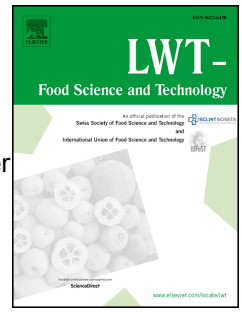

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PII: S0023-6438(20)31418-3

DOI: https://doi.org/10.1016/j.lwt.2020.110430

Reference: YFSTL 110430

To appear in: *LWT - Food Science and Technology*

Received Date: 5 August 2020

Revised Date: 12 October 2020

Accepted Date: 20 October 2020

Please cite this article as: Elhalis, H., Cox, J., Frank, D., Zhao, J., Microbiological and biochemical performances of six yeast species as potential starter cultures for wet fermentation of coffee beans, *LWT - Food Science and Technology*, https://doi.org/10.1016/j.lwt.2020.110430.

Credit author statement

HE designed and conducted experiments, performed the data analysis and wrote the manuscript. JZ and DF supervised the experiments, interpreted the scientific values of the obtained data and edited the manuscript. JC supervised work. All authors read and approved the manuscript.

Microbiological and biochemical performances of six yeast species as potential starter cultures for wet fermentation of coffee beans

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Abstract

This study investigated the microbiological and biochemical characteristics of six endogenous yeast species isolated from spontaneous wet coffee fermentation for their potential as starter cultures. The yeasts were cultured under elevated temperature, osmotic pressure, ethanol and acid concentrations to assess their tolerance to these coffee fermentation-related stress conditions. Their ability to produce hydrolytic enzymes for pectin, protein, cellulose and starch were evaluated using plate assays. *Hanseniaspora uvarum* and *Pichia kudriavzevii* were the most stress-tolerant species and also exhibited high pectinase, amylase, cellulase and protease activities compared with *P. fermentans*, *Candida railenensis*, *C. xylopsoci* and *Wickerhamomyces anomalus*. When the yeasts were inoculated in a synthetic coffee pulp extract medium all the isolates grew and produced several important aromatic compounds, including isoamyl alcohol, 2-phenylethyl alcohol, ethanol, ethyl acetate, acetaldehyde, and 2-propanone. The concentrations of these volatiles produced by the yeast species differed significantly. *H. uvarum* and *P. kudriavzevii* produced significantly higher concentration of total alcohols (42.5, 57.6 mg/l), esters (31.0, 33.7 mg/l) and aldehydes (0.7, 1.9 mg/l). Overall, *H. uvarum* and *P. kudriavzevii* demonstrated the strongest potential as starter cultures for wet coffee fermentation.

Keywords: Coffee, *Hanseniaspora uvarum*, *Pichia kudriavzevii*, Flavour, Pectinase.

1..Introduction

Coffee is one of the most popular non-alcoholic beverages consumed worldwide, with an estimated annual consumption of over 148 million cups (International Coffee Organization, 2018). The quality of coffee beverages is influenced by both of pre-harvest factors such as genotype, geographic location, climate, and agronomic practices, and post-harvest factors including primary processing, drying, roasting and storage conditions (Pereira et al., 2017b). Wet fermentation of coffee beans is one of the major primary processing methods and, if done properly, can produce coffee of high sensory qualities (Agate & Bhat, 1966; Amorim & Amorim, 1977; Evangelista et al., 2015; Goto & Fukunaga, 1986; Pereira et al., 2015; Pereira et al., 2014; Silva, 2014). Wet coffee fermentation involves submerging de-pulped coffee beans underwater and subjecting them to microbial fermentation for 24-48 h (Cleves, 2009; Gonzalez-Rios et al., 2007; Schwan & Wheals, 2004). Despite the large volume of coffee production, the fermentation is still performed in traditional, uncontrolled processes where indigenous microorganisms originated from the beans, environment and processing equipment perform spontaneous fermentation (Selvamurugan et al., 2010). To transform the fermentation process to a controlled, industrial process, it is crucial to develop microbial starter cultures with appropriate microbial and biochemical properties that can perform the fermentation efficiently and produce coffee with consistent high quality.

Wet coffee fermentation involves a complex microbiological ecology that includes yeasts, bacteria and filamentous fungi (Avallone et al., 2001a; Elhalis et al., 2020a; Evangelista et al., 2015; Silva et al., 2008). Microorganisms are believed to perform at least two crucial functions in the fermentation. The first is the breakdown of the mucilage layer of coffee beans as the mucilage components negatively affect coffee quality (Agate & Bhat, 1966; Masoud &

Jespersen, 2006). The mucilage of coffee beans consists mainly of proteins (14.2%), sugars (50%), cellulose (9.1%) and pectic substances (32.2%) (Avallone et al., 2001a). Mucilage degradation during wet fermentation is closely related to the capacity of the microbes to produce extracellular mucilage degrading enzymes such as pectinase, protease and cellulase (Haile & Kang, 2019; Pereira et al., 2014; Silva et al., 2013). The second function is the production of microbial metabolites which can migrate into the interior of the bean and contribute to the taste and aroma characteristics of coffee products. Microbial metabolites such as higher alcohols, esters, aldehydes, ketones and organic acids have been identified in coffee beans and are believed to be important to the sensory quality of coffee beverages (Evangelista et al., 2015; Pereira et al., 2015; Pereira et al., 2014). Yeasts are a major group of microorganisms in the microbial ecology of wet coffee fermentation and are believed to play a critical role in both mucilage degradation and production of desirable metabolites (Evangelista et al., 2014a; Martins et al., 2019; Pereira et al., 2015). The most frequently isolated yeasts during the fermentation were *Saccharomyces cervices*, *Pichia kluyveri*, *P. anomala*, *Hanseniaspora uvarum*, and *Debaryomyces hansenii delbrueckii* (Masoud et al., 2004; Silva et al., 2008; Vilela et al., 2010). In previous studies we have investigated the microbial ecology of wet coffee fermentation under Australian conditions and demonstrated the crucial roles of yeasts in the process, including their contributions to coffee taste and aroma (Elhalis et al., 2020a; Elhalis et al., 2020b). These findings confirmed similar conclusions reported for coffee fermentation conducted in other regions of the world (Avallone et al., 2001b; Masoud et al., 2004; Pereira et al., 2014). Several studies have used yeasts isolated from spontaneous fermentations of coffee beans to conduct inoculated fermentations (Evangelista et al., 2014a; Martins et al., 2019; Pereira et al., 2015; Ribeiro et al., 2017). However, there is generally a lack of systematic examination of the

79 microbiological and biochemical properties of yeasts for their suitability as potential starter
80 cultures for wet coffee fermentation. In this study, we systematically evaluated six yeast species,
81 isolated from natural wet coffee fermentation, for their tolerance to fermentation stresses and
82 capacity to produce mucilage degrading enzymes and desirable non-volatile and volatile
83 metabolites. The objective of the study was to find the yeast species with optimum
84 microbiological and biochemical characteristics that can be developed into starter cultures for
85 wet coffee fermentation.
86

87 **2. Materials and methods**

88 This study was divided into three steps, to evaluate yeasts growth under coffee fermentation
89 related stresses, to conduct basic plate enzyme assays and to identify the metabolite profiles
90 produced during synthetic coffee pulp extract medium fermentations.

91 **2.1. Yeast cultivation**

92 Yeasts were isolated from the spontaneous wet fermentation of coffee beans by plating the
93 fermenting mass on yeast extract agar (YEA) and identified by genetic sequencing. Details of
94 yeast isolation and identification were described in Elhalis et al. (2020a). Inocula of
95 *Hanseniaspora uvarum* (accession No. MF574306.1), *Pichia kudriavzevii* (accession No.
96 CP021092.1), *P. fermentans* (accession No. KM402059.1), *Candida railenensis* (accession No.
97 HQ438305.1), *C. xylopsoci* (accession No. KJ706861.1) and *Wickerhamomyces anomalus*
98 (accession No. KY587120.1) were prepared individually by selecting one pure colony for each
99 yeast from YEA and transferring it aseptically into 50 ml YE broth, which was incubated at 30
100 °C for 24 h in a shaking water bath. The broth was transferred to 100 ml YE broth and grown for
101 another 24 h under the same incubation conditions. Yeast cells were harvested by centrifugation
102 (10 min at 10, 000 g) and washed twice with sterile physiological saline.

2.2. Evaluation of yeasts growth under stress factors

Yeasts were evaluated for their capability to grow under individual stress conditions following the method described by Pereira et al. (2014) with some modifications. The harvested yeast cells were re-suspended and diluted in physiological saline to a cell concentration of 10^4 CFU /ml, determined by using a Neubauer chamber, and confirmed by plate counting on YEA. Aliquots (5 µl) of the cell suspensions of each strain was spot inoculated on the surface of the stress test agar plates, and after the required incubation time, the plates were visually checked to see if colonies appeared or not. Appearance of colonies indicated positive growth and if no colonies developed, this was considered as negative growth. All the test plates had a basal medium consisting of 5% glucose, 0.3% peptone, 0.05% beef extract and 2% agar. For the sugar stress plates, the sugar concentration of the medium was adjusted by adding glucose and fructose to the basal medium to 15, 30, and 50% (w/w) hexose equivalent. Acid stress was imposed by adjusting the pH of the basal medium to 6.0, 4.0 and 2.0 with 1 M H_2SO_4. Tolerance to acetic acid was tested by adding acetic acid to the basal medium to a final concentration of 1.0, 2.0, and 3.0%, while ethanol tolerance was performed by adjusting the ethanol concentration of the basal medium to 2.0, 4.0, 8.0, and 10.0%. These plates were incubated at 30 °C for 36 h. Thermotolerance test was performed by incubating the inoculated plates of basal medium at 25, 30, 35 and 40 °C for 36 h. Furthermore, yeasts were assessed under combined stresses of temperature, sugar, ethanol, pH and acetic acid. A control was included where the basal medium plates were inoculated with the yeasts and incubated at 30 °C for 36 h. All operations were done aseptically. All tests were conducted in triplicate, and the results were expressed as average with standard deviations of less than 0.05.

2.3. Enzymatic assays

126 The yeast isolates were screened for pectinase, protease, lipase, amylase and cellulase activities
127 using plate assays as follow.

128 **2.3.1. Assay of pectinolytic activity**

129 Pectinolytic activity of the yeast isolates was assayed according to the method of the
130 Compendium of Methods for Microbiological Examination of Foods (Speck, 1984). Pectin was
131 added to the basal medium (0.5 %, w/v), as described above, and the pH of the medium was
132 adjusted to 5.0. All the plates were surface spotted with 10 µl of 10^4 CFU/ml of each isolate and
133 incubated at 30 °C for 48 h. After incubation, the plates were flooded with 50 mM potassium
134 iodide-iodine solution and the hydrolysis zone surrounding the colonies, which appeared as a
135 clear halo around the colonies, was measured and used as an indication of the yeast's pectinase
136 activity.

137 **2.3.2. Assay of cellulolytic activity**

138 Cellulolytic activities of the yeast isolates were assayed following the method in the
139 Compendium of Methods for Microbiology Examination of Foods (Speck, 1984).
140 Carboxymethylcellulose was added to the basal medium at a concentration of 0.2% and the
141 medium was adjusted to pH 7.0. The plates were surface spot inoculated and incubated as
142 described above. After incubation, the plates were flooded with 7 N hydrochloric acid, and the
143 clear zone surrounding the colonies measured which indicated the yeast's cellulolytic activity.

144 **2.3.3. Proteolytic activity**

145 Proteolytic activity of the yeast isolates was determined according to the method described in
146 Harrigan and McCance (2014). Skim milk was added to nutrient agar (1%, pH 5). The plates
147 were surface spot inoculated and incubated as described above, and the enzyme activity was
148 determined by measuring the clear zone around the colonies.

149 **2.3.4. Amylase activity**

150 Amylolytic activity of the yeast isolates was determined according to the method described in
151 Harrigan and McCance (2014) with some modifications. Starch was added to nutrient agar (1%,
152 pH 5), and the plates were surface inoculated and incubated as described above. Amylase activity
153 was determined by measuring the clear zone surrounding the colonies after flooding with 50 mM
154 potassium iodide-iodine solution.

155 **2.4. Assessment of yeast metabolism on artificial coffee pulp medium**

156 Yeast isolates were evaluated for their capability to produce non-volatile and volatile metabolites
157 in synthetic coffee pulp extract medium (SCM) following the method of Pereira et al. (2014)
158 with slight modifications. The SCM was made by adding pectin (2 g/l), fructose (10 g/l) and
159 glucose (10 g/l) to coffee cheery pulp extract, pH 4.7. The coffee pulp extract was prepared by
160 boiling 400 g of coffee cherry pulp in 1 L Milli-Q water for 1 h, which was then filtrated through
161 glass wool. The SCM was autoclaved at 121 °C for 15 min, and after cooling to ambient
162 temperature, inoculated with yeast inoculums at 10^4 cells/ml. The inoculated media were allowed
163 to ferment at 25 °C for 36 h. Samples (20 ml) were taken every 12 h for microbiological and
164 chemical analyses. Yeasts in the fermenting mass were enumerated by spread plating on YEA
165 and the pH of the fermenting mass was measured using a portable pH meter (pH Cube, TPS Pty
166 Ltd, Brisbane, QLD, Australia). The analysis of each sample was performed in triplicate and the
167 results were expressed as an average.

168 **2.4.1. Chemical analyses**

169 The samples taken during fermentation were centrifuged at 18,920 x *g* for 15 min at 4 °C using
170 an Avanti J-E Centrifuge (Beckman Coulter, Indianapolis, IN, USA), and the supernatant was
171 retained and stored at -20 °C until analysis. Sugars, glycerol and organic acids were determined
172 by a Shimadzu High-Performance Liquid Chromatography (HPLC) system as described in

Elhalis et al. (2020a). The non-volatile metabolite concentrations were calculated by comparison with standard curves constructed from standard solutions of known concentrations of glucose, sucrose, fructose, mannitol, glycerol, citric, lactic, malic, acetic, quinic and succinic acids Volatiles were analyzed using head space solid phase microextraction gas chromatography mass spectrum (HS-SPME/GCMS) as described in Elhalis et al. (2020b). In brief, 4 ml of the samples with 4-methyl-1-pentanol as an internal standard (IS) was placed in 20 ml gas-tight vials. The headspace was extracted using DVB/Carboxen/PDMS solid-phase microextraction (SPME) fibers (50/30 μm, 23-gauge, 2 cm, Sigma-Aldrich, Castle Hill, Australia) for 40 min at 40 °C. The SPME was performed using an autosampler (Shimadzu AOC-5000, Rydalmere, Australia). The fiber was desorbed into an injector in splitless mode for at 250 ℃ for 5 min and analysed by a Shimadzu QP-2010-Plus GC-MS system. Samples were separated on a Sol-Gel Wax column (30 m ×0.25 mm id, 0.25 μm film, SGE, Ringwood, Australia). Linear retention indices (LRI) were calculated using a standard alkane mix (C7-C40, Supleco) and **v**olatiles were identified by comparing linear retention indices with published values in the NIST and PubChem websites. Further confirmation was done by matching their electron impact mass spectra with those of reference compounds in the NIST mass spectral library and, in some cases, using pure reference standards. The concentration of the volatiles was determined semi-quantitatively using the Shimadzu proprietary software "LabSolutions" (Version 2.53).The analysis of each sample was performed in triplicate and the results were expressed as an average.

2.5. Statistical analysis

One-way ANOVA was carried out to compare means between samples and Tukey's HSD post-hoc test was used to separate means of significant differences. Differences were regarded as

195 statistically significant at $P < 0.05$. All statistical analyses were performed with GenStat® (16th
196 Edition, VSN International, Hemel Hempstead, UK),

197 **3. Results**

198 **3.1. Growth of yeasts under different stress conditions**

199 The growth and survival of the different yeast species under individual and combined stressors
200 are given in Table 1. As expected, the viability of the yeast species was affected by the degree of
201 the stresses. All the yeasts grew at pH 4 and 6; however, at pH 2, only some of the isolates of
202 *Hanseniaspora uvarum*, *Pichia kudriavzevii* and *P. fermentans* grew while no isolates of
203 *Candida* or *Wickerhamomyces* species were able to grow. Similarly, under osmotic pressure
204 induced by high concentrations of glucose and fructose, the growth of *C. railenensis*, *C.*
205 *xylopsoci* and *W. anomalus* were suppressed, while *H. uvarum*, *P. kudriavzevii* and *P.*
206 *fermentans* were more resistant. All the isolates, except some of *C. xylopsoci* and *W. anomalus*,
207 tolerated high ethanol stresses. Acetic acid at the concentration of 3% strongly inhibited the
208 growth of *P. fermentans*, *C. xylopsoci* and *W. anomalus*, while *H. uvarum*, *P. kudriavzevii* and *P.*
209 *fermentans* were tolerant. Growth was observed with all the yeasts at the temperature range 25-
210 35 °C but their growth was inhabited at 40 °C, except *H. uvarum* and *P. kudriavzevii* which
211 continued to grow at this temperature. Regarding their tolerance to combined stresses, the
212 highest tolerance was observed with *H. uvarum* and *P. kudriavzevii*, followed by *P. fermentans*
213 and *C. railenensis*, while *C. xylopsoci* and *W. anomalus* showed the lowest tolerance as no
214 growth was detected for these two yeasts under the combined stresses.

215 **3.2. Enzymatic activities of yeasts for degrading macromolecules**

216 The pectinolytic, cellulolytic, proteolytic and amylase activities of the yeast species were
217 investigated using the plating technique and the results are shown in Table 2. The data showed

that *H. uvarum* and *P. kudriavzevii* were the species with the highest pectinolytic activities ($p < 0.05$), *C. xylopsoci*, *W. anomalus* and *C. railenensis* showed low pectinase activities, while all *P. fermentans* isolates had no pectinolytic activities. High levels of protease and cellulase activities were detected with *W. anomalus* ($p < 0.05$), followed by *H. uvarum* and *C. xylopsoci*, while such activities were absent with *P. fermentans* and *C. railenensis*. Amylase activity was also relatively high with *W. anomalus*, *H. uvarum* and *C. xylopsoci* compared to the other yeast species ($p < 0.05$).

3.3. Yeast growth and pH changes during SCM fermentation

Figure 1 shows the changes in pH values and the growth of yeasts during fermentations with SCM. The initial pH value of the fermenting mass was 5.6 which gradually declined with fermentation to pH 3.8 at the end after 36 h. No significant differences in the final pH values were observed among the fermentations with different yeast species. *H. uvarum* showed an initial population of 4.7 log CFU/ml which grew to a maximum population of 11.6 log CFU/ml in 36 h. All the remaining yeast species showed similar growth behavior with approximately 1 log difference in the population.

3.4. Changes in non-volatile metabolites during SCM fermentation

As expected, glucose and fructose were the main reducing sugars found in the SCM medium with an initial concentration of 16.9 and 13.4 g/l, respectively (Table 3). During fermentations with *P. kudriavzevii* and *P. fermentans*, glucose declined to about 3.0 g/l while it was almost totally consumed with the remaining yeasts ($p < 0.05$). Fructose concentration also decreased, but with slower rates leaving high levels of fructose residues in the medium with all the yeast species except *P. kudriavzevii*, in which fructose was totally used up. Glycerol was not detected initially in any of the fermentations, but subsequently identified with a maximum concentration

of about 0.13 g/l without significant differences among the fermentations with different yeasts ($p > 0.05$). Five main organic acids were identified during the fermentations, namely citric, gluconic, pyruvic, succinic and fumaric acids. Citric acid was the most abundant acid detected with an initial concentration of 4.01 g/l which declined significantly to 0.03-0.06 g/l among fermentations with all the yeasts. Gluconic, pyruvic and fumaric were not identified initially in all fermentations but detected in fermentations with *C. railenensis* and *C. xylopsoci* in the concentration range of 0.01- 0.20 g/l at the end. In terms of succinic acid, it was detected at the start of fermentation at 0.06 g/l, which varied after fermentation with different yeasts as its level increased by more than 2-fold with *P. kudriavzevii*, *C. railenensis* and *C. xylopsoci* ($p < 0.05$), declined with *H. uvarum* and *P. fermentans* and was not detected with *W. anomalus* at the end.

3.5. Changes in volatiles during SCM fermentation

A total of 27 volatiles were detected using the HS-SPME-GC-MS method during the fermentations. The most significant compounds were shown in Table 4 and 5 where they were grouped according to their chemical class: alcohols, aldehydes, ketones, esters, phenols and organic acids.

3.5.1. Alcohols

At the start of fermentation, five main alcohols were identified at low concentrations, which were ethanol, isoamyl alcohol, 3-methyl-2-butanol, 1- nonanol and phenylethyl alcohol. After fermentation, a huge increase in the concentration of alcohols, i.e., 700-1200-fold higher than the initial level ($p < 0.05$), was observed. The highest total amount was found in fermentation with *P. fermentans*, followed by *P. kudriavzevii* and *H. uvarum*. Isoamyl alcohol was the most abundant alcohol in all the fermentations with similar concentrations in *P. fermentans*, *P. kudriavzevii* and *C. xylopsoci* fermentations, which were approximately two-fold higher than in

those of other yeasts ($p < 0.05$). The highest level of ethanol was detected in *P. fermentans* and *P. kudriavzevii* fermentations, followed by *C. xylopsoci* and *H. uvarum* fermentations. Furthermore, significant accumulations of 2-phenylethyl alcohol were observed in all the fermentations, where the highest levels were found in fermentations with *P. kudriavzevii* and *P. fermentans*, followed by *C. xylopsoci*, which were about 2-3 times higher than those with the others fermentations ($p < 0.05$). 1-Nonanol was detected in the fermentations of *H. uvarum* and *W. anomalus* with a maximum concentration of 100.4 and 81.4 µg/kg, respectively, while its concentration was low in fermentations with the remaining yeasts ($p < 0.05$).

3.5.2. Aldehydes

Prior to fermentation, low concentrations of aldehydes were detected with a maximum concentration of 93.4 µg/kg, which subsequently increased by more than 20-fold after fermentations with *P. kudriavzevii* and *P. fermentans*, but smaller increases with the other yeasts. Acetaldehyde was the most abundant aldehyde, with the highest concentration detected in fermentations with *P. fermentans* and *P. kudriavzevii* ($p < 0.05$), followed by *Candida* species. Low levels of 3-methylbutanal and benzaldehyde were found in the medium before fermentation at 8.9 and 77.3 µg/kg, respectively. After fermentation, the level of 3-methylbutanal remained relatively unchanged, while the concentration of benzaldehyde declined. Furthermore, 2,2-dimethylhexanal was tentatively identified after fermentation with the highest concentration found with *H. uvarum* ($p < 0.05$), followed by *P. fermentans*, *P. kudriavzevii* and *Wickerhanomyces anomalus*.

3.5.3. Esters

Two main ester compounds were detected initially, ethyl acetate and methyl butanoate, with a concentration of 14.5 and 33.7 µg/kg, respectively. A substantial increase in the level of ethyl

acetate was observed after all fermentations, most notably for *C. xylopsoci* fermentation which produced the highest concentration, ~ 3000-fold higher than the initial amount ($p < 0.05$). The levels of ethyl acetate detected after fermentation with *H. uvarum*, *P. kudriavzevii*, *P. fermentans* and *W. anomalus* were similar ($p > 0.05$), and ~ 2000 times higher than the initial concentration, while a lower concentration was found with *C. railenensis* fermentation. In contrast, methyl butanoate declined to about half of its initial concentration in all the fermentations ($p > 0.05$).

3.5.4. Acids and phenols

The main volatile acid detected initially and after fermentation was acetic acid with an initial concentration of 136.9 µg/kg. After the completion of fermentation, the level of acetic acid increased ~ 7-10 times with higher concentrations in fermentations with *C. railenensis*, *H. uvarum* and *W. anomalus* than the other yeasts ($p < 0.05$). 2-Methoxy-4-vinylphenol (4-vinylguaiacol) was the main phenolic compound identified with an initial concentration of 3.7 µg/kg that increased slightly ($p < 0.05$) during fermentation without significant differences between the yeasts ($p > 0.05$).

3.5.5. Ketones

The main ketone detected at the start of the fermentation was 2-propanone with an average concentration of 7.4 µg/kg. After fermentation, the concentration increased ~ 400 times with *P. fermentans*, *P. kudriavzevii* and *C. xylopsoci*, which were almost 10 times higher than with the remaining yeasts ($p < 0.05$).

4. Discussion

In this study, we assessed six yeast species for their potential as starter cultures for coffee wet fermentation by evaluating their performance in a number of criteria. The yeasts were isolated from spontaneous wet coffee fermentations conducted in our laboratory (Elhalis et al., 2020a).

The mucilage layers of the coffee bean contain about 30% reducing sugars, mainly glucose and fructose (Avallone et al., 2001a; Murthy & Naidu, 2011). During coffee fermentation, the sugars are consumed by microorganisms, producing secondary metabolites such as organic acids, and alcohols, which leads to a decline in pH and rise in temperature (Avallone et al., 2001a; Elhalis et al., 2020a). These stressful environmental conditions can strongly affect the growth and metabolic activities of microflora during coffee fermentation. Thus, selecting yeasts that have the capability to survive and adapt to such environmental stresses is vital for them to become successful starter cultures. Our data showed that acetic acid was the most potent growth inhibitor among the tested stress factors, followed by osmotic pressure. Among the isolates tested, *H. uvarum* and *P. kudriavzevii* were able to tolerate most of the stressful conditions, whereas *Candida* species and *W. anomalus* displayed weak tolerance. *H. uvarum* and *P. kudriavzevii* have been found in relatively high counts during the fermentation of several foods and beverages such as coffee, cocoa, cheese, bread and wine (del Monaco et al., 2014; Fleet, 1993; Pereira et al., 2017b; Pereira et al., 2014; Verheyen et al., 2015; Zheng et al., 2018). Yeast cells were reported to response to these stresses by activating different stress regulatory genes, most of the studies were conducted on *Saccharomyces cerevisiae*. For example, hyperosmotic stress induces high osmolarity glycerol response pathway and accumulate either glycerol or trehalose as a compatible solute (Babazadeh et al., 2017; Hohmann, 2009; Saito & Posas, 2012). High temperature and ethanol induce heat shock proteins (HSP), which facilitate disaggregation of the denatured protein and synthesis of membrane-associated protein capable of protecting the

integrity of liposomal membrane against heat and ethanol, in addition to increases in the rate of ATP, trehalose and NADPH synthesis (Glover & Lindquist, 1998; Pereira et al., 2018; Sales et al., 2000). One of the most adaptive mechanisms of tolerance to acids is by reducing their the cell wall and /or plasm membrane permeability by altering their chemical structure and, thereby reducing their acid diffusion (Guerreiro et al., 2016; Simoes et al., 2006), in addition to the catabolism of the acids by yeast cells in some cases (Casal et al., 1996; Vilela-Moura et al., 2011). Adaptation to low pH may occur by changes in gene expression in membrane conductivity to H^+ and extrusion of protons through changing fatty acid profile of the cell membrane (Brandao et al., 2014; Chen et al., 2009; Eraso & Gancedo, 1987; Kawahata et al., 2006). The high viability of *H. uvarum* and *P. kudriavzevii* and their stress responses in our study might also be regulated by similar mechanisms.

There is a general agreement that during coffee fermentation microbial activities contribute to the breakdown of mucilage components such as polysaccharides (pectin), proteins, cellulose and starch, thus facilitating their removal (Agate & Bhat, 1966; Avallone et al., 2001a; Frank & Cruz, 1964; Masoud & Jespersen, 2006; Pereira et al., 2014; Silva, 2014). Thus, the enzymatic capability of the selected isolates to hydrolyze such components is an important indicator of their suitability as starter cultures as mucilage removal is one of the key functions of coffee wet fermentation. Of the tested isolates, *P. fermentans* and *C. railenensis* showed poor enzymatic activities, especially for protease, cellulase and pectinase. *C. xylopsoci* and *W. anomalus* showed relatively high activities for protease, amylase and cellulose, but poor pectinolytic activity. In contrast, *H. uvarum* and *P. kudriavzevii* displayed good activities on all the enzymes. This agrees with literature reports that *H. uvarum* exhibits activities for several enzymes such as protease, lipase glucosidase and xylosidase (Capece et al., 2005; Charoenchai et al., 1997; Manzanares et

355 al., 1999), while *P. kudriavzevii* has *β*□galactosidase, aminopeptidase, lipase, xylanase and
356 pectinase activities (Amoikon et al., 2019; Elahi & Rehman, 2018; Haile & Kang, 2019;
357 Verheyen et al., 2015; Zheng et al., 2018). Overall, based on these observations, *H. uvarum* and
358 *P. kudriavzevii* have shown a superior potential as starter cultures for coffee fermentation in both
359 their tolerance to environmental stresses and production of mucilage degrading enzymes,
360 compared to the other yeast species tested.

361 The assessment of the selected yeasts to produce desirable metabolites was achieved by
362 conducting inoculated fermentations using SCM, an approach that has been used by other
363 researchers (Hibbing et al., 2010). All the yeast species grew substantially during the
364 fermentation, thus, demonstrating that the medium was able to support their growth.
365 Furthermore, all treatments exhibited reduction in pH values at the end of the fermentation,
366 which was in line with the growth and metabolic activities of the inoculated yeasts. This shifting
367 in pH to a more acidic value was most likely related to sugar fermentation, mainly glucose and
368 fructose, into organic acids. The sugar consumption was accompanied by accumulation of
369 secondary metabolites such as glycerol and organic acids. Glycerol was the most abundant
370 metabolite detected during the fermentations with similar concentrations among all the species.
371 Glycerol was identified during the yeast fermentation of several food and beverage products,
372 including coffee beans, and was characterized by a sweet taste and smooth mouthfeel (Swiegers
373 et al., 2005). Regarding the production of organic acids, *C. railenensis* was the only species that
374 produced fumaric acid, while succinic acid was generated by *P. kudriavzevii*, *C. railenensis* and
375 *C. xylopsoci*. In contrast, citric acid was consumed by all the yeasts during the fermentations.
376 The levels and types of the organic acids present in green coffee beans might affect the sensory
377 quality of the final products. For instance, organic acids interfere with the perceived acidity and

sweetness of coffee beverages (Oestreich-Janzen, 2013; Ribeiro et al., 2017). Furthermore, the level of these acids in the green beans may affect the formation of important volatiles during the roasting of coffee beans such as pyrazines and furans (Liu et al., 2019; Yu & Zhang, 2010). Succinic acid is one of the main end metabolites produced by yeasts in alcoholic fermentations such as wine production (Boulton & Singleton, 1998; Webb et al., 1964). It has a potential impact on the final product acidity and is involved in the formation of ester compounds such as ethyl succinate (Webb et al., 1964). Gluconic, pyruvic and fumaric acids are well known microbial metabolites that may strongly affect the final product quality if present in green coffee beans (Lee et al., 2016a; Yeretzian et al., 2002).

Isoamyl alcohol, 2-phenylethyl alcohol, ethanol, ethyl acetate, acetaldehyde, 2-propanone and acetic acid were the major volatiles identified from the fermenting mass. Overall, these volatiles are known yeast derived metabolites produced during fermentations (Evangelista et al., 2015; Gonzalez-Rios et al., 2007; Sauer, 2016). 2-Phenylethyl alcohol, isoamyl alcohol and ethanol are characterized by their desirable sweet, alcoholic and fruity flavors and impact on the viscosity of coffee beverages (Carballo, 2012; Gamero et al., 2019; Tamang & Fleet, 2009). The presence of esters such as ethyl acetate, even at low concentrations, can impart fruity aromas to the final coffee products (Saerens & Swiegers, 2016). Similarly, acetaldehyde and 2-propanone were related to floral and fruity aromas (Nollet & Toldrá, 2010; Sanz et al., 2002). Low concentrations of acetic acid contribute to a pleasant clean and sweet taste, while high levels (more than 1 mg/ml) give a undesirable vinegary taste (Bertrand et al., 2012). The data showed that *Pichia* species produced relatively high levels of total alcohols and aldehydes while greater amounts of esters were found with *C. xylopsoci*, *Pichia* species and *H. uvarum*. In addition, *H. uvarum* produced relatively high levels of 1-nonanol, ethanol and acetic acid. The total concentrations of

401 such volatiles were higher with *C. railenensis, C. xylopsoci*, *P. fermentans* and *W. anomalus* than
402 with other yeasts; however, their fermentation power was weak as discussed above. It should be
403 mentioned that the concentration of volatile compounds was obtained by the internal standard
404 method in the present study. As different types of volatile compounds may have different
405 response factors to that of the internal standard in the MS detector, caution should be taken in
406 interpreting data on the absolute concentrations of the volatiles although comparison of the data
407 between different treatments should still be valid. Previous reports indicated that *H. uvarum* was
408 one of the dominate yeasts in several food and beverage products, and produced ethanol (Caridi
409 & Ramondino, 1999), primary metabolites such as acetaldehyde, glycerol (Ciani & Maccarelli,
410 1997; Romano et al., 1992), and secondary metabolites such as ethyl acetate (Romano et al.,
411 1997). Similarly, *P. kudriavzevii* was reported to improve the sensory quality of fermented foods
412 and beverages by increasing the level of volatile compounds such as higher alcohols and esters
413 (del Monaco et al., 2014; Pereira et al., 2017a; Shi et al., 2019; Zheng et al., 2018). These
414 findings agree with our observations. Pereira et al. (2014) tested nine endogenous yeast strains to
415 be used as starter cultures in coffee fermentation. *Saccharomyces sp*. was found to be the highest
416 pectinase producing strain, while high concentrations of flavour compounds, such as isoamyl
417 acetate, ethyl acetate, were detected with *P. fermentans*, which lead the authors to recommend
418 using co-culture of both strains in the inoculation. However, when the mixed culture was
419 inoculated into the synthetic coffee extract medium, the growth rate of *P. fermentans* was
420 reduced. The current study showed that *H. uvarum* and *P kudriavzevii* exhibited high production
421 features of both pectinases and aromatic metabolites in single strain inoculation, which would
422 make them more promising candidates for inoculated coffee fermentation.

423

5. Conclusion

This study investigated six yeast species isolated from spontaneous coffee fermentations for their potential as stater cultures for wet fermentations of coffee beans. *H. uvarum* and *P. kudriavzevii* were found to be the most promising candidates as they were tolerant to temperature, pH and acid stresses, exhibited high pectinase, protease, lipase and amylase activities that will enable them to facilitate mucilage degradation of the beans. They also produced a high mount of desirable primary and secondary metabolites such as organic acids, glycerol, alcohols, esters and aldehydes which are important to the sensory quality of coffee, during inoculated fermentation using coffee pulp medium. Further research should evaluate the suitability of these two yeasts as starter cultures in inoculated coffee fermentation and their impact on the sensory characteristics of coffee beverages.

Conflict of Interest

HE declares that he has no conflict of interest. JC declares that he has no conflict of interest. DF declares that he has no conflict of interest. JZ declares that he has no conflict of interest.

Compliance with ethics requirements

"This article does not contain any studies with human participants or animals performed by any of the authors."

Authors' contribution statement

HE designed and conducted experiments, performed the data analysis and wrote the manuscript. JZ and DF supervised the experiments, interpreted the scientific values of the obtained data and edited the manuscript. JC supervised work. All authors read and approved the manuscript.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgement

Not applicable.

References

Agate, A. D., & Bhat, J. V. (1966). Role of pectinolytic yeasts in the degradation of mucilage layer of Coffea robusta cherries. *Applied and Environmental Microbiology, 14*(2), 256-260.

Amoikon, T. L. S., Aké, M. D. F., Djéni, N. T., Grondin, C., Casaregola, S., & Djè, K. M. (2019). Diversity and enzymatic profiles of indigenous yeasts isolated from three types of palm wines produced in Côte d'Ivoire. *Journal of Applied Microbiology, 126*(2), 567-579.

Amorim, H. V., & Amorim, V. L. (1977). Coffee enzymes and coffee quality. In R. L. O. A. J. StAngelo (Ed.), *Enzymes in food and beverages processing* (Vol. 47, pp. 27-56). American Chemical Society. https://doi.org/10.1021/bk-1977-0047.ch003

Avallone, S., Guiraud, J. P., Guyot, B., Olguin, E., & Brillouet, J. M. (2001a). Fate of mucilage cell wall polysaccharides during coffee fermentation. *Journal of Agricultural and Food Chemistry, 49*(11), 5556-5559.

Avallone, S., Guyot, B., Brillouet, J.-M., Olguin, E., & Guiraud, J.-P. (2001b). Microbiological and biochemical study of coffee fermentation. *Current Microbiology, 42*(4), 252-256.

Babazadeh, R., Lahtvee, P.-J., Adiels, C. B., Goksör, M., Nielsen, J. B., & Hohmann, S. (2017). The yeast osmostress response is carbon source dependent. *Scientific reports, 7*(1), 1-11.

Bertrand, B., Boulanger, R., Dussert, S., Ribeyre, F., Berthiot, L., Descroix, F., & Joët, T. (2012). Climatic factors directly impact the volatile organic compound fingerprint in green Arabica coffee bean as well as coffee beverage quality. *Food Chemistry, 135*(4), 2575-2583.

Boulton, R. B., & Singleton, V. L. (1998). The role of sulfur dioxide in wine. In *Principles and Practices of Winemaking* (pp. 448-473). Springer US.

Brandao, R. L., Rosa, J. C. C., Nicoli, J. R., Almeida, M. V. S., do Carmo, A. P., Queiros, H. T., & Castro, I. M. (2014). Investigating acid stress response in different *Saccharomyces* strains. *Journal of Mycology, 2014*.

Capece, A., Fiore, C., Maraz, A., & Romano, P. (2005). Molecular and technological approaches to evaluate strain biodiversity in *Hanseniaspora uvarum* of wine origin. *Journal of Applied Microbiology, 98*(1), 136-144.

Carballo, J. (2012). The role of fermentation reactions in the generation of flavor and aroma of foods. In A. K.-E. Bhavbhuti M. Mehta, Robert Z. Iwanski (Ed.), *Fermentation: Effects on food properties* (pp. 51-83). CRC Press Boca Raton, Florida.

Caridi, A., & Ramondino, D. (1999). Biodiversità fenotipica in ceppi di *Hanseniaspora* di origine enologica. *L'Enotecnico, 35*, 71-76. http://hdl.handle.net/20.500.12318/53991

Casal, M., Cardoso, H., & Leao, C. (1996). Mechanisms regulating the transport of acetic acid in *Saccharomyces cerevisiae*. *Microbiology, 142*(6), 1385-1390.

Charoenchai, C., Fleet, G. H., Henschke, P. A., & Todd, B. (1997). Screening of non‐*Saccharomyces* wine yeasts for the presence of extracellular hydrolytic enzymes. *Australian Journal of Grape and Wine Research, 3*(1), 2-8.

Chen, A. K.-L., Gelling, C., Rogers, P. L., Dawes, I. W., & Rosche, B. (2009). Response of *Saccharomyces cerevisiae* to stress-free acidification. *The Journal of Microbiology, 47*(1), 1-8.

Ciani, M., & Maccarelli, F. (1997). Oenological properties of non-*Saccharomyces* yeasts associated with wine-making. *World Journal of Microbiology and Biotechnology, 14*(2), 199-203.

Cleves, S. (2009). Ecological processing of coffee and use of byproducts. In Wintgens J. N. (Ed.), *Coffee: growing, processing, sustainable production. A guidebook for growers, processors, traders and researchers* (pp. 724-738). Wiley-VCH.

del Monaco, S. M., Barda, N. B., Rubio, N. C., & Caballero, A. C. (2014). Selection and characterization of a Patagonian *Pichia kudriavzevii* for wine deacidification. *Journal of Applied Microbiology, 117*(2), 451-464.

Elahi, A., & Rehman, A. (2018). Bioconversion of hemicellulosic materials into ethanol by yeast, *Pichia kudriavzevii* 2-KLP1, isolated from industrial waste. *Revista Argentina de microbiologia, 50*(4), 417-425.

Elhalis, H., Cox, J., & Zhao, J. (2020a). Ecological diversity, evolution and metabolism of microbial communities in the wet fermentation of Australian coffee beans. *International Journal of Food Microbiology, 321*, 108544.

Elhalis, H., frank, D., Cox, J., & Zhao, J. (2020b). The crucial role of yeasts in the wet fermentation of coffee beans and quality. *International Journal of Food Microbiology.* https://doi.org/10.1016/j.ijfoodmicro.2020.108796

Eraso, P., & Gancedo, C. (1987). Activation of yeast plasma membrane ATPase by acid pH during growth. *FEBS Letters, 224*(1), 187-192.

Evangelista, S. R., Miguel, M. G. d. C. P., de Souza Cordeiro, C., Silva, C. F., Pinheiro, A. C. M., & Schwan, R. F. (2014a). Inoculation of starter cultures in a semi-dry coffee (Coffea arabica) fermentation process. *Food Microbiology, 44*, 87-95.

Evangelista, S. R., Miguel, M. G. d. C. P., Silva, C. F., Pinheiro, A. C. M., & Schwan, R. F. (2015). Microbiological diversity associated with the spontaneous wet method of coffee fermentation. *International Journal of Food Microbiology, 210*, 102-112.

Fleet, G. M. (1993). Yeasts-growth during fermentation. In *Wine microbiology and biotechnology* (pp. 27-54). Harword Academic Publishers.

Frank, H. A., & Cruz, A. S. D. (1964). Role of incidental microflora in natural decomposition of mucilage layer in Kona coffee cherries. *Journal of Food Science, 29*(6), 850-853.

552 Gamero, A., Ren, X., Lamboni, Y., de Jong, C., Smid, E. J., & Linnemann, A. R. (2019).
553 Development of a low-alcoholic fermented beverage employing cashew apple juice and
554 non-conventional yeasts. *Fermentation, 5*(3), 71.

555

556 Glover, J. R., & Lindquist, S. (1998). Hsp104, Hsp70, and Hsp40: a novel chaperone system that
557 rescues previously aggregated proteins. *Cell, 94*(1), 73-82.

558

559 Gonzalez-Rios, O., Suarez-Quiroz, M. L., Boulanger, R., Barel, M., Guyot, B., Guiraud, J.-P., &
560 Schorr-Galindo, S. (2007). Impact of "ecological" post-harvest processing on the volatile
561 fraction of coffee beans: I. Green coffee. *The Journal of Food Composition and Analysis,*
562 *20*(3-4), 289-296.

563

564 Goto, Y. B., & Fukunaga, E. T. (1986). *Coffee: harvesting and processing for top quality coffee.*
565 *A cup of Aloha: The Kona coffee epic. University of Hawaii Press.* Hawaii Agricultural
566 Experiment Station, University of Hawaii.

567

568 Guerreiro, J. F., Muir, A., Ramachandran, S., Thorner, J., & Sá-Correia, I. (2016). Sphingolipid
569 biosynthesis upregulation by TOR complex 2–Ypk1 signaling during yeast adaptive
570 response to acetic acid stress. *Biochemical Journal, 473*(23), 4311-4325.

571

572 Haile, M., & Kang, W. H. (2019). Isolation, identification, and characterization of pectinolytic
573 yeasts for starter culture in coffee fermentation. *MicroorganismS, 7*(10), 401.

Harrigan, W. F., & McCance, M. E. (2014). *Laboratory methods in microbiology*. Academic press.

Hibbing, M. E., Fuqua, C., Parsek, M. R., & Peterson, S. B. (2010). Bacterial competition: surviving and thriving in the microbial jungle. *Nature Reviews Microbiology, 8*(1), 15-25.

Hohmann, S. (2009). Control of high osmolarity signalling in the yeast *Saccharomyces cerevisiae*. *FEBS Letters, 583*(24), 4025-4029.

International Coffee Organization. (2018). *Total production by all exporting countries (In thousand 60 kg bags)*. Retrieved 23 December 2019 from http://www.ico.org/prices/po-production.pdf

Kawahata, M., Masaki, K., Fujii, T., & Iefuji, H. (2006). Yeast genes involved in response to lactic acid and acetic acid: acidic conditions caused by the organic acids in *Saccharomyces cerevisiae* cultures induce expression of intracellular metal metabolism genes regulated by Aft1p. *FEMS Yeast Research, 6*(6), 924-936.

Lee, L. W., Cheong, M. W., Curran, P., Yu, B., & Liu, S. Q. (2016a). Modulation of coffee aroma via the fermentation of green coffee beans with *Rhizopus oligosporus*: I. Green coffee. *Food Chemistry, 211*, 916-924.

Liu, C., Yang, Q., Linforth, R., Fisk, I. D., & Yang, N. (2019). Modifying Robusta coffee aroma by green bean chemical pre-treatment. *Food Chemistry, 272*, 251-257.

Manzanares, P., Ramón, D., & Querol, A. (1999). Screening of non-*Saccharomyces* wine yeasts for the production of β-D-xylosidase activity. *International Journal of Food Microbiology, 46*(2), 105-112.

Martins, P. M. M., Ribeiro, L. S., Miguel, M. G. d. C. P., Evangelista, S. R., & Schwan, R. F. (2019). Production of coffee (Coffea arabica) inoculated with yeasts: impact on quality. *Journal of the Science of Food and Agriculture, 99*(13), 5638-5645.

Masoud, W., Bjørg Cesar, L., Jespersen, L., & Jakobsen, M. (2004). Yeast involved in fermentation of Coffea arabica in East Africa determined by genotyping and by direct denaturating gradient gel electrophoresis. *Yeast, 21*(7), 549-556.

Masoud, W., & Jespersen, L. (2006). Pectin degrading enzymes in yeasts involved in fermentation of Coffea arabica in East Africa. *International Journal of Food Microbiology, 110*(3), 291-296.

Murthy, P. S., & Naidu, M. M. (2011). Improvement of robusta coffee fermentation with microbial enzymes. *European Journal of Applied Sciences, 3*(4), 130-139.

Nollet, L. M. L., & Toldrá, F. (2010). *Sensory analysis of foods of animal origin*. CRC press. 383-398.

Oestreich-Janzen, S. (2013). Chemistry of coffee. In *Reference module in chemistry, molecular sciences and chemical engineering* (pp. 1-28). Elsevier. https://doi.org/https://doi.org/10.1016/B978-0-12-409547-2.02786-4

Pereira, G. V. d. M., Alvarez, J. P., Neto, D. P. d. C., Soccol, V. T., Tanobe, V. O. A., Rogez, H., Góes-Neto, A., & Soccol, C. R. (2017a). Great intraspecies diversity of *Pichia kudriavzevii* in cocoa fermentation highlights the importance of yeast strain selection for flavor modulation of cocoa beans. *LWT-Food Science and Technology*, *84*, 290-297.

Pereira, G. V. d. M., Neto, E., Soccol, V. T., Medeiros, A. B. P., Woiciechowski, A. L., & Soccol, C. R. (2015). Conducting starter culture-controlled fermentations of coffee beans during on-farm wet processing: Growth, metabolic analyses and sensorial effects. *Food Research International*, *75*, 348-356.

Pereira, G. V. d. M., Soccol, V. T., Brar, S. K., Neto, E., & Soccol, C. R. (2017b). Microbial ecology and starter culture technology in coffee processing. *Critical Reviews in Food Science and Nutrition*, *57*(13), 2775-2788.

640 Pereira, G. V. d. M., Soccol, V. T., Pandey, A., Medeiros, A. B. P., Lara, J. M. R. A., Gollo, A.
641 L., & Soccol, C. R. (2014). Isolation, selection and evaluation of yeasts for use in
642 fermentation of coffee beans by the wet process. *International Journal of Food*
643 *Microbiology, 188*, 60-66.

644

645 Pereira, T., Vilaprinyo, E., Belli, G., Herrero, E., Salvado, B., Sorribas, A., Altés, G., & Alves,
646 R. (2018). Quantitative operating principles of yeast metabolism during adaptation to
647 heat stress. *Cell reports, 22*(9), 2421-2430.

648

649 Ribeiro, L. S., Miguel, M. G. d. C. P., Evangelista, S. R., Martins, P. M. M., van Mullem, J.,
650 Belizario, M. H., & Schwan, R. F. (2017). Behavior of yeast inoculated during semi-dry
651 coffee fermentation and the effect on chemical and sensorial properties of the final
652 beverage. *Food Research International, 92*, 26-32.

653

654 Romano, P., Suzzi, G., Comi, G., & Zironi, R. (1992). Higher alcohol and acetic acid production
655 by apiculate wine yeasts. *Journal of Applied Bacteriology, 73*(2), 126-130.

656

657 Romano, P., Suzzi, G., Comi, G., Zironi, R., & Maifreni, M. (1997). Glycerol and other
658 fermentation products of apiculate wine yeasts. *Journal of Applied Microbiology, 82*(5),
659 615-618.

660

661 Saerens, S., & Swiegers, J. H. (2016). Production of low-alcohol or alcohol-free beer with *Pichia*
662 *kluyveri* yeast strains. ." U.S. Patent No. 10,415,007. 17 Sep. 2019.

663

664 Saito, H., & Posas, F. (2012). Response to hyperosmotic stress. *Genetics, 192*(2), 289-318.

665

666 Sales, K., Brandt, W., Rumbak, E., & Lindsey, G. (2000). The LEA-like protein HSP 12 in
667 *Saccharomyces cerevisiae* has a plasma membrane location and protects membranes
668 against desiccation and ethanol-induced stress. *Biochimica et Biophysica Acta (BBA)-*
669 *Biomembranes, 1463*(2), 267-278.

670

671 Sanz, C., Maeztu, L., Zapelena, M. J., Bello, J., & Cid, C. (2002). Profiles of volatile compounds
672 and sensory analysis of three blends of coffee: influence of different proportions of
673 Arabica and Robusta and influence of roasting coffee with sugar. *Journal of the Science*
674 *of Food and Agriculture, 82*(8), 840-847.

675

676 Sauer, M. (2016). Industrial production of acetone and butanol by fermentation—100 years later.
677 *FEMS Microbiology Letters, 363*(13), fnw134.

678

679 Schwan, R. F., & Wheals, A. E. (2004). The microbiology of cocoa fermentation and its role in
680 chocolate quality. *Critical Reviews in Food Science and Nutrition, 44*(4), 205-221.

681

682 Selvamurugan, M., Doraisamy, P., & Maheswari, M. (2010). An integrated treatment system for
683 coffee processing wastewater using anaerobic and aerobic process. *Ecological*
684 *Engineering*, *36*(12), 1686-1690.

685

686 Shi, W.-K., Wang, J., Chen, F.-S., & Zhang, X.-Y. (2019). Effect of *Issatchenkia terricola* and
687 *Pichia kudriavzevii* on wine flavor and quality through simultaneous and sequential co-
688 fermentation with *Saccharomyces cerevisiae*. *LWT-Food Science and Technology*, *116*,
689 108477.

690

691 Silva, C. (2014). Microbial activity during coffee fermentation. In *Cocoa and Coffee*
692 *Fermentations* (pp. 368-423). CRC Press Boca Raton, FL, USA.

693

694 Silva, C., Batista, L. R., Abreu, L. M., Dias, E. S., & Schwan, R. F. (2008). Succession of
695 bacterial and fungal communities during natural coffee (Coffea arabica) fermentation.
696 *Food Microbiology*, *25*(8), 951-957.

697

698 Silva, C., Vilela, D. M., de Souza Cordeiro, C., Duarte, W. F., Dias, D. R., & Schwan, R. F.
699 (2013). Evaluation of a potential starter culture for enhance quality of coffee
700 fermentation. *World Journal of Microbiology and Biotechnology*, *29*(2), 235-247.

701

702 Simoes, T., Mira, N. P., Fernandes, A. R., & Sá-Correia, I. (2006). The SPI1 gene, encoding a
703 glycosylphosphatidylinositol-anchored cell wall protein, plays a prominent role in the

development of yeast resistance to lipophilic weak-acid food preservatives. *Applied and Environmental Microbiology, 72*(11), 7168-7175.

Speck, M. L. (1984). *Compendium of methods for the microbiological examination of foods*. American Public Health Association.

Swiegers, J. H., Bartowsky, E. J., Henschke, P. A., & Pretorius, I. S. (2005). Yeast and bacterial modulation of wine aroma and flavour. *Australian Journal of Grape and Wine Research, 11*(2), 139-173.

Tamang, J. P., & Fleet, G. H. (2009). Yeasts diversity in fermented foods and beverages. In *Yeast Biotechnology: Diversity and Applications* (pp. 169-198). Springer.

Verheyen, C., Albrecht, A., Elgeti, D., Jekle, M., & Becker, T. (2015). Impact of gas formation kinetics on dough development and bread quality. *Food Research International, 76*, 860-866.

Vilela-Moura, A., Schuller, D., Mendes-Faia, A., Silva, R. D., Chaves, S. R., Sousa, M. J., & Côrte-Real, M. (2011). The impact of acetate metabolism on yeast fermentative performance and wine quality: reduction of volatile acidity of grape musts and wines. *Applied Microbiology and Biotechnology, 89*(2), 271-280.

726 Vilela, D. M., Pereira, G. V. d. M., Silva, C. F., Batista, L. R., & Schwan, R. F. (2010).
727 Molecular ecology and polyphasic characterization of the microbiota associated with
728 semi-dry processed coffee (Coffea arabica L.). *Food Microbiology, 27*(8), 1128-1135.

729

730 Webb, A. D., Kepner, R. E., & Galetto, W. G. (1964). Comparison of the aromas of flor sherry,
731 baked sherry, and submerged-culture sherry. *American Journal of Enology and*
732 *Viticulture, 15*(1), 1-10.

733

734 Yeretzian, C., Jordan, A., Badoud, R., & Lindinger, W. (2002). From the green bean to the cup
735 of coffee: investigating coffee roasting by on-line monitoring of volatiles. *European*
736 *Food Research and Technology, 214*(2), 92-104.

737

738 Yu, A.-N., & Zhang, A.-D. (2010). The effect of pH on the formation of aroma compounds
739 produced by heating a model system containing L-ascorbic acid with L-threonine/L-
740 serine. *Food Chemistry, 119*(1), 214-219.

741

742 Zheng, X., Li, K., Shi, X., Ni, Y., Li, B., & Zhuge, B. (2018). Potential characterization of yeasts
743 isolated from Kazak artisanal cheese to produce flavoring compounds.
744 *MicrobiologyOpen, 7*(1), e00533.

745

746 **Figure caption**

747 **Figure 1.** Changes in pH values (a), and yeast species growth rates (b) during SCM
748 fermentations. *Hanseniaspora uvarum* (), *Pichia kudriavzevii* (), *P. fermentans* (),
749 *Candida railenensis* (), *C. xylopsoci* (), *Wickerhamomyces anomalus* (). Data are
750 the means of triplicate analyses with ± 0.03 for pH values and ± 0.05 CFU/ml for yeat
751 populations.

752

Table 1 Growth and survival of yeast isolates under individual and combined fermentation stress conditions

Growth at	Y1*	Y2	Y3	Y4	Y5	Y6
pH 6	+	+	+	+	+	+
pH 4	+	+	+	+	+	+
pH 2	1/5#	2/5	1/5	-	-	-
Ethanol (10%)	+	+	+	+	3/5	2/4
Ethanol (8%)	+	+	+	+	+	+
Ethanol (4%)	+	+	+	+	+	+
Ethanol (2%)	+	+	+	+	+	+
Glucose (50%)	2/5	2/5	1/5	-	-	-
Glucose (25%)	+	+	+	+	+	+
Glucose (15%)	+	+	+	+	+	+
Fructose (50%)	3/5	2/5	2/5	-	-	-
Fructose (25%)	+	+	+	+	+	+
Fructose (15%)	+	+	+	+	+	+
40 °C	+	+	1/5	-	-	-
35 °C	+	+	+	+	+	3/5
30 °C	+	+	+	+	+	+
25 °C	+	+	+	+	+	+
Acetic acid (3%)	3/5	1/5	-	4/5	-	-
Acetic acid (2%)	+	3/5	1/5	+	1/5	2/5
Acetic acid (1%)	+	+	+	+	+	-
Combined stresses	+	+	1/5	1/5	-	-

*Y1 *Hanseniaspora uvarum*; Y2 *Pichia kudriavzevii*; Y3 *P. fermentans*; Y4 *Candida railensis*; Y5 *C. xylopsoci*; Y6 *Wickerhamomyces anomalus*.

+, all isolates showed positive growth; −, all isolates showed negative growth; #, figures indicate number of isolates that showed positive growth /number of isolates tested, Combined stresses: ethanol 8%, pH 4, acetic acid 2%, glucose/fructose 25%, 37 °C. Values are means of triplicate.

Table 2 Production of pectinase, cellulase, amylase and protease by yeast species on plate assays

Enzymes	Y1	Y2	Y3	Y4	Y5	Y6
Protease	**++ (5)**	**+ (4)**	**-**	**-**	**+++ (4)**	**++++ (5)**
Cellulase	**+++ (5)**	**+ (4)**	**-**	**-**	**+ (2)**	**+++ (5)**
Amylase	**+++ (5)**	**++ (4)**	**++ (4)**	**+ (5)**	**+++ (5)**	**++++ (5)**
Pectinase	**++++(5)**	**++++ (5)**	**-**	**+ (5)**	**++ (2)**	**+ (1)**

++++ high enzyme activity (hydrolysis zone >5 mm); +++ good enzyme activity (hydrolysis zone 3-5 mm); ++ average enzyme activity (hydrolysis zone 2-3 mm); + low enzyme activity (no or 1-2 mm hydrolysis zone); − no enzyme activity (no hydrolysis halos). Data in parentheses indicate the number of isolates giving reaction. Abbreviations; Y1 *Hanseniaspora uvarum*; Y2 *Pichia kudriavzevii*; Y3 *P. fermentans*; Y4 *Candida railensis*; Y5 *C. xylopsoci*; Y6 *Wickerhamomyces anomalus*.

Table 3 Changes in the concentrations of non-volatile metabolites during fermentation of different yeasts with synthetic coffee pulp extraction medium (SCM).

Comp. (g/l)	Initial	Y1*	Y2	Y3	Y4	Y5	Y6
Glucose	16.90^{a}	ND^{d}	3.52^{b}	3.10^{b}	ND^{d}	0.06^{c}	ND^{d}
Fructose	13.40^{a}	4.71^{c}	ND^{e}	2.30^{d}	7.12^{b}	4.56^{c}	6.23^{b}
Citric	4.01^{a}	0.03^{b}	0.05^{b}	0.03^{b}	0.06^{b}	0.03^{b}	0.06^{b}
Gluconic acid	ND^{b}	ND^{b}	0.13^{a}	ND^{b}	0.17^{a}	0.17^{a}	ND^{b}
Pyruvic acid	ND^{b}	ND^{b}	0.01^{a}	ND^{b}	0.02^{a}	0.02^{a}	0.03^{a}
Succinic acid	0.06^{b}	0.04^{b}	0.16^{a}	0.03^{b}	0.18^{a}	0.16^{a}	ND^{c}
Fumaric acid	ND^{b}	ND^{b}	ND^{b}	ND^{b}	0.01^{a}	ND^{b}	ND^{b}
Glycerol	ND^{b}	0.13^{a}	0.14^{a}	0.12^{a}	0.13^{a}	0.15^{a}	0.15^{a}

Values are means of three replicates. Data in each row with different lower-case letters (a–e) indicate significant differences among the yeast species ($\boldsymbol{p}$<0.05). Abbreviations: Y1 *Hanseniaspora uvarum*; Y2 *Pichia kudriavzevii*; Y3 *Pichia fermentans*; Y4 *Candida railensis*; Y5 *Candida xylopsoci*; Y6 *Wickerhamomyces anomalus*, Comp. compounds; ND not detected.

Table 4 Analytical parameters for target volatiles in coffee beans

Volatiles	Functional group	m/z	LRI	Sensory descriptors*	Identification method
Acetic acid	Acid	43	1458	Vinegar, pungent	L, MS, ST
Ethanol	Alcohol	45	948	Alcoholic	L, MS, ST
Isoamyl alcohol	Alcohol	55	1226	Banana and pear	L, MS, ST
3-Methyl-2-buten-1-ol	Alcohol	71	1330	Fruity	L, MS
1-Nonanol	Alcohol	70	1652	Floral	L, MS
2-Phenylethyl alcohol	Alcohol	91	1933	Floral, fruity	L, MS, ST
Acetaldehyde	Aldehyde	44	701	Fruity, pungent	L, MS, ST
2-Methyl Butanal	Aldehyde	44	921	Coffee-like	L, MS, ST
2,2-dimethyl hexanal	Aldehyde	57	1456	__	L, MS
Benzaldehyde	Aldehyde	77	1534	Fatty almond	L, MS, ST
Ethyl acetate	Ester	43	893	Fruity	L, MS, ST
Methyl butanoate	Ester	74	992	Fruity	L, MS, ST

2-Propanone	Ketone	86	1477	Fruity	L, MS
2-Methoxy-4-vinylphenol	Phenol	135	2192	Clove	L, MS, ST

RT, retention time; LRI = linear retention index; Identification method (MS = mass spectrum compared to NIST database, L = literature LRI; ST = standard compound). literature LRI and Sensory descriptors are taken from (Czerny & Grosch, 2000; Febrianto et al., 2016; Flament et al.; Liu et al., 2019; Mottram, 2005; G. V. d. M. Pereira et al., 2014; Sunarharum et al., 2014).

Table 5 Changes in the volatile concentrations in fermentation of synthetic coffee pulp extraction medium (SCM) with different yeasts

		Fermentation (25 °C for 36 h)					
Volatile (µg/l)	Initial (0 h)	Y1*	Y2	Y3	Y4	Y5	Y6
Acetic acid	136.9^{d}	1,293.7^{a}	458.3^{c}	404.0^{c}	1,298.2^{a}	673.1^{c}	998.7^{b}
Total acids	136.9^{d}	1,293.7^{a}	458.3^{c}	404.0^{c}	1,298.2^{a}	673.1^{c}	998.7^{b}
Ethanol	17.1^{d}	3,3640.4^{b}	37,347.5^{a}	37,719.4^{a}	27,831.8^{c}	35,632.4^{b}	31,234.3^{b}
Isoamyl alcohol	1.8^{c}	4,944.5^{b}	10,848.3^{a}	11,020.6^{a}	5455.8^{b}	10844.4^{a}	4,377.3^{b}
2-Butanol, 3-methyl-	4.0^{b}	14.1^{a}	14.2^{a}	13.7^{a}	19.4^{a}	15.8^{a}	12.7^{a}
1-Nonanol	2.8^{e}	100.4^{a}	31.1^{c}	41.2^{c}	10.9^{d}	15.1^{d}	81.4^{b}
Phenylethyl Alcohol	22.8^{d}	3,658.8^{c}	9,387.0^{a}	9,117.0^{a}	2,838.9^{c}	7,881.1^{b}	2,507.8^{c}
Total alcohols	48.5^{e}	42,358.2^{c}	57,628.1^{a}	57,911.9^{a}	36,156.8^{d}	54,388.8^{b}	38,213.6^{d}
Acetaldehyde	7.3^{d}	573.2^{c}	1,775.0^{a}	2,176.3^{a}	1,304.7^{b}	1,091.0^{b}	347.3^{c}
3-Methylbutanal	8.9^{a}	18.6^{a}	2.4^{a}	4.5^{a}	15.7^{a}	9.1^{a}	11.2^{a}
2,2-Dimethyl hexanal	ND^{e}	72.4^{a}	54.0^{b}	55.7^{b}	9.4^{d}	37.5^{c}	52.8^{b}
Benzaldehyde	77.3^{a}	19.6^{b}	19.2^{b}	13.3^{b}	11.5^{b}	19.5^{b}	18.2^{b}

Total aldehydes	93.4^{d}	683.9^{c}	1,850.6^{a}	2,249.9^{a}	1,341.3^{b}	1,157.2^{b}	429.5^{c}
Ethyl acetate	14.5^{e}	30,968.5^{b}	33,631.2^{b}	32,719.4^{b}	1,278.8^{d}	40,691.4^{a}	26,175.9^{c}
Methyl butanoate	33.7^{a}	13.1^{b}	19.2^{b}	15.9^{b}	14.0^{b}	14.8^{b}	13.1^{b}
Total esters	48.3^{e}	30,981.7^{b}	33,650.5^{b}	32,735.3^{b}	1,292.8^{d}	40,706.2^{a}	26,189.0^{c}
2-Propanone	7.4^{e}	253.7^{c}	2,837.7^{a}	2,977.2^{a}	67.7^{d}	2,073.3^{b}	189.2^{c}
Total ketones	7.4^{e}	253.7^{c}	2,837.7^{a}	2,977.2^{a}	67.7^{d}	2,073.3^{b}	189.2^{c}
4-Vinylguaiacol	3.7^{b}	10.4^{a}	12.0^{a}	10.7^{a}	6.9^{a}	10.9^{a}	9.8^{a}
Total phenols	3.7^{b}	10.4^{a}	12.0^{a}	10.7^{a}	6.9^{a}	10.9^{a}	9.8^{a}

Data are the means of three replicates at the start and end of fermentations. Mean values in each row with different lower-case letters (a–c) indicate significant differences among the yeast species (p<0.05). Abbreviations: Y1 *Hanseniaspora uvarum*; Y2 *Pichia kudriavzevii*; Y3 *P. fermentans*; Y4 *Candida railensis*; Y5 *C. xylopsoci*; Y6 *Wickerhamomyces anomalus*. ND not detected.

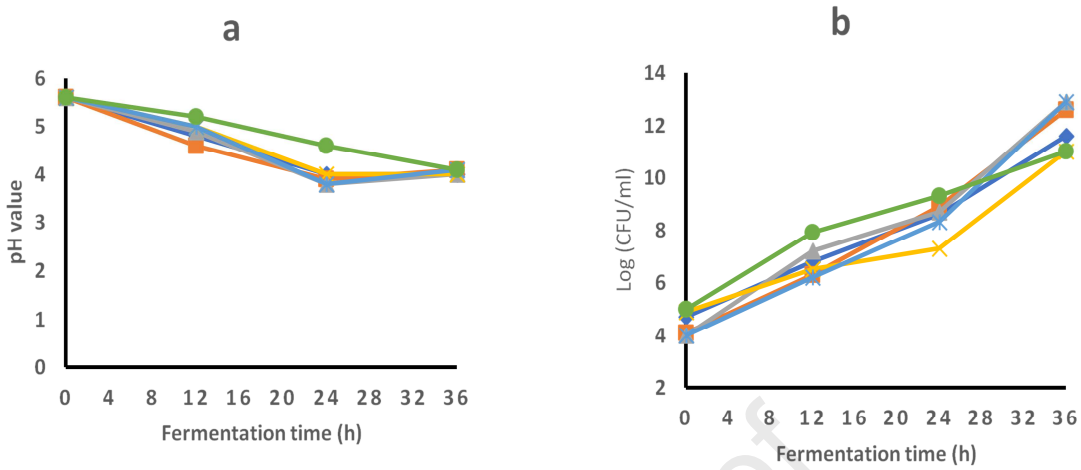

Figure 1 Changes in pH values (a), and yeast species growth rates (b) during SCM fermentations. *Hanseniaspora uvarum* (), *Pichia kudriavzevii* (), *P. fermentans* (), *Candida railenensis* (), *C. xylopsoci* (), *Wickerhamomyces anomalus* (). Data are the means of triplicate analyses with ± 0.03 for pH values and ± 0.05 CFU/ml for yeat populations.

Highlights

- Six endogenous yeasts isolated from a coffee fermentation were tested as starter cultures.
- Their viability, enzymatic activities and production of desirable volatiles were evaluated.
- *H. uvarum* and *P. kudriavzevii* had high viability and pectinolytic activities.
- Both yeasts produced high amounts of organic acids, alcohols, esters and aldehydes.
- Both yeasts had the potential to be developed into starter cultures for coffee fermentation.

Conflict of Interest:

HE declares that he has no conflict of interest. JC declares that he has no conflict of interest. DF declares that he has no conflict of interest. JZ declares that he has no conflict of interest.