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Effect of Co-Inoculation with *Pichia fermentans* and *Pediococcus acidilactici* on Metabolite Produced During Fermentation and Volatile Composition of Coffee Beans

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Abstract: Removal of the mucilage layer of coffee fruits by a fermentation process has became an interesting strategy to improve coffee quality, which is able to assist the formation of flavored molecules. In this study, four sets of inoculation protocols were evaluated using ripe and immature coffee fruits, respectively, including (i) pure culture fermentation with *Pichia fermentans*, (ii) pure culture fermentation with *Pediococcus acidilactici*, (ii) combined fermentation with *P. fermentans* and *P. acidilactici*, and (iv) spontaneous, non-inoculated control. The initial pulp sugar concentration of ripe coffee fruits (0.57 and 1.13 g/L glucose and fructose content, respectively) was significantly higher than immature coffee pulp (0.13 and 0.26 g/L glucose and fructose content, respectively). Combined inoculation with *P. fermentans* and *P. acidilactici* of ripe coffee beans increased pulp sugar consumption and production of metabolites (lactic acid, ethanol, and ethyl acetate), evidencing a positive synergic interaction between these two microbial groups. On the other hand, when immature coffee fruits were used, only pure culture inoculation with *P. fermentans* was able to improve metabolite formation during fermentation, while combined treatment showed no significant effect. Altogether, 30 volatile compounds were identified and semi-quantified with HS- solid phase microextraction (SPME)-gas chromatography coupled to mass spectrophotometry (GC/MS) in fermented coffee beans. In comparison with pure cultures and spontaneous process, combined treatment prominently enhanced the aroma complexity of ripe coffee beans, with a sharp increase in benzeneacetaldehyde, 2-heptanol, and benzylalcohol. Consistent with the monitoring of the fermentation process, only *P. fermentans* treatment was able to impact the volatile composition of immature coffee beans. The major impacted compounds were 2-hexanol, nonanal, and D-limonene. In summary, this study demonstrated the great potential of the combined use of yeast and lactic acid bacteria to improve fermentation efficiency and to positively influence the chemical composition of coffee beans. Further studies are still required to investigate the mechanisms of synergism between these two microbial groups during the fermentation process and influence the sensory properties of coffee products.

Keywords: coffee processing; coffee fermentation; starter culture; coffee beverage; yeast

1. Introduction

Coffee plants are cultivated in more than 80 countries around the world, providing raw materials for a global industry valued at an excess of 10 billion US$ [1]. Production conditions and post-harvest

operations, such as fruit harvesting, depulping, drying, and storage, have a direct impact on the quality of coffee products. Fruit harvesting is the first step in postharvest coffee processing. The heterogeneous development of coffee fruits leads to a simultaneous presence of different maturation stages in the same coffee tree, namely: (i) Green (immature) coffee fruits, presenting incomplete endosperm development and low reducing sugars content in the mucilaginous layer; (ii) cherry (ripe) fruits, presenting mucilage rich in reducing sugars, complete development of the endosperm and red or yellow exocarp color; and (iii) raisin (overripe), which are fruits showing the initial characteristics of the senescence cycle with metabolic pathway deviation for the catabolism of the nutrients accumulated in the beans [2]. In Brazil, the largest coffee producer in the world, it is estimated that 31% of the coffee fruits are harvested in the immature stage [3]. Immature coffee beans have a high content of chlorogenic acids (caffeine, trigonelline) and lower sugar content due to incomplete cycle of maturation, attributing astringency and depreciating the quality of coffee products [4,5].

After harvesting, coffee processing must begin as quickly as possible to prevent fruit spoilage by unfavorable fermentation or mold formation [6,7]. The outer layers of the coffee fruit (skin and pulp) are easily removed, while the mucilage, parchment, and silver skin are firmly attached to the beans [8]. The way that coffee growers use to remove the mucilage layer attached to the fruits classifies coffee in the international market: 'Natural coffee', where mucilage is removed by a simple method of sun-drying, known as dry processing; 'washed coffee', produced from coffee beans that undergo a relatively complex series of steps, including depulping, fermentation, and sun-drying known as wet processing; and "pulped natural coffee", which the fruits are mechanically husked and the mucilage is removed by a sun-drying process, known as semi-dry processing [9].

In the wet processing, coffee beans are submitted to underwater tank fermentation for mucilage breakdown and removal. The sugars present in the mucilage support microbial, especially yeasts and lactic acid bacteria [10]. Recent studies have been dedicated to the use of yeast and lactic acid bacteria (LAB) as pure starter cultures in post-harvest processing, in order to reduce the time required for fermentation and modulate the chemical and sensory characteristics of coffee beans [11–14]. Among the selected microorganisms, it is possible to highlight the yeast *Pichia fermentans* YC5.2 and the lactic acid bacteria *Pediococcus acidilactici* LPBC161, which are cultures with characteristics of efficient consumption of coffee pulp-sugars and adaptability to the stress factors of coffee processing [15,16]. Despite that the use of pure cultures offers advantages, recent studies in wine, meat, and dairy fermentations demonstrate that mixed starters are able to improve the sensorial and safety proprieties of the final product [17,18]. In this regard, the aim of this study was to evaluate the effects of co-inoculation with *Pichia fermentans* YC5.2 and *Pediococcus acidilactici* LPBC161 on metabolites produced during fermentation and the volatile composition of coffee beans.

2. Material and Methods

2.1. Microorganism and Inoculum Preparation

The selected yeast (*Pichia fermentans* YC5.2) and lactic acid bacteria (*Pediococcus acidilactici* LPBC161) strains used in this study were previously isolated and selected from spontaneous coffee fermentations, as detailed in Muynarsk et al. [15] and Pereira et al. [16]. The *P. fermentans* YC5.2 and *P. acidilactici* LPBC161 were reactivated in MRS (Merck Millipore, Burlington, MA) and YEPG broth (Himedia, Marg, India), respectively, at 28 °C during 24 h. Each microorganism was then grown up to a concentration of 10^9 CFU/mL. To reach this concentration, *P. acidilactici* LPBC161 was cultivated in Erlenmeyer containing 4 L of sugar cane molasses 3% (w/v) medium, enriched with yeast extract 0.5% (w/v), ammonium citrate 0.5% (w/v), ammonium phosphate 0.5% (w/v), sodium acetate 0.5% (w/v), Tween 80 0.1% (v/v), and manganese sulfate 0.005% (w/v) [19], and *P. fermentans* YC5.2 was grown in Erlenmeyer containing 4 L of sugar cane molasses 3% (w/v) medium enriched with yeast extract 0.5% (w/v). After incubation, the yeast and lactic acid bacteria (LAB) cells were separated from the medium by centrifugation at 5000 × *g* during 5 min, washed twice with sterile saline-peptone solution (0.1% [w/v]

bacteriological peptone (Himedia), 0.8% (w/v; NaCl (Merck)), and resuspended in sterile saline solution (0.9% (w/v) NaCl).

2.2. Farm Experiments

The field experiments were conducted at the Fazenda Baobá (21°42′42.8′′ S, 46°49′42.2′′ W; 1400 m above sea level) situated in São Sebastião da Grama, São Paulo state, Brazil. Ripe and immature coffee fruits (10 kg) were, respectively, deposited in 20-L plastic buckets with 5 L of water. Four sets of inoculation protocols were performed in triplicate: (i) Pure culture fermentation with *P. fermentans*, (ii) pure culture fermentation with *P. acidilactici*, (ii) combined fermentation with *P. fermentans* and *P. acidilactici*, and (iv) spontaneous, non-inoculated control. Prior to inoculation, yeast and LAB cells were counted by a Thoma hemocytometer chamber using methylene blue dye as a marker of cell viability. Then, appropriate amounts of inoculum were used to reach an initial cell population of about 7 log CFU/mL. At the end of fermentation, coffee beans were sun dried until the value of 12% of moisture was reached.

2.3. Sampling and pH Measurement

Samples (50 mL) of the liquid fraction of the fermenting coffee pulp were collected in triplicate at intervals of 12 h to monitor sugars consumption and organic acids, ethanol, and volatile compounds production. At each sampling point, the pH was measured using a digital pH meter (Requipal, Curitiba, Brazil).

2.4. HPLC Analysis of Fermenting Coffee Pulp

The concentration of reducing sugars (glucose and fructose), organic acids (citric, succinic, lactic, acetic, and propionic acids), and ethanol in coffee pulp (liquid fraction) was determined in intervals of 12 h. Aliquots of 2 mL were centrifuged at 6000 × g for 15 min and filtered through 0.22 µm pore size filter (Millipore Corp., Billerica, MA, USA). Analysis parameters were performed according to Carvalho Neto et al. [20]. The filtered samples were injected into high-performance liquid chromatograph (HPLC) system equipped with an Aminex HPX 87 H column (300 × 7.8 mm; Bio-Rad, Richmond, CA, USA) and a refractive index (RI) detector (HPG1362A; Hewlett–Packard Company, Palo Alto, CA, USA). The column was eluted in an isocratic mode with a mobile phase of 5 mM H2SO4 at 60 °C and a flow rate of 0.6 mL/min.

2.5. GC Analysis of Fermenting Coffee Pulp

The formation of major volatile compounds was determined in intervals of 12 h by gas chromatography. For sample preparation, aliquots (4 mL) from the liquid fraction of the fermenting coffee pulp were placed in 20 mL hermetically sealed flasks containing NaCl 5% (w/v), followed by heating during 10 min at 60 °C. The headspace was then collected using a glass syringe (Hamilton, Bonaduz, Switzerland) and injected into a gas chromatograph (model 17A; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector at 230 °C. 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 [13]. A standard curve was constructed using authentic analytical standards purchased from Sigma and concentration of the compounds was expressed as µmol/L of headspace

2.6. GC/MS Analysis of Fermented Coffee Beans

The volatile aroma compound composition of spontaneous and inoculated coffee beans was determined by gas chromatography coupled to mass spectrophotometry (GC-MS) according to Carvalho Neto et al. [20]. The extraction of volatile compounds from the beans samples (2 g) was performed using a headspace vial coupled to a solid phase microextraction (SPME) fiber DVB/CAR/PDMS fiber (Supelco Co., Bellefonte, PA, USA). The flasks were heated at 70 °C for 10 min without agitation,

followed by 15 min of exposition of the fiber in a COMBI-PAL system. The compounds were desorbed into the gas chromatograph injection system gas phase (CGMS-gun TQ Series 8040 and 2010 Plus GC-MS; Shimadzu, Tokyo, Japan) at 260 °C. The column oven temperature was maintained at 60 °C during 10 min, followed by two heating ramps of 4 and 10 °C /min until reaching the temperatures of 100 and 200 °C, respectively. The compounds were separated on a column 95% PDMS/5% PHENYL (30 m × 0.25 mm × 0.25 mm film thickness). The GC was equipped with an HP 5972 mass selective detector (Hewlett Packard, Palo Alto, CA, USA). Helium was used as carrier gas at a rate of 1.0 mL/min. Mass spectra were obtained by electron impact at 70 eV and a start and end mass-to-charge ratio (m/z) of 30 and 200, respectively. The compounds were identified by comparison to the mass spectra from library databases (Nist'98 and Wiley7N).

2.7. Statistical Analysis

The data obtained of target metabolite analysis were analyzed by post-hoc comparison of means by Duncan's test and a principal component analysis (PCA). Statistical analyses were performed using the SAS program (Statistical Analysis System Cary, NC, USA). Level of significance was established in a two-sided p-value <0.05.

3. Results and Discussion

3.1. Field Experiment

The use of mixed fermentation instead a single culture is a practice widely applied in winemaking in order to improve the aroma complexity or mouth-feel of wines [21,22]. It offers a number of advantages over conventional single-culture fermentations, including higher microbial growth rate and metabolite yield, better utilization of the substrate, and complex formation of aromatic compounds [23]. This work represents the first study on a mixed culture in the coffee beans fermentation process. We experimentally tested the impact of the combination of two selected cultures (*P. fermentans* YC5.2 and *P. acidilactici* LPBC161) in terms of the fermentation efficiency and volatile composition of coffee beans. The experiments were performed with individual and combined inoculations in ripe and immature coffee beans, compared to a spontaneous process. The changes in major non-volatiles (sugars, organic acids, and ethanol) and volatiles metabolites were quantified in the course of the fermentation time. The initial pulp sugar concentration of ripe coffee fruits (0.57 and 1.13 g/L glucose and fructose content, respectively) was significantly higher than immature coffee pulp (0.13 and 0.26 g/L glucose and fructose content, respectively; Figure 1). The low levels of sugars in the case of immature coffee are the consequence of incomplete maturation cycle fruits [24]. In all fermentation processes, the sugar concentration showed an increase during the initial 12 h. This phenomenon can be associated with the hydrolysis of pectin, cellulose, sucrose, and other coffee pulp complexes carbohydrates, into monomers of glucose and fructose [25,26]. After this increase, both glucose and fructose were partially consumed, resulting in a residual concentration of around 0.37 and 1.51 g/L (ripe coffee pulp) and 0.19 and 0.74 g/L (immature coffee pulp) of glucose and fructose, respectively. Residual pulp sugars are generally observed after the coffee fermentation process, mainly associated with the short fermentation cycle [14,27,28]. However, fructose consumption was more efficient in the treatments that the yeast was used (i.e., *P. fermentans*-pure culture and combined treatment with *P. fermentans* and *P. acidilactici*) than *P. acidilactici* pure culture and spontaneous assay (Figure 1). The yeast's ability to withstand stress tolerance factors and the production of pectinolytic enzymes confer advantages in comparison to lactic acid bacteria [16,29]. In addition, the low availability of initially willing nutrients in immature coffee pulp [24] may have restricted the development of *P. acidilactici*, showing poor sugar consumption (Figure 1B). Lactic acid bacteria are further distinguished by their limited biosynthetic abilities, being unable to synthesize multiple cofactors, vitamins, purines, pyrimidines, and other nutrients [30].

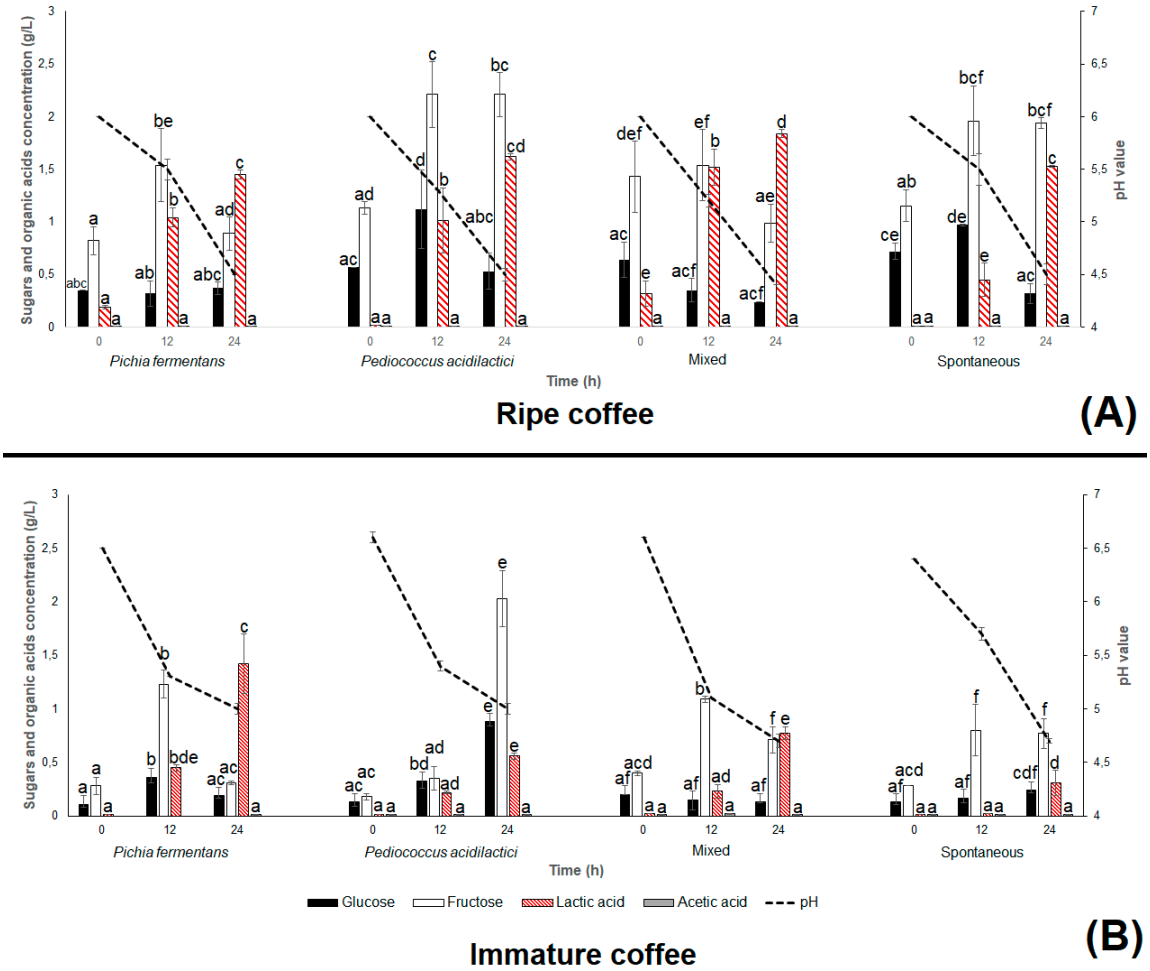

Figure 1. Pulp sugar consumption, organic acid production, and pH monitoring of inoculated (pure culture with *Pichia fermentans*, pure culture with *Pediococcus acidilactici*, and combined fermentation with *P. fermentans* and *P. acidilactici*) and spontaneous fermentation of ripe (**A**) and immature (**B**) coffee fruits. The significance of the results was assessed using an ANOVA with Duncan's post-hoc test at $p < 0.05$. Different lowercase letters (a, b, c, d, e, f) indicate significant differences within the same process (ripe and immature coffee beans) over fermentation time.

Lactic acid showed a significant increase through fermentative processes, reaching maximum concentration of ≥1.45 and ≥0.53 g/L in the ripe and immature treatments, respectively (Figure 1). Basal concentrations of acetic acid (≤0.01 g/L) were detected in all the processes, which can be associated with yeast metabolism or the heterofermentative nature of *P. acidilactici* [31,32]. Overfermentation acids, such as propionic and butyric acids, were not detected in both ripe and immature treatments. In this sense, the acidification of fermenting coffee pulp can be attributed mainly to lactic acid content. As expected, while the lactic acid concentration increased, the pH decreased progressively during all fermentation processes (Figure 1). Lactic acid is an important end-metabolite associated with coffee fermentation, which assists in the coffee-pulp acidification process without interference in the final product (Figure 1). The pH monitoring is a crucial parameter, since pH values below 4.5 are used as to indicate the end of the coffee fermentation process [33–35]. In immature coffee treatments, a pH higher than 4.5 was reported, which may be attributed to the insufficient development of *P. acidilactici*.

Ethanol and ethyl acetate were the major volatile compounds detected during fermentation processes (Figure 2). Interestingly, combined inoculations with *P. acidilactici* and *P. fermentans* resulted in a significant increase in the production of these metabolites when compared to pure cultures and a spontaneous process. The higher values of ethanol (27.04 and 14.8 µmol/L) and ethyl acetate (1.63 and 1.21 µmol/L) were reached after 24 h of ripe and immature combined fermentations, respectively.

This agrees with the findings of Sun et al. [36] and Cañas et al. [37] that demonstrated an increase of ethyl- and acetate esters as a result of the co-inoculation of LAB and yeasts in wine fermentation. The significant sugar consumption and lactic acid, ethyl acetate, and ethanol production in treatments with combined inoculations indicated an ecological interaction between these two microbial groups. The complex nature of this interaction is highlighted by the observations that (i)the autolysis of yeasts release nutrients, such as amino acids, polysaccharides and riboflavin, favorable for bacterial growth, and that (ii) the acidification of the fermentation media by LAB creates a prone environment for yeast development [9,38,39]. These positive interactions have been shown to promote desired sensory attributes in wine, sourdough, and yogurt. However, information about these mechanisms in coffee fermentation is scarce [40].

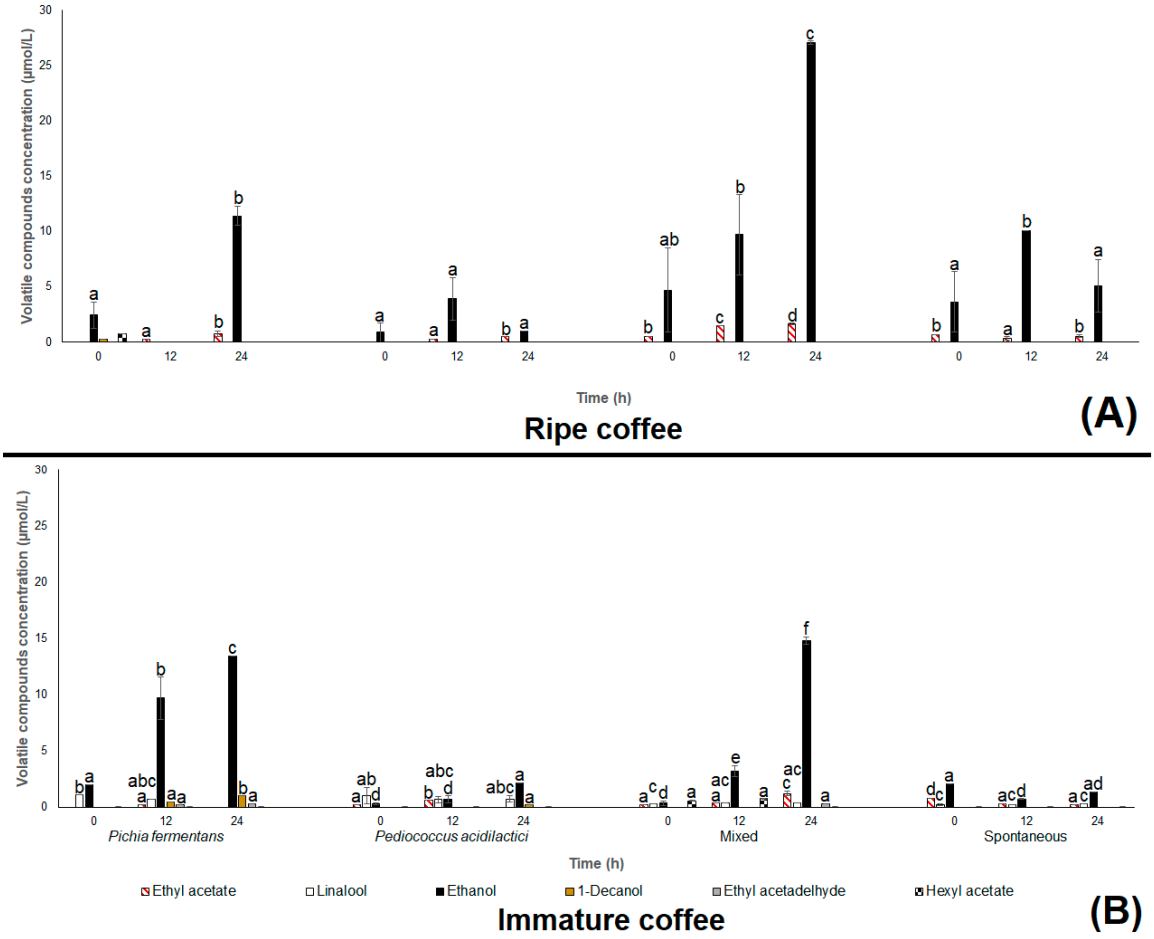

Figure 2. Concentration of volatile compounds produced in the course inoculated (pure culture with *P. fermentans*, pure culture with *P. acidilactici*, and combined fermentation with *P. fermentans* and *P. acidilactici*) and spontaneous fermentation of ripe (**A**) and immature (**B**) coffee fruits. The significance of the results was assessed using an ANOVA with Duncan's post-hoc test at $p < 0.05$. Different lowercase letters (a, b, c, d, e, f) indicate significant differences within the same process (ripe and immature coffee beans) over fermentation time.

Other minor volatile compounds that increased during the fermentation processes were 1-decanol, ethyl-acetaldehyde, and hexyl acetate (Figure 2). Yeast and lactic acid bacteria generate ethyl-acetaldehyde by a condensation reaction between fatty acids and an alcohol molecule [41], while 1-decanol can be derived from amino acid catabolism via the Ehrlich pathway [16,42]. The presence of higher concentrations of linalool using immature fruits can be associated with the inferior maturation stage of the coffee beans, since this compound has been considered a volatile marker of coffee beverages produced from immature fruits [43].

3.2. Coffee Beans Chemical Composition

Over 900 volatile compounds have already been identified in green and roasted coffee beans [27,44]. Among the major volatiles found, pyrazines, furans, ketones, aldehydes, higher alcohols, esters, and sulphur compounds can be highlighted [45–47]. Although some of these flavor-active compounds originate from the beans itself, recent studies have revealed that microbial-derived metabolites can also diffuse into the beans [11,14,27,33,48–50]. Upon characterization of the volatile composition of fermented coffee beans, it was observed that inoculation of *P. fermentans* and *P. acidilactici*, both in pure and combined treatments, resulted in the modulation of the volatile constitution of coffee beans. A total of 30 compounds were identified in fermented ripe coffee beans, including higher alcohols (seven compounds), aldehydes (six compounds), and terpenes (three compounds; Table 1). Among these, 1-hexanol, 2-heptanol, phenylethyl alcohol, and benzeneacetaldehyde were the major volatiles found. Single inoculation of *P. fermentans* and *P. acidilactici* resulted in the formation and diffusion of some volatile compounds, such as 3-octanol, 2-heptenal, benzaldehyde, dodecanal, and D-limonene, that were not detected in a spontaneous process. These compounds are strictly related to both yeast and LAB metabolism, such as aldehydes and higher alcohols formed from the catabolism of coffee pulp amino acids, and terpenes through mevalonic acid pathway or released from glycoside precursors during fermentation [10,51,52].

Table 1. Concentration of volatile compounds (Area*10^5) in ripe coffee beans after single cultures, a combined treatment, and a spontaneous assay. Means of triplicate in each row bearing the same lowercase letters (a, b) are not significantly different ($p > 0.05$) from one another using Duncan's Test (mean ± standard variation).

Compounds	Aroma Perception	Fermented Ripe Coffee Beans			
		Spontaneous	*P. fermentans*	*P. acidilactici*	*P. fermentans* + *P. acidilactici*
Organic acids (3)					
Butanoic acid, 3-methyl	-	7.02 ± 1.00 [a]	6.88 ± 1.48 [a]	6.10 ± 1.74 [a]	6.91 ± 0.18 [a]
Butanoic acid, 2-methyl	Fruity, dirty, acidic with a dairy buttery	1.77 ± 0.35 [a]	1.98 ± 0.28 [a]	ND	1.79 ± 0.28 [a]
Hexanoic acid	Sour, fatty, sweat, cheesy	ND	1.34 ± 0.61 [a]	0.99 ± 0.23 [a]	1.67 ± 0.88 [a]
Higher alcohols (7)					
2-Heptanol	Fresh, lemon grass, herbal	10.21 ± 0.00 [a]	ND	ND	12.12 ± 0.10 [b]
5-Methyl-2-Hexanol	-	6.09 ± 2.66	ND	ND	ND
1-Hexanol	Green, fruity, apple-skin and oily	15.90± 2.36 [a]	16.95 ± 2.23 [a]	17.10 ± 2.36 [a]	18.24 ± 0.17 [a]
1-Octen-3-ol	Earthy, green, oily, umami sensation	0.82 ± 0.00 [a]	0.80 ± 0.17 [a]	ND	0.86 ± 0.03 [a]
3-Octanol	Musty, mushroom, earthy, creamy dairy	ND	0.50 ±0.14 [a]	0.58 ± 0.00 [a]	0.67 ± 0.00 [a]
Benzylalcohol	Sweet, fruity with balsamic nuances	2.58 ± 0.05 [a]	3.26 ± 0.36 [a]	3.20 ± 0.68 [a]	4.31 ± 0.17 [b]
Phenylethyl alcohol	Floral, sweet and bready	9.52 ± 0.73 [a]	10.34 ± 0.41 [a]	12.17 ± 0.25 [b]	12.60 ± 1.31 [b]
Esters (3)					
Butanoic acid, 2-methyl, ethyl ester	-	0.58 ± 0.26 [a]	0.34 ± 0.00 [a]	ND	0.54 ± 0.20 [a]
Butanoic acid, 3-methyl- ethyl ester	-	4.62 ± 0.59 [a]	3.66 ± 1.71 [a]	ND	4.70 ± 0.57 [a]
Methyl salicylate	Wintergreen, mint-like	ND	0.28 ± 0.01	ND	ND
Aldehydes (6)					
2-Heptenal	Sweet, fresh fruity apple skin nuances	ND	0.80 ± 0.00 [a]	ND	0.80 ± 0.00 [a]
Benzaldehyde	Fruity, cherry	ND	0.32 ± 0.00 [a]	2.15 ± 0.84 [a]	2.56 ± 0.69 [a]
Dodecanal	Soapy, waxy, citrus, orange mandarin	ND	ND	0.23 ± 0.14	ND
Nonanal	With a fresh green lemon peel-like nuance	0.95 ± 0.05 [a]	0.93 ± 0.35 [a]	0.81 ± 0.06 [a]	0.84 ± 0.27 [a]
Benzeneacetaldehyde	Almond, fruity, powdery, nutty	1.83 ± 0.29 [a]	2.80 ± 0.73 [a]	2.28 ± 0.04 [a]	6.94 ± 0.00 [b]
Decanal	Sweet, aldehydic, orange, waxy and citrus rind	ND	0.37 ± 0.11 [a]	0.37 ± 0.09 [a]	0.34 ± 0.07 [a]
Ketone (1)					
2-Heptanone	Fruity, spice, herbal	3.49 ± 0.69 [a]	2.92 ± 0.28 [a]	2.29 ± 0.90 [a]	4.38 ± 1.75 [a]

Table 1. *Cont.*

Compounds	Aroma Perception	Fermented Ripe Coffee Beans			
		Spontaneous	***P. fermentans***	***P. acidilactici***	***P. fermentans* + *P. acidilactici***
Pyridine (2)					
Pyridine, 2,3-dimethyl	-	ND	1.55 ± 0.94 [a]	1.44 ± 0.26 [a]	1.25 ± 0.63 [a]
Pyridine, 2,6-Lutidine	Nutty, amine, woody, bready and vegetable-like	1.85 ± 0.19	ND	ND	ND
Lactone (1)					
Butyrolactone	Creamy, oily, fatty, caramellic	5.28 ± 0.10 [a]	6.17 ± 1.04 [a]	4.86 ± 1.13 [a]	6.09 ± 0.13 [a]
Terpenes (3)					
Linalool	Citrus, orange, lemon	2.36 ± 0.29 [a]	2.27 ± 0.21 [a]	2.22 ± 0.82 [a]	2.92 ± 0.17 [b]
D-Limonene	Sweet, orange, citrus	ND	1.29 ± 0.60 [a]	1.37 ± 0.49 [a]	1.42 ± 0.35 [a]
Anethole	-	4.10 ± 1.00 [b]	1.87 ± 0.26 [a]	1.96 ± 0.66 [a]	3.13 ± 0.20 [a,b]
Hydrocarbons (2)					
Styrene	Sweet, balsamic, floral	ND	2.89 ± 0.00 [a]	ND	3.15 ± 0.00 [a]
Tetradecane	Waxy	0.91 ± 0.07 [a]	0.82 ± 0.06 [a]	0.88 ± 0.16 [a]	0.80 ± 0.06 [a]
Pyrzine (1)					
Pyrazine, 2-methoxy-3-(2-methylpropyl)	Roasted almond hazelnut peanut	0.92 ± 0.04 [a]	ND	0.93 ± 0.08 [a]	ND
Furan (1)					
Furan, 2-pentyl	Waxy, with musty, cooked caramellic nuances	1.16 ± 0.04	ND	ND	ND

Interestingly, coffee beans generated from combined treatments showed significantly increased ($p < 0.05$) of specific volatile compounds, such as benzeneacetaldehyde, 2-heptanol, and benzylalcohol. These findings are in accordance with Englezos et al. [18] and Plessas et al. [53], which evidences that mixed treatments of yeast and LAB starter cultures enable higher production of esters, aldehydes, and higher alcohols in sourdough and wine fermentations when compared to single inoculations. However, further studies are required to evidence the metabolic pathways associated with the positive interaction between these microorganisms in coffee products.

Chemical analysis of immature coffee beans revealed a composition with lower diversity and concentration of volatile compounds (Table 2). A total of 19 compounds were detected, including higher alcohols (four compounds), organic acids (three compounds), and aldehydes (three compounds). *P. fermentans*-single inoculation resulted in coffee beans with significantly higher concentrations ($p < 0.05$) of 2-hexanol, nonanal, and D-limonene when compared to the spontaneous process. These compounds are commonly attributed to *Pichia* metabolism [10,54,55]. This corroborates with results from fermentation process monitoring, which demonstrated intense microbial activity of *P. fermentans* in immature coffee pulp. On the other hand, coffee beans derived from *P. acidilactici*-pure culture and combined treatment showed no significant increase ($p > 0.05$) in the volatile constituents when compared to the control (spontaneous process). This fact can be correlated to the insufficient growth of the LAB starter culture in the nutrient-scarce environment from the pulp of immature coffee beans. The auxotrophism of several amino acids turns LAB directly dependent on a rich growth medium for its full development [31].

Table 2. Concentration of volatile compounds (Area*10^5) in immature coffee beans after single cultures, a combined treatment, and a spontaneous assay. Means of triplicate in each row bearing the same lowercase letters (a, b, c) are not significantly different ($p > 0.05$) from one another using Duncan's Test (mean ± standard variation).

Compounds	Aroma Perception	Fermented Immature Coffee Beans			
		Spontaneous	***P. fermentans***	***P. acidilactici***	***P. fermentans* + *P. acidilactici***
Organic acids (3)					
Butanoic acid, 3-methyl	-	7.77 ± 1.05 [a]	5.99 ± 2.31 [a]	8.56 ± 1.57 [a,b]	5.15 ± 0.98 [a,c]
Butanoic acid, 2-methyl	Fruity, acidic with a dairy buttery	1.32 ± 0.36 [a]	1.24 ± 0.86 [a]	1.65 ± 0.13 [a,b]	0.69 ± 0.15 [a,c]
Hexanoic acid	Sour, fatty, sweat, cheesy	ND	0.63 ± 0.15 [a]	0.50 ± 0.11 [a]	ND
Higher alcohols (4)					
1-Hexanol	Green, fruity, apple-skin and oily	10.02 ± 0.26 [a]	10.57 ± 1.33 [a]	9.14 ± 1.51 [a]	9.85 ± 1.17 [a]
2-Hexanol	-	0.36 ± 0.05 [a]	0.75 ± 0.01 [b]	ND	0.45 ± 0.10 [a]
Benzylalcohol	Sweet, fruity with balsamic nuances	0.54 ± 0.15 [a]	0.62 ± 0.08 [a]	0.51 ± 0.20 [a]	0.61 ± 0.04 [a]
Phenylethyl alcohol	Floral, sweet and bready	2.54 ± 0.38 [a]	2.78 ± 0.17 [b]	2.24 ± 0.12 [a]	2.20 ± 0.18 [a]
Aldehydes (3)					
Nonanal	Citrus, with a fresh green lemon peel-like nuance	0.30 ± 0.08 [a]	0.74 ± 0.04 [b]	0.46 ± 0.07 [c,d]	0.48 ± 0.05 [d]
Benzeneacetaldehyde	Almond, fruity, powdery, nutty	0.21 ± 0.00 [a]	0.28 ± 0.01 [a]	0.27 ± 0.13 [a]	0.38 ± 0.00 [a]
Decanal	Sweet, orange, waxy and citrus rind	0.24 ± 0.09 [a]	0.43 ± 0.06 [a]	0.30 ± 0.10[a]	0.42 ± 0.08 [a]
Pyridines (2)					
Pyridine, 2,3-dimethyl	-	ND	1.45 ± 0.34 [a]	0.62 ± 0.14 [a]	1.10 ± 0.36 [a]
Pyridine, 2,6-Lutidine	Nutty, woody, bready and vegetable-like	0.95 ± 0.35	ND	ND	ND
Lactone (1)					
Butyrolactone	Creamy, oily, fatty, caramellic	0.92 ± 0.07 [a]	0.46 ± 0.20 [a]	0.66 ± 0.26 [a]	0.49 ± 0.17 [a]
Terpenes (1)					
D-Limonene	Sweet, orange, citrus	0.36 ± 0.06 [a]	0.72 ± 0.16 [b]	0.16 ± 0.09 [c]	0.41 ± 0.19 [a]

Table 2. *Cont.*

Compounds	Aroma Perception	Fermented Immature Coffee Beans			
		Spontaneous	***P. fermentans***	***P. acidilactici***	***P. fermentans* + *P. acidilactici***
Furans (2)					
Furan-2-pentyl	Waxy, cooked caramellic nuances	0.57 ± 0.17 [a]	0.74 ± 0.12 [a]	0.58 ± 0.15 [a]	0.69 ± 0.13 [a]
2(3)-Furanone, dihydro-5-methyl	Creamy, waxy with a citrus fruity nuance	0.55 ± 0.14 [a]	0.38 ± 0.16 [a]	ND	0.45 ± 0.15 [a]
Hydrocarbons (2)					
Hexadecane	-	ND	0.55 ± 0.01	ND	ND
Tetradecane	Waxy	0.62 ± 0.02 [a]	0.24 ± 0.07 [b]	0.30 ± 0.06 [c]	0.76 ± 0.10 [a,c]
Pyrzine (1)					
Pyrazine, 2-methoxy-3-(2-methylpropyl)	Roasted almond hazelnut peanut	0.78 ± 0.07 [a]	0.80 ± 0.01 [a]	0.85 ± 0.12 [a]	0.92 ± 0.08 [a]

In order to explain the chemical characteristics and grouping of the samples, the parameters in Tables 1 and 2 were analyzed by a PCA (Figure 3). The first and second principal components explained, together, 73.66% of the total variability within the data. The samples were categorized into two clusters, *viz.*, ripe and immature coffee beans. This distinction was mainly related to the richer constitution of volatiles of ripe coffee beans relative to immature treatments. In addition, the presence of specific compounds (benzyl alcohol, phenylethyl alcohol, benzeneacetaldehyde, decanal, and D-limonene in ripe coffee beans, and furan-2-pentyl, 2-methyl-butanoic acid, and pyrazine, 2-methoxy-3-(2-methylpropyl) in immature coffee beans) also contributed to the separation of ripe and immature coffee beans in the PCA analysis. Interestingly, only the treatment with *P. fermentans* grouped immature coffee beans in the positive axis, which corroborates with the better yeast' adaptation and generation of volatiles in immature coffee pulp.

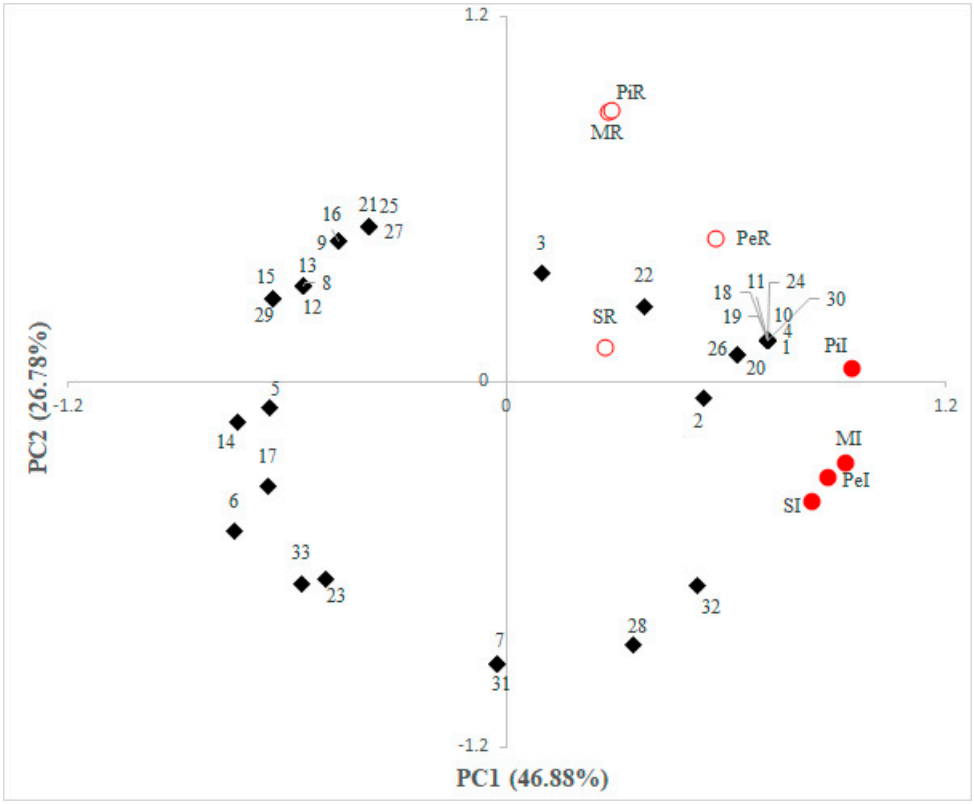

Figure 3. Principal component analysis (PCA) of volatile compounds (lozenges) identified in the different treatments of ripe (open circles) and immature (closed circles) coffee beans. Abbreviations: *SR*—spontaneous, ripe control; *PiR*—*Pichia* inoculation in ripe coffee beans; *PeR*—*Pediococcus* inoculation in ripe coffee beans; *MR*—mixed (*Pichia* plus *Pediococcus*) inoculation in ripe coffee beans; *SI*—spontaneous, immature control; *PiI*—*Pichia* inoculation in immature coffee beans; *PeI*—*Pediococcus* inoculation in immature coffee beans; *MI*—mixed (*Pichia* plus *Pediococcus*) inoculation in immature coffee beans. 1—butanoic acid, 3-methyl; 2—butanoic acid, 2-methyl; 3—hexanoic acid; 4—1-hexanol; 5—2-heptanol; 6—5-methyl-2-hexanol; 7—2-hexanol; 8—1-octen-3-ol; 9—3-octanol; 10—benzylalcohol; 11—phenylethyl alcohol; 12—butanoic acid, 2-methyl, ethyl ester; 13 - butanoic acid, 2-methyl, ethyl ester; 14—methyl salicylate; 15—2-heptenal; 16—benzaldehyde; 17—dodecanal; 18—nonanal; 19—benzeneacetaldehyde; 20—decanal; 21—2-heptanone; 22—pyridine, 2,3-dimethyl; 23—pyridine, 2,6-lutidine; 24—butyrolactone; 25—linalool; 26—D-limonene; 27—anethole; 28—furan-2-pentyl; 29—styrene; 30—tetradecane; 31—2(3)-furanone, dihydro-5-methyl; 32—pyrazine, 2-methoxy-3-(2-methylpropyl); 33—hexadecane.

4. Conclusions

This is the first study investigating the impact of co-inoculation with yeast and LAB on the fermentation of ripe and immature coffee fruits. Among the different treatments, combined inoculations with *Pichia fermentans* YC5.2 and *Pediococcus acidilactici* LPBC161 in ripe coffee fruits showed interesting features. It was possible to reach increased coffee pulp-sugar consumption and production of metabolites (lactic acid, ethanol, and ethyl acetate), evidencing a positive synergic interaction between these two microbial groups. On the other hand, when using immature coffee fruits, only *Pichia fermentans* was able to improve metabolite formation during fermentation and impact volatile composition of resulting coffee beans. This may be due to the high nutritional requirement of LAB species and poor adaptability in immature coffee pulp. Howsoever, since immature coffee beans usually have a low quality because the formation of flavored precursors is incomplete, yeast metabolism has great potential to add flavor quality to these beans.

Chemical analysis revealed a more complex volatile composition of fermented coffee beans from combined treatment in relation to pure inoculations and spontaneous process. The major compounds impacted were benzeneacetaldehyde, 1-hexanol, benzylalcohol, 2-heptanol, and phenylethyl alcohol, which are reported as important aroma-impacting compounds. Thus, this study shows the great potential of combined inoculation for the formation of desirable aroma compounds and production of specialty coffees. Further studies are still required to investigate the mechanisms of synergism between yeast and LAB and influence on sensory properties of coffee products.

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