isolates under tested conditions. The criteria used were based on the physical and chemical changes that yeast cells face through the fermentation process. Coffee pulp and mucilage are high density substrates that consistof 27–30% fermentable sugars, in particular glucose and fructose (Wrigley, 1988; Avallone et al., 2001; Murthy and Naidu, 2011). After inoculation, the yeast can experience hypertonic conditions, which lead to an efflux of water from the cell, diminished turgor pressure and reduced water availability (Bauer and Pretorius, 2000). Throughout coffee fermentation process, approximately 60% of the sugars are utilized as substrate for microbial growth which produces significant amounts of ethanol and acetic and lactic acids, resulting in lowered pH (from 5.5–6.0 to 3.5–4.0) (Avallone et al., 2001; Jackels and Jackels, 2005). Thus, when the cell has adapted to the new environment and the fermentation begins, other stressors become relevant as organic acids and alcohols accumulate, the temperature changes and the environment acidifies (Avallone et al., 2000; Silva et al., 2008; Velmourougane, 2013). Following a successful inoculation of coffee beans, the ability of the yeast to adapt and to cope with the hostile environment and stress conditions prevailing in coffee fermentation matrix are of vital importance to fermentation performance. Based on this, nine stress-tolerant strains, possessing the following characteristics, were pre-selected for further investigation: (i) growth capacity in a typical pH range of coffee fermentation (pH 2.0 to pH 8.0); (ii) osmotic pressure tolerance (growth detected in the presence of up to 50% glucose and fructose); (iii) heat tolerance (ability to grow at temperatures of 37 to 43 °C); and (iv) metabolite accumulation tolerance (growth capacity up to 12 to 15% ethanol, 2% lactic acid and 2% acetic acid). These included YC9.15, YC9.13 and YC.8.10 (classified as *Sacharomyces* sp.); YC5.2 and YC8.8 (classified as *P. fermentans*); YH7.16 (classified as *P. kluyveri*); YC1.2 (classified as *Pichia guilliermondii*); YC1.4 (classified as *H. opuntiae*); and YH1.5 (classified as *C. glabrata*).

3.3. Polygalacturonase activity screening

Secretion of polygalacturonase by the nine pre-selected yeast strains was investigated in a synthetic pectin medium. The results showed that *Saccharomyces* sp. YC9.15, *Saccharomyces* sp. YC8.10 and *P. fermentans* YC8.8 were the strains with higher polygalacturonase activity under the assay conditions ($p < 0.05$), which produced 2.03, 1.90 and 1.72 U/ml polygalacturonase after 48 h of fermentation. The other yeast strains produced less than 1 U/ml (data not shown). Previous studies also reported the pectinolytic activity of indigenous coffee yeasts, such as species of *Kluyveromyces*, *Saccharomyces*, *Pichia*, and *Candida* (Masoud and Jespersen, 2006; Silva et al., 2012). These pectinolytic strains appeared to have potential to be used as starter cultures for mucilage degradation during coffee fermentation; nevertheless, further studies are needed to investigate the ability of these yeasts to degrade the mucilage in vivo (i.e., during coffee processing). The removal of the mucilage by microorganisms facilitates bean drying and produces metabolites that diffuse into the interior of the coffee beans and react with substances responsible for the flavor of the final beverage (Silva et al., 2012).

3.4. Production of volatile aroma compounds by pre-selected yeast strains

The nine pre-selected yeast strains were inoculated into coffee pulp simulation medium and the volatile aroma compound production was quantified after 48 h of fermentation. The composition of the coffee

pulp simulation medium supported good growth of the investigated yeasts because all isolates grew about 3 log cfu/ml within 48 h, relative to the initial population inoculated (data not shown). Fourteen compounds were quantified in the headspace of the inoculated fermentations. These included acetaldehyde, benzaldehyde, caprylic acid, ethanol, ethyl acetate, ethyl laurate, isoamyl acetate, 2,3-butanedione, 1-decanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 2-hexanol, 2-octanol and 1-octanol. The most important volatile compounds (i.e., acetaldehyde, ethanol, isoamyl acetate and ethyl acetate) of the different fermentations are listed in Table 1. As expected, *Saccharomyces* strains produced higher amounts of ethanol compared to non-*Saccharomyces* species; *Saccharomyces* sp. YC8.10 produced the highest ethanol levels (126.9 μmol/l), followed by *Saccharomyces* sp. YC9.13 and *Saccharomyces* sp. YC9.15, which produced 125.0 and 121.8 μmol/l, respectively. Isoamyl acetate and ethyl acetate were produced in significant amounts only by *P. fermentans* YC5.2 and *P. kluyveri* YC7.16. *P. fermentans* YC5.2 produced the highest concentrations of ethyl acetate (pineapple-like aroma) and isoamyl acetate (banana-like aroma), reaching concentrations of 79.04 and 23.04 μmol/l, respectively. As these compounds are related to fruity aroma, which participates in aromatic complexity, this makes *P. fermentans* YC5.2 an attractive yeast to enhance the aromatic value of coffee beans and was chosen to evaluate its behavior in co-culture with the pectinolytic *Saccharomyces* sp. YC9.15 at different temperatures. In addition to pectinolytic production, the *Saccharomyces* sp. YC9.15 was selected also for its high production of acetaldehyde in relation to other pectinolytic strains. Acetaldehyde has been correlated with sensory attributes as it gives a floral and fruity note to the final beverage (Sanz et al., 2002).

3.5. Culture of selected yeasts in coffee pulp simulation medium

The growth and volatile compound production of *P. fermentans* YC5.2 in pure and mixed cultures with pectinolytic *Saccharomyces* sp. YC9.15 are shown in Tables 2 and 3, respectively. The viable count of *P. fermentans*'s single-culture fermentations peaked at a maximum population exceeding 7 log cfu/ml. On the other hand, in the mixed-culture fermentations, the viable counts of *P. fermentans* from ~5.8 log cfu/ml increased to 6.4 and 6.3 log cfu/ml at 15 and 28 °C, respectively, and declined to 4.5 log cfu/ml at 37 °C, while the viable counts of *Saccharomyces* sp. were over 7 log cfu/ml (7.3, 8.4 and 7.7 log cfu/ml at 15, 28 and 37 °C, respectively) (Table 2). The highest isoamyl acetate and ethyl acetate concentrations (59.5 and 171.8 μmol/l, respectively) were determined in pure culture of *P. fermentans* at 28 °C, whereas the production of acetaldehyde and ethanol was greater in mixed fermentations at 28 and 37 °C, respectively (Table 3). Thus, it can be see that growth and volatile compound production of *P. fermentans* were reduced by the presence of *Saccharomyces* sp. in mixed-culture fermentations at all tested temperatures. It should be noted, however, that the metabolism of *P. fermentans* was encouraged when fermentations were carried out at low temperature, and even when in co-culture with *Saccharomyces* sp., a high production of isoamyl acetate was observed (Table 3). Several studies also have indicated a higher persistence of some non-*Saccharomyces* yeasts and different fermentation behaviors at low temperatures (Ciani et al., 2006; Erten, 2002; Gao and Fleet, 1988; Heard and Fleet, 1988). This can provide light in future studies aiming to balance the metabolisms of both these species in an attempt to optimize the production of pectinolytic enzymes while maintaining high production of aromatic compounds.

3.6. Inoculation of selected yeasts in wet fermentation of coffee beans and sensory evaluation of coffee beverages

To respond to the new challenges of consumer demands for coffees with high complexity of flavors and stylistic distinction, the time-temperature profile used during roasting is the most used (Wang and Lim, 2013). However, no method of imparting yeast-derived volatile aroma compounds to coffee during wet processing has been established. In order to evaluate the impact of the starter cultures developed in this study on the final beverage flavor, wet fermentation trials at laboratory scale were carried out by inoculating coffee beans. The results presented in Table 4 show the yeast counts and volatile aroma compounds produced after 48 h of fermentation, as well as the sensory flavor profiles of coffee beverages produced thereof. Except for *P. fermentans* in mixed fermentation, which had its growth strongly reduced to 3.26 log cfu/ml, the starter cultures were able to grow under wet processing conditions, as observed in the increasing log cfu/ml after 48 h of fermentation compared to their original numbers at time 0.

Ethanol, acetaldehyde, ethyl acetate, isoamyl acetate, 2,3-butanedione and hexanal were the major volatile compounds released in the headspace of spontaneous and inoculated coffee fermentations (Table 4). These volatile compounds are proposed to derive from two main sources—namely, those resulted from yeast metabolism (i.e., ethanol, acetaldehyde, ethyl acetate, and isoamyl acetate) and those that derived from thermal reactions during fermentation (i.e., hexanal and 2,3-butanedione) (Gonzalez-Rios et al., 2007). Some of these compounds are known to play a role in aroma development during coffee fermentation (e.g., ethanol, ethyl acetate, isoamyl acetate and acetaldehyde) (Czerny and Grosch, 2000; Evangelista et al., 2014; Gonzalez-Rios et al., 2007). The use of the culture starters developed in this study significantly increased ($p < 0.05$) the production of these compounds during the fermentation process (Table 4).

There were no statistically significant differences among any treatments for the descriptor caramel aroma, chocolate aroma and acidic (Table 4). On the other hand, the inoculated fermentations produced beverages with higher sensory scores ($p < 0.05$) for fruity, buttery and fermented aroma compared to the un-inoculated control. Coffee beverage produced from beans inoculated with *P. fermentans* single-culture was rated as having the highest intensity of fruit, which is usually associated with esters produced in the fermentation process (e.g., ethyl acetate and isoamyl acetate). In addition, the significantly more intense

Table 1
Concentrations of major volatile compounds produced by pre-selected yeast strains after 48 h of fermentation in coffee pulp simulation medium.

Compounds (μmol/l)[b]	Yeast strain[a]								
	SC9.15*	SC8.10	SC9.13	PF8.8	PF5.2*	CG1.5	HO1.4	PG1.2	PK7.16
Acetaldehyde	22.04	0.47	0.86	ND	0.53	0.87	0.73	11.5	ND
Ethanol	121.8	126.9	125.0	40.5	60.7	49.1	47.4	72.8	30.4
Ethyl acetate	0.52	0.37	ND	ND	79.4	ND	ND	0.25	0.72
Isoamyl acetate	ND	ND	ND	ND	23.4	ND	ND	ND	4.48

[a] Abbreviations: SS9.15: *Saccharomyces* sp. YC9.15; SC8.10: *Saccharomyces* sp. YC8.10; SC9.13: *Saccharomyces* sp. YC9.13; PF8.8: *P. fermentans* YC8.8; PF5.2: *P. fermentans* YC5.2; CG1.5: *C. glabrata*; HO1.4: *H. opuntiae* YC1.4; PG1.2: *P. guilliermondii* YC1.2; PK7.16: *P. kluyveri* YH7.16.
[b] Means of triplicate fermentations expressed in μmol/l of ethanol equivalent. Minor volatile compounds, such as benzaldehyde, caprylic acid, ethyl laurate, 2,3-butanedione, 1-decanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 2-hexanol, 2-octanol and 1-octanol were produced in amounts below 1 μmol/l.
* Strains labeled with an asterisk were selected for further investigation.

Table 2
Growth (log cfu/ml) of *P. fermentans* YC5.2 in pure or mixed culture with *Saccharomyces* sp. YC9.15 at 15, 28 and 37 °C after 48 h of fermentation in coffee pulp simulation medium.

		Temperature incubation					
		15 °C (h)		28 °C (h)		37 °C (h)	
		0	48	0	48	0	48
Pure culture	*P. fermentans* YC5.2	5.6[a] ± 0.2	7.8[b,c] ± 0.2	5.7[a] ± 0.1	8.3[c] ± 0.2	5.8[a] ± 0.3	7.0[b] ± 0.1
Mixed culture[d]	*P. fermentans* YC5.2	5.8[a] ± 0.1	6.4[b] ± 0.2	5.7[a] ± 0.1	6.3[b] ± 0.2	5.8[a] ± 0.2	4.5[c] ± 0.3
	Saccharomyces sp. YC9.15	5.7[a] ± 0.1	7.3[b] ± 0.4	5.6[a] ± 0.2	8.4[c] ± 0.3	5.9[a] ± 0.1	7.7[b,c] ± 0.4

[d] In mixed culture, the dominance was verified by DNA approaches, as described in the supplementary material. Means of triplicate fermentations in each row bearing the same letters are not significantly different ($p > 0.05$) from one another, using Duncan's test. (Mean ± standard deviation).

Table 3
Mean concentrations[f] of volatile compounds produced by *P. fermentans* YC5.2 in pure or mixed culture with *Saccharomyces* sp. YC9.15 at 15, 28 and 37 °C after 48 h of fermentation in coffee pulp simulation medium.

Coumpounds	Pure culture			Mixed culture		
	15 °C	28 °C	37 °C	15 °C	28 °C	37 °C
Ethanol	114.0[a] ± 12.4	158.6[a,b] ± 1.7	53.0[c] ± 2.0	145.4[a,b] ± 2.6	163.8[b] ± 15.4	151.5[a,b] ± 24.0
Isoamyl acetate	25.8[a] ± 1.8	59.5[b] ± 4.5	22.5[a] ± 3.1	26.5[a] ± 0.2	24.2[a] ± 1.4	2.4[c] ± 0,4
Ethyl acetate	14.0[a] ± 1.9	171.8[b] ± 27.5	93.6[c] ± 4.2	35.8[d] ± 4.0	22.1[a,d] ± 2.3	0.3[e] ± 0.04
Acetaldehyde	1.6[a,b] ± 0.2	0.2[b] ± 0.1	ND	11.32[c] ± 0.8	21.8[d] ± 0.8	4.2[a] ± 0.03
N-butyl acetate	0.8[a] ± 0.1	3.8[b] ± 0.7	3.4[b] ± 0.5	ND	ND	ND
Isobutyl acetate	ND	ND	0.9[a] ± 0.2	ND	ND	ND
Ethyl isobutyrate	ND	ND	0.7 ± 0.1	ND	ND	ND
3-Methyl-1-butanol	0.6[a] ± 0.2	1.2[b] ± 0	ND	ND	ND	ND
1-Pentanol	0.4[a] ± 0.1	ND	0.3[a] ± 0.1	ND	ND	ND
1-Octanol	ND	0.3[a] ± 00.07	ND	0.2[a] ± 0.1	0.4[a] ± 0.3	ND
2-Hexanol	ND	2.0 ± 0.3	ND	ND	ND	ND
1-Decanol	ND	ND	0.4[b] ± 0.1	ND	ND	ND
Caprylic acid	5.9[a] ± 0.1	0.3[b] ± 0.04	0.4[b] ± 0.1	ND	0.5[b] ± 0.3	ND
2,3-Butanedione	6.0[a] ± 1.7	ND	1.3[b] ± 3.4	ND	ND	ND
2-Hexanone	ND	ND	ND	0.4 ± 0.05	ND	ND
Diethyl succinate	ND	ND	ND	0.32 ± 0.02	ND	ND
Ethyl octonoate	ND	ND	ND	0.20 ± 0.08	ND	ND
2-Methyl-1-butanol	ND	ND	ND	ND	1.31 ± 0.41	ND

ND: not detected. Means in each row bearing the same letters are not significantly different ($p > 0.05$) from one another, using Duncan's test. (Mean ± standard deviation).
[f] Values expressed in µmol/l of ethanol equivalent as means of triplicate fermentations (mean ± standard deviation).

Table 4
Volatile aroma compounds and yeast counts after 48 h of wet fermentation of coffee beans and sensory analysis of coffee beverages.

Parameters	Fermentation assay[d]		
	Single-culture	Mixed-culture	Un-inoculated control
Aroma compounds (µmol/l)			
Ethanol	43.08[a] ± 23.81	88.50[b] ± 61.72	1.29[c] ± 0.19
Acetaldehyde	0.48[a] ± 0.01	12.85[b] ± 0.57	0.49[a] ± 0,07
Ethyl acetate	42.42[a] ± 8.38	5.37[b] ± 2.16	ND
Isoamyl acetate	10.18[a] ± 1.59	0.32[b] ± 0.11	ND
Hexanal	21.59[a] ± 9.30	24.91[a] ± 6,17	12.59[b] ± 2.38
2,3-Butanediona	17.86[a] ± 0.74	17.30[a] ± 3.01	8.15[b] ± 1.45
Cell growth (log cfu/ml)			
Pichia fermentans YC5.2	7.74[a] ± 0.61	3.26[b] ± 0.32	ND
Saccharomyces sp. YC9.15	ND	7.40 ± 0.54	ND
Sensory flavor profiles			
Fruity	7.83[a] ± 0.14	6.16[b] ± 0.28	5.83[b] ± 0.30
Buttery	6.25[a] ± 0.25	6.30[a] ± 0.14	5.40[b] ± 0.53
Caramel aroma	6.66[a] ± 0.14	6.58[a] ± 0.14	6.75[a] ± 0.25
Chocolate aroma	5.83[a] ± 0.14	5.91[a] ± 0.14	5.75[a] ± 0.25
Fermented aroma	6.40[a] ± 0.38	6.87[a] ± 0.17	5.16[b] ± 0.14
Acidic	5.83[a] ± 0.14	5.91[a] ± 0.28	5.8[a] ± 0.14

In mixed culture, the dominance was verified by DNA approaches, as described in the supplementary material. Means of triplicate fermentations in each row bearing the same letters are not significantly different ($p > 0.05$) from one another, using Duncan's test. (Mean ± standard deviation).
[d] Single-culture = coffee fermentation conducted with a pure culture of *Pichia fermentans* YC5.2; mixed-culture = coffee fermentation conducted with a mixed culture of *Pichia fermentans* YC5.2 and *Saccharomyces* sp. YC9.15; un-inoculated control = spontaneous fermentation process carried out by indigenous microorganisms from the coffee fruit.

buttery and fermented aroma in inoculated coffees could be linked to the total volatile concentration produced in the fermentation processes, such as 2,3-butanedione (buttery flavor), acetaldehyde (fruity flavor), hexanal (green beans flavor), ethanol (alcoholic flavor) and esters (fruity flavor).

4. Conclusion

This study demonstrated a useful approach for target selection of aromatic coffee yeasts and their use as starter cultures during the fermentation step of wet processing. The strong flavor producing

P. fermentans YC5.2 and the pectinolytic *Saccharomyces* sp. YC9.15 (acetaldehyde producing) were selected as starter cultures for coffee fermentation. The use of these starter cultures in wet processing resulted in coffee beverages with modified flavors, which reveals that yeasts have a complementary role when associated with coffee quality through the synthesis of yeast-specific volatile constituents. A coffee with a distinctive aroma of fruits could be produced using the starter cultures in coffee processed by the wet method. The selected yeast strains *P. fermentans* YC5.2 and *Saccharomyces* sp. YC9.15 have a great potential for use as starter cultures in wet processing of coffee and may possibly help to control and standardize the fermentation process and produce coffee beverages with novel and desirable flavor profiles. Further studies should be directed toward the implementation of these yeast strains under on-farm coffee processing conditions and their interaction with other microorganisms, such as lactic and acetic acid bacteria and Gram-negative bacteria present during fermentation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2014.07.008.

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